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(54) Title: IMMUNOSTIMULATORY BACTERIA-BASED VACCINES, THERAPEUTICS, AND RNA DELIVERY PLATFORMS

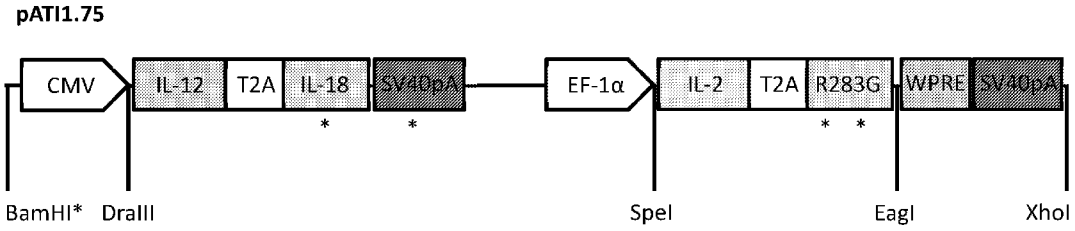


FIG. 1A

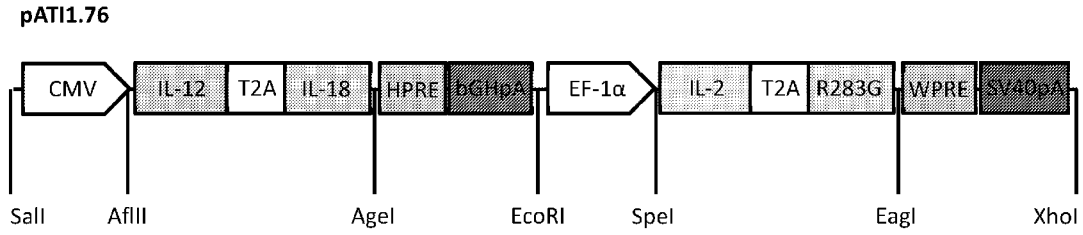


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FIG. 1B

(57) Abstract: Provided are attenuated immunostimulatory bacteria with genomes that are modified to, for example, reduce toxicity and improve the anti-tumor activity, such as by increasing accumulation in the tumor microenvironment, particularly in tumor- resident myeloid cells, improving resistance to complement inactivation, reducing immune cell death, promoting adaptive immunity, and enhancing T-cell function. Also provided are immunostimulatory bacteria for use as vaccines, and for delivery of mRNA. The increase in colonization of phagocytic cells improves the delivery of encoded therapeutic products to the tumor microenvironment and to tumors and permits, among other routes, systemic administration of the immunostimulatory bacteria. The increase in colonization of phagocytic cells also provides for use of immunostimulatory bacteria for direct tissue administration for use as vaccines.

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[Continued on next page]

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# IMMUNOSTIMULATORY BACTERIA-BASED VACCINES, THERAPEUTICS, AND RNA DELIVERY PLATFORMS

## RELATED APPLICATIONS

Benefit of priority is claimed to U.S. Provisional Application Serial No. 63/064,869, filed on August 12, 2020, entitled "IMMUNOSTIMULATORY BACTERIA DELIVERY PLATFORM," to Applicant Actym Therapeutics, Inc., and inventors Laura Hix Glickman, Christopher D. Thanos, Alexandre Charles Michel Iannello, Chris Rae, and Haixing Kehoe.

Benefit of priority also is claimed to U.S. Provisional Application Serial No. 63/188,443, filed May 13, 2021, entitled "IMMUNOSTIMULATORY BACTERIA DELIVERY PLATFORM" to Applicant Actym Therapeutics, Inc., and inventors Laura Hix Glickman, Christopher D. Thanos, Alexandre Charles Michel Iannello, Chris Rae, and Haixing Kehoe.

Benefit of priority also is claimed to U.S.Application No. 17/320,200, filed May 13, 2021, entitled "IMMUNOSTIMULATORY BACTERIA DELIVERY PLATFORMS AND THEIR USE FOR DELIVERY OF THERAPEUTIC PROTEINS," to Applicant Actym Therapeutics, Inc., and inventors Laura Hix Glickman, Christopher D. Thanos, Alexandre Charles Michel Iannello, Chris Rae, Haixing Kehoe, Bret Nicholas Peterson, and Chingnam Cheung.

This application is related to co-pending International Patent Application No. PCT/US2020/060307, filed on November 12, 2020 and published as WO 2021/097144 on May 20, 2021, entitled "IMMUNOSTIMULATORY BACTERIA DELIVERY PLATFORMS AND THEIR USE FOR DELIVERY OF THERAPEUTIC PRODUCTS," to Applicant Actym Therapeutics, Inc., and inventors Christopher D. Thanos, Laura Hix Glickman, Alexandre Charles Michel Iannello, Chris Rae, Haixing Kehoe, Bret Nicholas Peterson, and Chingnam Cheung

This application is related to co-pending International Patent Application No. PCT/US2020/020240, filed on February 27, 2020 and published as WO 2020/176809 on September 03, 2020, and to co-pending U.S. Patent Application Serial No. 16/824,500, filed on March 19, 2020 and published as U.S. Publication No. US 2020-0270613 A1 on August 27, 2020, each entitled "IMMUNOSTIMULATORY BACTERIA ENGINEERED TO COLONIZE TUMORS, TUMOR-RESIDENT

IMMUNE CELLS, AND THE TUMOR MICROENVIRONMENT," to Applicant Actym Therapeutics, Inc., and inventors Christopher D. Thanos, Laura Hix Glickman, Justin Skoble, Alexandre Charles Michel Iannello, and Haixing Kehoe.

This application also is related to International Patent Application No. PCT/US2018/041713, filed on July 11, 2018 and published as WO 2019/014398 on January 17, 2019, and to co-pending U.S. Patent Application Serial No. 16/033,187, now allowed, filed on July 11, 2018 and published as U.S. Publication No. 2019/0017050 A1 on January 17, 2019, each entitled "ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF."

This application also is related to International Patent Application No. PCT/US2019/041489, filed on July 11, 2019, and published as WO 2020/014543, on January 16, 2020, entitled "ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF."

The immunostimulatory bacteria provided in each of these applications can be modified as described in this application, and such bacteria are incorporated by reference herein. Where permitted, the subject matter of each of these applications is incorporated by reference in its entirety.

**INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED ELECTRONICALLY**

An electronic version of the Sequence Listing is filed herewith, the contents of which are incorporated by reference in their entirety. The electronic file was created on August 12, 2021, is 868 kilobytes in size, and is titled 1708SEQPC0.txt.

**FIELD OF THE INVENTION**

Provided are attenuated immunostimulatory bacteria with genomes that are modified to, for example, reduce undesirable inflammatory responses and toxicity, and improve the anti-tumor activity and/or immunostimulatory activity by increasing resistance to complement inactivation, by reducing immune cell death, by promoting adaptive immunity, and by enhancing T-cell function. The increase in colonization of phagocytic cells, for anti-cancer uses improves the delivery of encoded therapeutic products to the tumor microenvironment and to tumors, and permits, among other routes, systemic administration of the immunostimulatory bacteria. For use as vaccines, by appropriate administration, the bacteria can colonize phagocytic cells,

including in tissues colonized by pathogens.

## BACKGROUND

The field of cancer immunotherapy has made great strides, as evidenced by the clinical successes of anti-CTLA-4, anti-PD-1 and anti-PD-L1 immune checkpoint antibodies (see, *e.g.*, Buchbinder *et al.* (2015) *J. Clin. Invest.* 125:3377-3383; Hodi *et al.* (2010) *N. Engl. J. Med.* 363(8):711-723; and Chen *et al.* (2015) *J. Clin. Invest.* 125:3384-3391). Tumors have evolved a profoundly immunosuppressive environment. They initiate multiple mechanisms to evade immune surveillance, reprogram anti-tumor immune cells to suppress immunity, and continually mutate resistance to the latest cancer therapies (see, *e.g.*, Mahoney *et al.* (2015) *Nat. Rev. Drug Discov.* 14(8):561-584). Designing immunotherapies and cancer therapies that overcome immune tolerance and escape, while limiting the autoimmune-related toxicities of current immunotherapies, challenges the field of immuno-oncology. Hence, additional and innovative immunotherapies and other therapies are needed.

## SUMMARY

Provided herein are immunostimulatory bacteria that are cancer therapeutics by virtue of their ability to effectively colonize tumors, particularly tumor resident immune cells, and by virtue of the encoded payloads that result in an anti-tumor immune response. Because of their ability to colonize tumors and the tumor microenvironment, immunostimulatory bacteria provided herein can be used to treat immune desert (immune-excluded) tumors, which have scarce or absent T-cell infiltration in the tumor microenvironment and tumors. These include tumors with stromal barriers. The immunostimulatory bacteria provided herein can turn so-called "cold" tumors, which are resistant to or non-responsive to immunotherapy into "hot" tumors.

These immunostimulatory bacteria include genome modifications described herein, such that they are TLR2/4/5 attenuated, such as by virtue of elimination of flagella, the *msbB*$^-$/*pagP*$^-$ phenotype, as well as additional mutations, such as the elimination of curli fimbriae, and also other mutations, such as the *ansB*$^-$ phenotype. These bacteria can proliferate *in vivo*. The payloads encoded in the plasmid(s) in the bacteria are those that are part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN). In particular, these products include gain-of-function mutations to render expression of type I IFN constitutive. These immunostimulatory bacteria also can encode a cytokine or cytokines, such as an IL-15,

particularly as an IL-15/IL-15R alpha chain complex, and can encode tumor-associated antigens and/or bi-specific T-cell engager antibodies. For cancer therapy the bacteria can be administered systemically.

Also provided herein are immunostimulatory bacteria that can be used and/or formulated as vaccines for administration into tissues, such as by intramuscular injection, inhalation, and other such direct routes. These bacteria are designed to be non-replicating *in vivo*, and, thus, comprise a nutritional auxotrophy, such as a *thyA*$^-$ so that they do not express active thymidylate synthase, and they encode the payload under control of a promoter recognized in the bacterium. If they are intended to deliver protein payloads to a vacciniated host, the encoded payloads include sequences or are designed so that they are translated in the bacterial host. If they are intended to deliver RNA, then the encoding nucleic acids are designed so that the bacterial ribosomes cannot translate them, but so that eukaryotic ribosomes can translate them. This can be effected, for example, by include an IRES in the encoding nucleic acid. The payloads of the vaccines include nucleic acid encoding the immunizing antigen or protein, such as an antigen from a viral or bacterial pathogen. Payloads also can include immunostimulatory proteins, such as a product, such as STING, particularly modified STING, that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), and also, optionally, a cytokine, such as an IL-15, such as IL-15/IL-15R alpha chain complex. The vaccines are formulated for a suitable route of administration, and include aerosols and emulsions, tablets, and powders.

Provided are immunostimulatory bacteria that contain genome modifications and a plasmid that encodes one or more therapeutic products, such as anti-cancer therapeutics or associated treatments. The genome modifications result in immunostimulatory bacteria that accumulate in the tumor microenvironment and in tumor-resident immune cells, where they express the encoded therapeutic products. The immunostimulatory bacteria provided herein encoded one or a plurality of complementary products that stimulate or induce or result in a robust anti-cancer response in the subject.

Because of the similarity in the immune response between an anti-tumor response and an anti-viral response, immunostimulatory bacteria provided herein also can be used to treat infectious diseases. The immunostimulatory bacteria can encode an anti-viral or anti-bacterial therapeutic, such as an inhibitor of a viral or bacterial

product, or an inhibitor of the expression of a viral or bacterial product, or a viral or bacterial antigen. The combination of the immune response from the immunostimulatory bacteria and the therapeutic anti-pathogen product, and also to the immunostimulatory proteins and other such therapeutics, provides a therapeutic immunostimulatory bacterium for vaccinating against and/or for treating infectious diseases, particularly diseases associated with viral infections, such as chronic viral infections and latent viral infections. Of interest are chronic viral infections, such as infections by hepatitis viruses, herpes viruses, varicella zoster virus (VZV), Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), human T-cell leukemia virus (HTLV), Respiratory Syncytial Virus (RSV), measles virus, and other such viruses that chronically infect subjects. The immunostimulatory bacteria also can be used for treatment of acute infections as well, such as initial infections with chronic influenza, *P. gingivalis*, and coronaviruses, such as Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19). Targeted pathogenic bacteria also include, for example, species of *Escherichia*, *Staphylococcus*, *Pseudomonas*, and *Porphyromonas*.

The immunostimulatory bacteria provided herein can encode an antigen or antigens from a pathogen, such as a viral antigen, and are used as a vaccine to prevent infection, or to treat existing infections. Antigens include, but are not limited to, any that are known to those of skill in the art to elicit an immmunoprotective response or to ameliorate a disease resulting from the pathogen. These immunostimulatory bacteria, by virtue of the ability to accumulate in immune cells, such as antigen-presenting cells, can prime the T-cell response to a pathogen, such as a virus. For example, as described in detail herein, among the immunostimulatory bacteria provided herein are those that are deficient in asparaginase II, an enzyme that suppresses the function of T-cells. Any of the immunostimulatory bacteria described and provided herein can be used. For example, it is described and shown herein, that eliminating asparaginase II activity, such as by modifying the bacterial genome to eliminate expression of active enzyme, can be used to encode an antigen or combination of antigens. The resulting bacteria promote an anti-pathogen, such as anti-viral, T-cell response. The combination of expression of an antigen, such as from

a pathogen, bacterial or viral or other, with the ability to accumulate in immune cells, such as antigen-presenting cells, provides protection from infection by the pathogen. For example, the immunostimulatory bacteria can encode a viral antigen, such as an antigen from an essential viral core protein shared among a family of viruses or across viral families. For example, in the case of a coronavirus, such as SARS-COV2, an antigen from the nucleocapsid and/or non-structural M proteins that can enhance $CD8^+$ T-cell responses to heavily conserved and less mutated core proteins, thereby providing broad pan-coronavirus protection, to provide effective vaccines and treatments. Proteins and antigens from this corona virus and corona virus family that are used for immunization and/or treatment are known, and exemplary ones are described herein and known to those of skill in the art. In addition to spike proteins, portions thereof, and modified spike proteins, others proteins have been identified for this purpose. See, *e.g.*, Cohen *et al.*, (2021) *Cell Reports Medicine 2*:1000354.

The immunostimulatory bacteria can encode an anti-viral therapeutic or anti-bacterial therapeutic. Such therapeutics include inhibitors of viral genes and proteins, such as proteins required for replication and/or packaging, or the immunostimulatory bacteria can encode a therapeutic that prevents binding or interaction of a virus with a receptor or receptors that facilitate or provide for viral entry into a target cell. In some embodiments, expression of the encoded therapeutic protein, such as the antigen or antigenic protein, can be under the control of a prokaryotic promoter. In other embodiments, the protein can be expressed under control of a eukaryotic promoter. The choice of promoter depends upon whether it is to be expressed in the bacterium, such as before administration as described herein for delivery of mRNA that is translated in the host, or the protein is to be expressed in the host cells, such as immune cells, after delivery.

The immunostimulatory bacteria provided herein include genome modifications, such as deletions, disruptions, and other alterations that result in inactive encoded product, such as changing the orientation of all or part of the gene, so that functional gene products are not expressed. Among the immunostimulatory bacteria provided are those that are modified so that the resulting bacteria are *msbB*$^-$/*purI*$^-$. In some embodiments, the bacteria are *msbB*$^-$ and *purI*$^-$, whereby the full length of at least the coding portion of the *msbB* and/or *purI* genes are/is deleted. The

genome of the bacteria also can be modified so that the bacteria lack flagella. This is effected in bacteria that normally express flagella. In such bacteria, for example, the *fliC* and *fljB* genes in *Salmonella*, or equivalent genes in other species to *fliC* and *fljB*, can be deleted or otherwise modified so that functional gene product is not expressed. The bacteria also can be modified so that they are adenosine auxotrophs, and/or are *msbB*$^-$/*pagP*$^-$. Also provided are immunostimulatory bacteria and pharmaceutical compositions containing them, where the bacteria do not express L-asparaginase II, whereby the bacteria are *ansB*-. Elimination of the encoded asparaginase activity improves or retains T-cell viability/activity. Therapeutic bacteria, such as inactivated or attenuated bacteria that are used as vaccines, can be improved by modifying the bacterial genome to eliminate asparaginase activity. Exemplary of such vaccines is the BCG (Bacillus Calmette–Guérin) vaccine and related vaccines, which are used to immunize against tuberculosis. The BCG vaccine is known to have variable effectiveness; eliminating the asparaginase can improve the effectiveness of such vaccine, because the endogenous bacterial asparaginase inhibits or reduces T-cell activity.

Provided herein are immunostimulatory bacteria that contain a plasmid encoding a therapeutic product, or combinations of therapeutic products, under control of a eukaryotic promoter. The genomes of the bacteria can contain modifications, such as one, two, or more modifications, selected from among:

a) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has been modified to generate penta-acylated lipopolysaccharide (LPS), wherein:

the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has been modified to generate penta-acylated lipopolysaccharide; and

hexa-acylated lipopolysaccharide is substantially reduced, by at least 10-fold, compared to the wild-type bacterium, or is absent;

b) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has attenuated recognition by Toll-like Receptors (TLR) 2, TLR4, and/or TLR5;

c) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium does not activate the synthesis of curli fimbriae and/or cellulose;

d) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium does not activate the synthesis of secreted asparaginase;

e) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium is auxotrophic for purines, for adenosine, and/or for ATP;

f) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium lacks flagella;

g) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has been modified to specifically infect tumor-resident myeloid cells;

h) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has been modified to specifically infect tumor-resident myeloid cells, and is unable to replicate in tumor-resident myeloid cells; and

i) deletion or disruption of either or both of *lppA* and *lppB*, to decrease or eliminate lipoprotein expression in the membrane, whereby expression of an encoded therapeutic protein is increased in the tumor microenvironment and/or in tumor-resident immune cells.

For example, the immunostimulatory bacteria contain modifications, including deletions, insertions, and replacements, of a), d), and f), or modifications c) and d), or modifications a), c), d), e), and f), or modifications a), c), d), e), f), and i), or modifications a), d), f), and i), or modifications c), d), and i), or modifications f) and i), or modifications a)-i), or modifications a), b), d), and f), or modifications a), b), c), and d), and other combinations of modifications a)-i). Deletion or disruption includes any modification of a gene whereby active gene product is not expressed.

In particular, provided are immunostimulatory bacteria whose genomes are modified by deletion or disruption, including by insertion, of all or of a sufficient portion of a gene or genes, whereby the bacteria have attenuated recognition by TLR2, TLR4, and TLR5. Such bacteria have low toxicity and accumulate in/colonize the tumor microenvironment and tumor-resident myeloid cells, such as macrophages. These bacteria contain plasmids that encode therapeutic products, particularly

combinations of complementary products, such as a cytokine and a modified STING polypeptide, including gain-of-function/constitutively active STING proteins, STING chimeras, and chimeric STING proteins that include gain-of-function (GOF) mutations. The cytokines include, for example, IL-15/IL-15R alpha chain complex (also referred to herein as IL-15Rα/IL-15sc, or IL-15/IL-15Rα, or IL-15 complex), or IL-15, or IL-12, or other anti-tumor immune stimulating cytokines or chemokines. The bacteria can additionally encode other products, such as anti-tumor antibodies. Combinations of products are described and provided herein. The combinations of products that stimulate or promote an anti-tumor response and/or deliver a therapeutic product, are described throughout the disclosure herein, and they are delivered by the immunostimulatory bacteria whose genomes are modified so that the bacteria have low toxicity and effectively colonize tumors, the tumor microenvironment, and/or tumor-resident immune cells, such as macrophages. Exemplary of such bacteria are those of species, such as *Salmonella*, *Listeria*, and *E. coli*, that are modified so that they do not have flagella, and are modified so that they contain lipopolysaccharide (LPS) with penta-acylated lipid A, such as by rendering the bacteria *msbB*$^-$/*pagP*$^-$. The bacteria additionally can be modified by elimination of curli fimbriae and/or have reduced or eliminated cellulose production and biofilm formation, such as by modifying the bacteria so that they are *csgD*$^-$. It is shown herein that bacteria with these modifications have no maximum tolerated dose (MTD), and exhibit high tumor colonization.

In all embodiments, the immunostimulatory bacteria also can comprise or further comprise deletion of or disruption of the genes encoding the flagella, whereby the bacterium is flagellin$^-$ (such as *fliC*$^-$/*fljB*$^-$) and does not produce flagella, wherein the wild-type bacterium has flagella. The immunostimulatory bacteria can be auxotrophic for purines, such as auxotrophic for adenosine; or auxotrophic for adenosine, adenine, and/or ATP. The immunostimulatory bacteria also can be *purI*$^-$. The immunostimulatory bacteria also can be *pagP*$^-$. The immunostimulatory bacteria also can be aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$), such as where the bacterium is *asd*$^-$ by virtue of disruption or deletion of all or a portion of the endogenous gene encoding aspartate-semialdehyde dehydrogenase (*asd*), whereby endogenous *asd* is not expressed. The bacteria can encode aspartate-semialdehyde

dehydrogenase (*asd*) on the plasmid under control of a bacterial promoter. The immunostimulatory bacteria also can be *msbB*$^-$, or can be *pagP*$^-$/*msbB*$^-$. For example, the immunostimulatory bacteria can be *asd*$^+$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ (*fliC*$^-$/*fljB*$^-$), and *pagP*$^-$, or they can be *asd*$^+$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ (such as *fliC*$^-$/*fljB*$^-$), and *pagP*$^-$. In some embodiments, the immunostimulatory bacteria are *ansB*$^-$, *asd*$^+$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ (such as *fliC*$^-$/*fljB*$^-$), and *pagP*$^-$.

Provided are immunostimulatory bacteria that contain a plasmid encoding a therapeutic product under control of a eukaryotic promoter, or that encode a plurality of products under control of a plurality of eukaryotic promoters, or under control of a single promoter. The genome of the immunostimulatory bacteria is modified by deletion of a sufficient portion of a gene or genes, or by the disruption of a gene or genes, whereby the bacterium is one or more of *ansB*$^-$, *asd*$^+$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ (such as *fliC*$^-$/*fljB*$^-$), and *pagP*$^-$. The immunostimulatory bacteria provided herein also include those that have the genes *lppA* (*lpp1*) and/or *lppB* (*lpp2*), which encode major outer membrane lipoproteins Lpp1 (LppA) and Lpp2 (LppB), respectively, deleted or disrupted, to eliminate or substantially reduce expression of the encoded lipoprotein(s). In particular, the immunostimulatory bacteria are *lppA*$^-$ and *lppB*$^-$. Provided are immunostimulatory bacteria that contain a plasmid encoding an anti-cancer therapeutic, or an anti-pathogen therapeutic, under control of eukaryotic regulatory sequences, and that are *lppA*$^-$ and *lppB*$^-$. For example, the immunostimulatory bacteria can be *ansB*$^-$, *asd*$^+$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ (such as *fliC*$^-$/*fljB*$^-$), *pagP*$^-$, *lppA*$^-$, and/or *lppB*$^-$.

In embodiments herein, the therapeutic product is an anti-cancer therapeutic or a therapeutic used in cancer therapy. The encoded product(s) can be operably linked to nucleic acid encoding a secretion signal, whereby, when expressed, the therapeutic product is secreted, such as secreted from a tumor-resident immune cell.

Any of the immunostimulatory bacteria also can have one or more genes or operons, involved in *Salmonella* pathogenicity island 1 (SPI-1) invasion, deleted or inactivated, whereby the immunostimulatory bacteria do not invade or infect epithelial cells. For example, the one or more genes/operons are selected from among *avrA*, *hilA*, *hilD*, *invA*, *invB*, *invC*, *invE*, *invF*, *invG*, *invH*, *invI*, *invJ*, *iacP*, *iagB*,

*spaO*, *spaQ*, *spaR*, *spaS*, *orgA*, *orgB*, *orgC*, *prgH*, *prgI*, *prgJ*, *prgK*, *sicA*, *sicP*, *sipA*, *sipB*, *sipC*, *sipD*, *sirC*, *sopB*, *sopD*, *sopE*, *sopE2*, *sprB*, and *sptP*.

The plasmid in the immunostimulatory bacteria can be present in low copy number or medium copy number. The plasmid can contain a medium-to-low copy number origin of replication, such as a low copy number origin of replication. In some embodiments, the plasmid is present in higher copy number. Generally, medium copy number is less than 150 or less than about 150, and more than 20 or about 20, or is between 20 or 25 and 150; and low copy number is less than 25, or less than 20, or less than about 25, or less than about 20 copies. In particular, low to medium copy number is less than about 150 copies, or less than 150 copies; low copy number is less than about 25 copies, or less than 25 copies.

Encoded therapeutic products include nucleic acids and proteins. The plasmid can encode two or more therapeutic products. Exemplary products include, but are not limited to, a cytokine, a protein that constitutively induces a type I IFN, and a co-stimulatory receptor or ligand. Further exemplary combinations are described below. In some embodiments, the co-stimulatory molecule lacks all or a portion of the cytoplasmic domain for expression on an antigen-presenting cell (APC), whereby the truncated molecule is capable of constitutive immuno-stimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the antigen-presenting cell (APC), due to the deleted cytoplasmic domain or deleted portion thereof.

The encoded therapeutic products can be operatively linked to nucleic acid encoding regulatory sequences recognized by a eukaryotic host, such as, for example, secretion signals, to effect secretion from a cell comprising the bacterium or plasmid. In embodiments where the immunostimulatory bacteria encode two or more products, expression of each product can be under control of a separate promoter. Alternatively, two or more products can be expressed under control of a single promoter, and each product is separated by nucleic acid encoding, for example, an internal ribosomal entry site (IRES), or a 2A peptide, to effect separate expression of each encoded therapeutic product. Exemplary 2A peptides are T2A, F2A, E2A, or P2A, which can flank nucleic acids encoding the therapeutic products, to effect separate expression of the therapeutic products expressed under control of a single promoter. The therapeutic

products are expressed under control of a eukaryotic promoter, such as an RNA polymerase (RNAP) II promoter, or an RNA polymerase III promoter. These include an RNA polymerase II promoter that is a viral promoter, or a mammalian RNA polymerase II promoter, such as, but not limited to, as a cytomegalovirus (CMV) promoter, an SV40 promoter, an Epstein-Barr virus (EBV) promoter, a herpes virus promoter, an adenovirus promoter, an elongation factor-1 (EF-1) alpha promoter, a UBC promoter, a PGK promoter, a CAGG promoter, an adenovirus 2 or 5 late promoter, an EIF4A1 promoter, a CAG promoter, or a CD68 promoter. The plasmids further can include other eukaryotic regulatory sequences, such as terminators and/or promoters, selected from among SV40, human growth hormone (hGH), bovine growth hormone (bGH), MND (a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer), chicken beta-globulin, and rbGlob (rabbit globulin) genes, to control expression of the therapeutic product(s). Other regulatory sequences include a polyA tail, a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE), and a Hepatitis B virus Posttranscriptional Regulatory Element (HPRE).

The encoded therapeutic products include any described herein and in the original claims, such as nucleic acid encoding a protein that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), or a variant thereof. Type I IFNs include interferon-α and interferon-β. Variants include those that, when expressed in a subject, lead to constitutive expression of type I IFN. These include a gain-of-function (GOF) variant that does not require cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides (CDNs) to result in expression of type I IFN. Exemplary of these proteins is a protein selected from among STING, RIG-I, MDA-5, IRF-3, IRF-5, IRF-7, IRF-8, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200, and variants thereof that have increased activity, or that result in constitutive expression of type I interferon (IFN). Variants include a variant of STING, RIG-I, IRF-3, or MDA5, in which one or more serine (S) or threonine (T) residue(s) that is/are phosphorylated as a consequence of viral infection, is/are replaced with an aspartic acid (D) residue, whereby the resulting variant is a phosphomimetic that constitutively induces type I IFN, and any known to

those of skill in the art and/or described herein. Variants include, for example, those wherein the mutations are selected as follows: a) in STING, with reference to SEQ ID NOs: 305-309, one or more selected from among: S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E, S102P/F279L, F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R197A, D205A, R310A, R293A, T294A, E296A, R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q, S272A, R293A/T294A/E296A, D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, S358A, E360A, S366A, R238A, R375A, N154S/R284G, and S324A/S326A; b) in MDA5, with reference to SEQ ID NO:310, one or more of: T331I, T331R, A489T, R822Q, G821S, A946T, R337G, D393V, G495R, R720Q, R779H, R779C, L372F, and A452T; c) in RIG-I, with reference to SEQ ID NO:311, one or both of E373A and C268F; and d) in IRF-3, with reference to SEQ ID NO:312, S396D, such as a variant STING that contains one or more amino replacement(s) selected, with reference to SEQ ID NOs: 305-309, from among: S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E, S102P/F279L, F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R197A, D205A, R310A, R293A, T294A, E296A, R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q, S272A, R293A/T294A/E296A, D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, S358A, E360A, S366A, R238A, R375A, N154S/R284G, and S324A/S326A, and conservative replacements thereof, and combinations thereof.

The immunostimulatory bacteria also can encode an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment. These include, but are not limited to, a cytokine, a chemokine, or a co-stimulatory molecule. Exemplary of these is a protein selected from among one or more of: IL-2, IL-7, IL-12p70 (IL-12p40 + IL-12p35), IL-15, IL-36 gamma, IL-2 that has attenuated binding to IL-2Ra, IL-15/IL-15R alpha chain complex, IL-18, IL-21, IL-23, IL-2 that is modified so that it does not bind to IL-2Ra, CXCL9, CXCL10, CXCL11, interferon-α, interferon-β, interferon-γ, CCL3, CCL4, CCL5, proteins that are involved in or that effect or potentiate the recruitment and/or persistence of T cells, CD40, CD40 ligand (CD40L), CD28, OX40, OX40 ligand (OX40L), 4-1BB, 4-1BB ligand (4-1BBL), members of the B7-CD28 family, CD47 antagonists, an anti-

IL-6 antibody or an IL-6 binding decoy receptor, TGF-beta polypeptide antagonists, and members of the tumor necrosis factor receptor (TNFR) superfamily. The co-stimulatory molecule, selected from among CD40, CD40 ligand, CD28, OX40, OX40 ligand, 4-1BB, and 4-1BB ligand, can be truncated, such that the molecule lacks a cytoplasmic domain, or a portion thereof, for expression on an antigen-presenting cell (APC); and the truncated gene product is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the antigen-presenting cell (APC), due to the deleted, or partially deleted, or truncated cytoplasmic domain, which eliminates the immunosuppressive reverse signaling. Other such proteins are TGF-beta polypeptide antagonists, such as an anti-TGF-beta antibody or a fragment thereof, an anti-TGF-beta receptor antibody or a fragment thereof, a soluble TGF-beta antagonist polypeptide, or a TGF-beta binding decoy receptor.

The plasmids can encode a therapeutic antibody or antigen-binding fragment thereof, such as, for example, a Fab, Fab', $F(ab')_2$, single-chain Fv (scFv), Fv, dsFv, nanobody, diabody fragment, or a single-chain antibody. Examples include, but are not limited to, an antagonist of PD-1, PD-L1, CTLA-4, VEGF, VEGFR2, or IL-6.

The plasmids can encode complementary products whose expression results in enhanced anti-tumor or other activity. For example, the combination of a modified, such as a constitutively active and/or chimeric STING protein described herein, with a cytokine, such as IL-15/IL-15R alpha chain complex (IL-15Rα-IL-15sc), has synergistic activity.

As described herein, the immunostimulatory bacteria provided herein can be used as a vaccine by encoding an antigen against which an immune response, or immunization, or immunoprotection is desired. The immunostimulatory bacteria herein can be used to deliver RNA, such as mRNA or other forms, for use as a vaccine or for delivery of a therapeutic. As described herein, the bacteria contain a plasmid that encodes a product of interest, such as a therapeutic product, such as an antigen from a pathogen, under control of a bacterial or other prokaryotic promoter recognized by the bacterium. The encoding nucleic acid cassette includes a regulatory sequence or other sequence that blocks or inhibits or prevents translation by bacterial ribosomes, but that permits, or provides for, or enhances translation by eukaryotic,

such as those present in human cells, ribosomes. The bacteria are modified so that they cannot grow or replicate in eukaryotes, such as by rendering the bacteria *asd*$^-$ which require DAP for growth *in vitro*, or *ThyA*$^-$, which requires thymidine monophosphate precursors, for growth, but can be cultured *in vitro* so that they produce the encoded RNA. A skilled person can inactivate the gene or product by modifying the endogenous gene, such as by deletions, insertions, replacements, transpositions, or any such modification so that active enzyme is not produced. See, SEQ ID NO: 464 for an exemplary *ThyA* gene from *Salmonella*, and SEQ ID NO:465 for the encoded protein. RNA, encoding protein and/or antigen for immunization, is encoded in the plasmid, but the encoding nucleic acid includes translation signals/sequences so that bacteria cannot translate the this RNA. The resulting bacteria deliver the encoded RNA into the host phagocytic cells, where it is translated by host cell ribosomes. Immunostimulatory bacteria that are *ThyA*$^-$ have genome modifications, such insertions, deletions, replacements, or other changes, that result in inactive or eliminate production of thymidylate synthase, which catalyzes the reductive methylation of dUMP to dTMP, a DNA biosynthesis precursor (precursor to dTTP).

Δ*ThyA* Auxotrophies for other nutrients and essential products can be introduced in place of or in addition to the asd inactivation/deletion. Other deletions or inactivation of genes or gene products required for growth, such as genes that produce nutrients, can be used in place of or in addition to the asd, and include for example *thyA* (see, *e.g.*, Loessner *et al.* (2006) *FEBS Lett 265*:81-88). Elimination of expression or production or other attenuating mutations of the bacterial genome for production of such products results in release of encoded macromolecules upon bacterial cell death *in vivo* after administration. Asd is is an essential enzyme for bacterial cell wall synthesis; ThyA is an enzyme needed for DNA synthesis. Mutation of the respective genes renders the strain auxotrophic for diaminopimelic acid (DAP) or thymidine monophosphate precursors. Upon deprivation of the complementing substrates, such bacteria die by DAP-less or thymineless death, resulting in release of bacterial proteins and plasmid. Inactivation or elimination of Asd, results in release of macromolecules; elimination or inactivation of ThyA (to produce Δ*ThyA* bacteria) expression/activity does not result in release of macromolecules, including proteins

and plasmids, upon thymidine starvation (Leossner *et al.* (2006) *FEBS Lett 265*:81-88). Thus, Δ*ThyA* are advantageous for *in vivo* delivery of plasmids to host cells, since the bacteria will not prematurely release their contents. Since the bacteria provided here infect or accumulate in myeloid cells, such as phagocytic cells, such as macrophate, dendritic cells, monocytes and neutrophils, which consume bacteria, the intact Δ*ThyA* bacteria, release the plasmid encoding the therapeutic product inside the targeted cells.

The bacteria are genome-modified so that they are attenuated, such as the bacteria herein, where the response by toll-like receptors (TLR) 2, 4, and 5 is reduced, compared to bacteria without such genome modifications, and optionally, encode *rck* Rck (resistance to complement killing) to reduce inactivation by complement, and include modifications, as needed, so that they infect primarily or only phagocytic cells, such as tissue-resident macrophages. It is shown herein that genome modifications, such the combination of modifications that reduce responses by TLRs 2,4,5, are necessary for the production of type I IFN by human antigen-presenting cells.

Provided are bacteria that contain genome modifications, whereby the response by toll-like receptors (TLRs) 2, 4, and 5 is reduced, compared to the bacteria without the genome modifications. Such modifications include those that result in penta-acylated LPS and elimination of flagella, such as the *pagP*$^-$/*msbB*$^-$ bacteria that lack flagella, and also those that are deficient or do not produce or express asparaginase II, such as those that are Δ*asn*. The bacteria also can comprise further genomic modifications such as one or more modifications whereby they are auxotrophic for a required nutrient or for a factor, so that they are unable to replicate in a eukaryotic host, but can replicate *in vitro* when supplied with the nutrient or factor, such auxotrophic for thymidine (Δ*ThyA*), such as by genome modifications that render them unable to produce or express thymidylate synthase (Δ*ThyA*), or Asd.

The bacteria that are provided herein that combine some or all of these traits are used to express therapeutic products, including anti-cancer products, and antigens, depending upon their intended use. For administration to subjects with cancer, the bacteria, which accumulate in tumor-resident myeloid cells, encode anti-cancer therapeutics, such as products that result in stimulation of an immune response, and/or

that result in inhibiting immunosuppression, or that encode a product that treats the tumor, and those that encode a combination of products that can act synergistically to treat cancer. The bacteria provided herein that accumulate in or infect phagocytic cells, also can be used for subjects that do not have cancer, such as, as vaccines by delivering or encoding an antigen, or delivering RNA. The various embodiments and combinations of properties and products, and uses, are described throughout the disclosure herein.

In some embodiments, the bacteria comprise a plasmid containing nucleic acid encoding a product, or comprise RNA encoding the product, where the product encoded by the nucleic acid or RNA is an antigenic sequence or sequences from a pathogenic virus, bacterium, parasite, or is a tumor antigen, whereby, upon expression of the encoded antigen in the host, the host develops an immune-protective response or immunizing response against the pathogenic virus, bacterium, parasite, or tumor antigen, or the encoded product is a therapeutic product; expression of the antigenic sequence(s) is/are under control of a prokaryotic promoter so that RNA encoding the antigen(s) is produced in the bacteria; nucleic acid encoding the antigen comprises regulatory sequences that inhibit or prevent translation of encoded RNA by bacterial ribosomes, but that does not inhibit or prevent translation of the encoded RNA by eukaryotic host ribosomes, whereby translation is de-coupled from transcription in the bacteria; the resulting bacteria are selective for infecting phagocytic cells when administered to a eukaryotic subject, and deliver the nucleic acid into the phagocytic cells, wherein the RNA is translated.

The bacteria, which are cultured *in vitro* to produce the RNA, upon administration, infect the phagocytes and deliver their contents, but they are not viable and/or do not replicate, thereby providing the RNA, such as mRNA, to the host cells, which translate the RNA to produce the encoded product, such as an immunogenic protein or antigen. The RNA generally is mRNA, and also can be other forms of RNA, such as RNAi, or eRNA (circular RNA), and other therapeutic forms. The immunostimulatory bacteria that are used for this purpose can include the plasmids, which encode the RNA, in high or higher (generally 150 or greater) copy numbers, to increase the amount of RNA delivered. Various embodiments are described, claimed, and exemplified herein. The mRNA can encode pathogen proteins, pathogen antigens,

tumor-antigens, therapeutic products for treatment of tumors or infections, and combinations thereof. The mRNA can be synthetic, such as those designed for immunization (see, *e.g.*, US patent publication 20190351040, and others that describe mRNA for immunization or treatment). The resulting bacteria are vaccines for therapy or immunization. The payloads can include products that are adjuvants, that are immunostimulatory protein, that induce type I interferon (IFN) to activate T-cells in concert with the immunizing antigen/protein.

In some embodiments, the immunostimulatory bacteria provided herein contain a plasmid that encodes two or more therapeutic proteins selected from among: a) an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment; b) one or more of a protein that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), or a variant thereof that has increased activity to increase expression of type I IFN, or a variant thereof that results in constitutive expression of a type I IFN; and c) an anti-cancer antibody or antigen-binding portion thereof. For example, the immunostimulatory protein can be a co-stimulatory molecule that is one that lacks a cytoplasmic domain or a sufficient portion thereof, for expression on an antigen-presenting cell (APC), whereby the truncated co-stimulatory molecule is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the antigen presenting cell (APC). In some embodiments, the immunostimulatory bacteria encode at least two therapeutic products, selected from among a cytokine, a protein that constitutively induces a type I IFN, a co-stimulatory molecule, and an anti-cancer antibody or antigen-binding portion thereof, which can be under control of a single promoter. For example, expression of the nucleic acid encoding at least two or all of the products is under control of a single promoter, and the nucleic acid encoding each product is separated by nucleic acid encoding 2A polypeptides, whereby, upon translation, each product is separately expressed. The nucleic acid encoding each product can be operatively linked to nucleic acid encoding a sequence that directs secretion of the expressed product from a cell.

Provided are immunostimulatory bacteria that encode two or more therapeutic products, wherein at least one product is selected from a), and at least one is selected

from b), and a) is IL-2, IL-7, IL-12p70 (IL-12p40 + IL-12p35), IL-15, IL-23, IL-36 gamma, IL-2 that has attenuated binding to IL-2Ra, IL-15/IL-15R alpha chain complex (also referred to herein as IL-15/IL-15Rα, IL-15 complex, or other variations), IL-18, IL-2 that is modified so that it does not bind to IL-2Ra, CXCL9, CXCL10, CXCL11, interferon-α, interferon-β, CCL3, CCL4, CCL5, proteins that are involved in or that effect or potentiate the recruitment and/or persistence of T cells, CD40, CD40 Ligand (CD40L), OX40, OX40 Ligand (OX40L), 4-1BB, 4-1BB Ligand (4-1BBL), members of the B7-CD28 family, TGF-beta polypeptide antagonists, or members of the tumor necrosis factor receptor (TNFR) superfamily; and b) is STING, RIG-I, MDA-5, IRF-3, IRF-5, IRF-7, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200. They also can encode one or more of a TGF-beta inhibitory antibody, a TGF-beta binding decoy receptor, an anti-IL6 antibody, or an IL-6 binding decoy receptor.

Exemplary of combinations of encoded therapeutic products are any of the following combinations of therapeutic products: IL-2 and IL-12p70; IL-2 and IL-21; IL-2, IL-12p70, and a STING GOF variant; IL-2, IL-21, and a STING GOF variant; IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt), where Δcyt is a deleted cytoplasmic domain; IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-15/IL-15Rα, and a STING GOF variant; IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-15/IL-15Rα and IL-12p70; IL-15/IL-15Rα and IL-21; IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; IL-15/IL-15Rα, IL-21, and a STING GOF variant; IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-12p70 and IL-21; IL-12p70, IL-21, and a STING GOF variant; IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-12p70 and a STING GOF variant; IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-12p70 and IL-18; IL-12p70, IL-18, and a STING GOF variant; IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-2, and IL-12p70; a TGF-β decoy receptor, IL-2, and IL-21; a TGF-β decoy receptor, IL-2, IL-12p70, and a STING GOF variant; a TGF-β decoy receptor, IL-2, IL-21, and a

STING GOF variant; a TGF-β decoy receptor, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-15/IL-15Rα, and a STING GOF variant; a TGF-β decoy receptor, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-12p70; a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-21; a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, and a STING GOF variant; a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, a STING GOF variant and, 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-12p70, and IL-21; a TGF-β decoy receptor, IL-12p70, IL-21, and a STING GOF variant; a TGF-β decoy receptor, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor and IL-12p70; a TGF-β decoy receptor, IL-12p70, and a STING GOF variant; a TGF-β decoy receptor, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-12p70, and IL-18; a TGF-β decoy receptor, IL-12p70, IL-18, and a STING GOF variant; a TGF-β decoy receptor, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor and a STING GOF variant; an anti-CTLA-4 antibody, IL-2, and IL-12p70; an anti-CTLA-4 antibody, IL-2, and IL-21; an anti-CTLA-4 antibody, IL-2, IL-12p70, and a STING GOF variant; an anti-CTLA-4 antibody, IL-2, IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-15/IL-15Rα, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-12p70; an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-21; an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-

1BBLΔcyt); an anti-CTLA-4 antibody, IL-12p70, and IL-21; an anti-CTLA-4 antibody, IL-12p70, IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody and IL-12p70; an anti-CTLA-4 antibody, IL-12p70, and a STING GOF variant; an anti-CTLA-4 antibody, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-12p70, and IL-18; an anti-CTLA-4 antibody, IL-12p70, IL-18, and a STING GOF variant; an anti-CTLA-4 antibody, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody and a STING GOF variant; a CD40 agonist, IL-2, and IL-12p70; a CD40 agonist, IL-2, and IL-21; a CD40 agonist, IL-2, IL-12p70, and a STING GOF variant; a CD40 agonist, IL-2, IL-21, and a STING GOF variant; a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-15/IL-15Rα, and a STING GOF variant; a CD40 agonist, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-15/IL-15Rα, and IL-12p70; a CD40 agonist, IL-15/IL-15Rα, and IL-21; a CD40 agonist, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; a CD40 agonist, IL-15/IL-15Rα, IL-21, and a STING GOF variant; a CD40 agonist, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-12p70, and IL-21; a CD40 agonist, IL-12p70, IL-21, and a STING GOF variant; a CD40 agonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist and IL-12p70; a CD40 agonist, IL-12p70, and a STING GOF variant; a CD40 agonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-12p70, and IL-18; a CD40 agonist, IL-12p70, IL-18, and a STING GOF variant; a CD40 agonist, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a CD40 agonist and a STING GOF variant.

Other combinations of products include, for example, IL-15 and a STING gain-of-function variant, including STING chimeras with a gain-of-function mutation or mutations, as provided herein, or IL-15Rα-IL-15sc and a STING gain-of-function variant, including STING chimeras with a gain-of-function mutation or mutations.

Other products or combinations thereof include a bi-specific T-cell engager (BiTe®); a BiTe® and a STING protein, such as a modified GOF STING protein or a STING chimera, as described herein; a BiTe® and IL-15; a BiTe® and IL-15Rα-IL-15sc; a BiTe®, IL-15 and a STING protein, such as a modified GOF STING protein or chimeric STING protein; and a BiTe®, IL-15Rα-IL-15sc, and a STING protein, such as a modified GOF STING protein or chimeric STING protein, where the BiTe® targets include, for example, DLL3, EGFR, Her2, CEA, Mesothelin, PSMA, EpCAM, CD74, Folate Receptor, Nectin4, EphA2, CA-IX, B7H3, Siglec-15, Muc1, Lewis Y antigen, and other such tumor antigens/ tumor targets.

Also provided are therapeutic compositions containing a tumor antigen(s) and a STING gain-of-function variant or STING chimera; a therapeutic composition of a tumor antigen(s) and IL-15; a therapeutic composition of a tumor antigen(s) and IL-15Rα-IL-15sc; a therapeutic composition of a tumor antigen(s), IL-15, and a STING gain-of-function variant or STING chimera; and a therapeutic composition of a tumor antigen(s), IL-15Rα-IL-15sc, and a STING gain-of-function variant or STING chimera. These products can be encoded in the immunostimulatory bacteria. The tumor antigens can be any listed or described herein (for example, in Example 35), or known in the art.

Combinations of products also include combinations of antigens and immune stimulating proteins. The antigens can be tumor antigens, or they can be immunizing antigens, such as pathogenic antigens, where the pathogens include, for example, bacteria, protozoans, viruses, and prions, and other prion-like particles that cause diseases and disorders. The antigens include any described or listed herein, or known in the art. The combinations include, for example, combinations of one or more antigens and IFNα2; one or more antigens and IFN-β; one or more antigens, IFNα2, and IFN-β; one or more antigens and an IRF3 GOF variant with the mutation S396D; and one or more antigens, IFNα2, and an IRF3 GOF variant with the mutation S396D.

Other products and combinations of products that are encoded in the immunostimulatory bacteria provided herein, include, but are not limited to, the combination of IFNα2 and an IRF3 GOF variant with the mutation S396D; IFNα2 and IFN-β; FLT-3L (FMS-like tyrosine kinase 3 ligand; see, *e.g.*, SEQ ID NO:436); sialidase (see, *e.g.*, SEQ ID NO:435); the IL-12 p35 subunit of IL-12p70 only;

Azurin; a membrane anchored/tethered cytokine or molecule, such as, for example, IL-2, IL-12, IL-12p35, IL-21, IL-15, IL-15Rα-IL-15sc, or FLT-3L; or a TLR8 agonist, such as, for example, where the TL38 agonist is polyU or polyU/G, a microRNA, or is miR-21.

Also provided are modified non-human Stimulator of Interferon Genes (STING) proteins, and STING protein chimeras, as well as delivery vehicles, including any described herein, pharmaceutical compositions, cells encoding or containing these STING proteins, and uses thereof, and methods of treatment of cancers. In particular, the immunostimulatory bacteria provided herein encode the modified non-human STING proteins, non-human STING proteins, and STING chimeras, as described herein. These STING proteins that are encoded by the immunostimulatory bacteria are provided herein and described throughout. Provided herein are:

1. Modified non-human STING proteins, where the non-human STING protein is one that has lower NF-κB activation than the human STING protein, and, optionally, higher type I interferon activation activity compared to the wild-type (WT) human STING protein. These non-human STING proteins are modified to include a mutation or mutations so that they have increased activity, or act constitutively, in the absence of cytosolic nucleic acid signaling. The mutations are typically amino acid mutations that occur in interferonopathies in humans, such as those described above for human STING. The corresponding mutations are introduced into the non-human species STING proteins, where corresponding amino acid residues are identified by alignment. Also, in some embodiments, the TRAF6 binding site in the C-terminal tail (CTT) of the STING protein is deleted, reducing NF-κB signaling activity.

Provided are modified STING proteins, particularly human STING proteins, that are chimeras, in which the CTT (C-terminal tail) region in the STING protein from one species, such as human, is replaced with the CTT from a STING protein of another species that has lower NF-κB signaling activity and/or higher type I IFN signaling activity than human STING. Also, the TRAF6 binding site is optionally deleted in these chimeras.

The modified STING proteins also include the mutations as set forth throughout the disclosure herein.

Also provided delivery vehicles, such as immunostimulatory bacteria, and any provided herein or known to those of skill in the art, including, for example, exosomes, nanoparticles, minicells, cells, liposomes, lysosomes, oncolytic viruses, and other viral vectors, that encode the modified STING proteins of any of 1-3.

Also provided are delivery vehicles, such as immunostimulatory bacteria, any provided herein or known to those of skill in the art, including, for example, exosomes, nanoparticles, minicells, cells, liposomes, lysosomes, oncolytic viruses, and other viral vectors, that encode unmodified STING from a non-human species whose STING protein has reduced NF-κB signaling activity compared to that of human STING, and optionally, increased type I interferon stimulating/signaling activity compared to that of human STING.

Also provided are cells (non-zygotes, if human), such as cells used for cell therapy, such as T-cells and stem cells, and cells used to produce the STING proteins as described herein. Also provided are pharmaceutical compositions that contain the STING proteins, or the delivery vehicles, or the cells, or combinations thereof.

Uses and methods of treatment of cancer and vaccination against pathogens or cancer by administering any the immunostimulatory bacteria as described herein are provided.

Assays and methods to assess NF-κB activity (signaling activity), and type I interferon stimulating activity or interferon-β stimulating activity of STING are described herein, and also, are known to those of skill in the art. Methods include those described, for example, in de Oliveira Mann *et al.* (2019) *Cell Reports 27*:1165-1175, which describes, *inter alia*, the interferon-β and NF-κB signaling activities of STING proteins from various species, including human, thereby identifying STING proteins from various species that have lower NF-κB activity than human STING, and those that also have comparable or higher interferon-β activity than human STING. de Oliveira Mann *et al.* (2019) provides species alignments and identifies domains of STING in each species, including the CTT domain (see, also, the Supplemental Information for de Oliveira Mann *et al.* (2019)).

The non-human STING proteins can be, but are not limited to, STING proteins from the following species: Tasmanian devil (*Sarcophilus harrisii*; SEQ ID NO:349), marmoset (*Callithrix jacchus*; SEQ ID NO:359), cattle (*Bos taurus*; SEQ ID

NO:360), cat (*Felis catus*; SEQ ID NO:356), ostrich (*Struthio camelus australis*; SEQ ID NO:361), crested ibis (*Nipponia nippon*; SEQ ID NO:362), coelacanth (*Latimeria chalumnae*; SEQ ID NOs:363-364), boar (*Sus scrofa*; SEQ ID NO:365), bat (*Rousettus aegyptiacus*; SEQ ID NO:366), manatee (*Trichechus manatus latirostris*; SEQ ID NO:367), ghost shark (*Callorhinchus milii*; SEQ ID NO:368), and mouse (*Mus musculus*; SEQ ID NO:369). These vertebrate STING proteins readily activate immune signaling in human cells, indicating that the molecular mechanism of STING signaling is shared in vertebrates (see, de Oliveira Mann *et al.* (2019) *Cell Reports* 27:1165-1175).

It is shown herein that the immunostimulatory bacteria provided herein, by virtue of the ability to infect myeloid cells, such as tumor-resident and tissue-resident macrophages, and the ability to retain viability for at least a limited time, and/or to deliver plasmids that encode therapeutic products that result in expression of type I IFN and/or other immune-stimulating products, such as gain-of-function (GOF) variants that do not require cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides (CDNs) to result in expression of type I IFN, can convert macrophages that have the M2 phenotype into M1 or M1-like macrophages, with immunosuppressive properties reduced or eliminated, and immune-stimulating, anti-tumor or anti-viral properties enhanced or added. Provided are immunostimulatory bacteria that contain a plasmid encoding a therapeutic product, where infection of a macrophage, including human macrophages, by the bacterium, converts an M2 macrophage to an M1 phenotype or M1-like phenotype macrophage. Provided are immunostimulatory bacteria that contain a plasmid encoding a therapeutic product whose expression in a macrophage results in the conversion of, or converts, M2 macrophages, such as human M2 macrophages, to an M1 or M1-like phenotype. The immunostimulatory bacteria with such properties include any of the bacteria provided herein that contain genome modifications that result in infection of tumor-resident (in subjects with cancer), and tissue-resident myeloid cells. These genome modifications include those that result in a lack of flagella, wherein the wild-type bacterium has flagella, and others, such as those that result in bacteria that are *pagP*$^{-}$/*msbB*$^{-}$. Other modifications include those that result in the elimination of asparaginase activity, such as modifications that result in bacteria that are *ansB*$^{-}$, in the bacteria that infect

myeloid cells, which thereby enhances T-cell activities, and other modifications that alter the lipopolysaccharide (LPS). These immunostimulatory bacteria provided herein convert immunosuppressive phagocytic macrophages to immunostimulatory, phagocytic macrophages that are capable of *in situ* antigen cross-presentation to CD8+ T-cells, and of migration to lymph nodes to prime CD4+ and CD8+ T-cells.

Included are immunostimulatory bacteria that encode therapeutic products in macrophages, that facilitate or result in the conversion of, or that convert, M2 macrophages to an M1 or M1-like phenotype, which has a profile of some or all characteristics of M1 macrophage. Exemplary of the therapeutic products are those that are part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), particularly constitutive expression. This includes the gain-of-function (GOF) variants of therapeutic products that are part of the cytosolic DNA/RNA sensor pathway, and that do not require cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides (CDNs) to result in expression of type I IFN, such as the variant and non-human STING proteins, STING chimeras, and STING chimeras with gain-of-function mutations, as described and provided herein. The bacteria include any that can be modified as described herein, including the species listed herein, such as *Salmonella* species and strains.

Also provided are immunostimulatory bacteria that contain nucleic acid operatively linked to a prokaryotic promoter, where: the nucleic acid comprises RNA that lacks sequences necessary for translation by a prokaryotic cell, whereby the RNA is produced in the bacterium, but is not translated into protein. For example, the RNA lacks a Shine-Dalgarno sequence, and comprises an Internal Ribosome Entry Site (IRES) and/or a translational read-through 2A peptide. The IRES sequence prevents translation by prokaryotic ribosomes, but provides for translation by eukaryotic ribosomes. The bacteria include immunostimulatory bacteria in which the 2A peptide is one or more of T2A, P2A, E2A, or F2A, to produce discrete products from polycistronic constructs.

Also provide are immunostimulatory bacteria as described herein that can be a delivery vehicle for delivering RNA to eukaryotic cells, such as myeloid cells. These bacteria include nucleic acid operatively linked to a prokaryotic promoter, where: the nucleic acid and prokaryotic promoter generally are encoded on a plasmid, but in

some embodiments, are encoded in the bacterial genome; the nucleic acid comprises RNA that lacks sequences necessary for translation by the bacterial ribosomes, whereby the RNA is produced in the bacterium, and where: the RNA lacks a Shine-Dalgarno sequence, and comprises an Internal Ribosome Entry Sequence (IRES), or a translational read-through 2A peptide. The prokaryotic promoter, when operatively linked to nucleic acid encoding a therapeutic protein (or non-bacterial protein), can be a bacterial promoter or a phage promoter, such as a bacteriophage promoter. The RNA polymerase that recognizes the phage promoter can be encoded in the bacterial genome, or on a plasmid for expression in the bacteria. Exemplary prokaryotic promoters include any known to those of skill in the art, including, but not limited to, those that comprise all or a sufficient portion of (sufficient to initiate transcription of an operatively linked nucleic acid) the promoters whose sequences are set forth in any of SEQ ID NOs: 393-396, respectively:

attatgtcttgacatgtagtgagtgggctggtataatgcagcaag (SEQ ID NO:393),

ttatgcttgacgctgcgtaaggtttttgttataatacaccaag (SEQ ID NO:394), or

attatgtcttgacatgtagtgagtgggctggtaaatgcagcaag (SEQ ID NO:395), or

gatcccggagttcatgcgtgatgcaatgaaagtgccgttctacttcggtgggacctcactgcttatcgttgttgtcgtgattatg
gactttatggctcaagtgcaaactctgatgatgtccagtcagtatgagtctgcattgaagaaggcgaacctgaaaggctacg
gccgttaattggtcgcctgagaagttacggagagtaaaaatgaaagttcgtgcttccgtcaagaaattatgccgtaactgcaa
aatcgttaagcgtgatggtgtcatccgtgtgatttgcagtgccgagccgaagcataaacagcgccaaggctgatttttcgca
tatttttcttgcaaagttgggttgagctggctagattagccagccaatcttttgtatgtctgtacgtttccatttgagtatcctgaaa
acgggcttttcagcatggtacgtacatattaaatagtaggagtgcatagtggcccgtatagcaggcattaacattcctgatca
gaaacacgccgtgatcgcgttaacttcgatctacggtgtcggcaagacccgttctaaagccatcctggctgcagcgggtat
cgctgaaaatgttaagatcctctagatttaagaaggagatatacat (*Salmonella* rpsm promoter; SEQ ID NO:396).

These immunostimulatory bacteria comprise genomic modifications, as described herein, whereby the bacteria infect tissue-resident myeloid cells, and/or tumor-resident myeloid cells, or, in subjects that do not have tumors, in phagocytic cells, such as macrophages. The bacteria infect the cells and deliver the RNA, which is translated in the eukaryotic host cell. Exemplary of such bacteria, are those that are modified to lack flagella, such as by deletion or disruption of the genes involved in producing flagella. The bacteria are species and strains that, without the genomic modifications, have flagella.

Also provided are immunostimulatory bacteria in which an encoded therapeutic product, such as a protein, is linked to a moiety that confers an improved

pharmacological property, such as a pharmacokinetic or pharmacodynamic property, such as increased serum half-life. Hence, provided are immunostimulatory bacteria, where an encoded therapeutic product comprises an Fc domain, or a half-life extending moiety, such as human serum albumin, or a portion thereof. Half-life extension modalities or methods, include, for example, PEGylation, modification of glycosylation, sialylation, PASylation (modification with polymers of PAS amino acids that are about 100-200 residues in length), ELPylation (see, *e.g.*, Floss *et al.* (2010) *Trends Biotechnol.*28(1):37-45), HAPylation (modification with a glycine homopolymer), fusion to human serum albumin, fusion to GLK, fusion to CTP, GLP fusion, fusion to the constant fragment (Fc) domain of a human immunoglobulin (IgG), fusion to transferrin, fusion to non-structured polypeptides, such as XTEN (also referred to as rPEG, which is a genetic fusion of non-exact repeat peptide sequences, containing A, E, G, P, S, and T; see, *e.g.*, Schellenberger *et al.* (2009) *Nat. Biotechnol.* 27(12):1186-1190), and other such modifications and fusions that increase the size, increase the hydrodynamic radius, alter the charge, or target to receptors for recycling rather than clearance, and combinations of such modifications and fusions.

Also provided are immunostimulatory bacteria, where the encoded therapeutic product comprises the B7 protein transmembrane domain, or where the therapeutic product is GPI-anchored by virtue of an endogenous or added GPI anchor. The encoded therapeutic product can comprise a fusion to collagen.

The immunostimulatory bacteria in any and all embodiments can be any suitable species. Where reference is made to particular genes and gene modifications, the genes and modifications are those that correspond to the genes and modifications referenced with respect to *Salmonella*, as an exemplary species. Species and strains include, for example, a strain of *Rickettsia*, *Klebsiella*, *Bordetella*, *Neisseria*, *Aeromonas*, *Francisella*, *Corynebacterium*, *Citrobacter*, *Chlamydia*, *Haemophilus*, *Brucella*, *Mycobacterium*, *Mycoplasma*, *Legionella*, *Rhodococcus*, *Pseudomonas*, *Helicobacter*, *Vibrio*, *Bacillus*, *and Erysipelothrix.* For example, *Rickettsia rickettsiae*, *Rickettsia prowazekii*, *Rickettsia tsutsugamuchi*, *Rickettsia mooseri*, *Rickettsia sibirica*, *Bordetella bronchiseptica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Aeromonas eucrenophila*, *Aeromonas salmonicida*, *Francisella*

*tularensis*, *Corynebacterium pseudotuberculosis*, *Citrobacter freundii*, *Chlamydia pneumoniae*, *Haemophilus somnus*, *Brucella abortus*, *Mycobacterium intracellulare*, *Legionella pneumophila*, *Rhodococcus equi*, *Pseudomonas aeruginosa*, *Helicobacter mustelae*, *Vibrio cholerae*, *Bacillus subtilis*, *Erysipelothrix rhusiopathiae*, *Yersinia enterocolitica*, *Rochalimaea quintana*, and *Agrobacterium tumerfacium*.

Provided herein are genome modified bacteria, comprising genome modifications, whereby TLR2, TLR4, and TLR5 signaling is reduced compared to the bacteria without the genome modifications, wherein:

the bacteria comprise further genomic modifications whereby they are auxotrophic for a required nutrient or factor so that they are unable to replicate in a eukaryotic host, but can replicate *in vitro* when supplied with the nutrient or factor;

the bacteria comprise a plasmid containing nucleic acid, or comprise RNA encoding an antigenic sequence or sequences from a pathogenic virus, bacterium, or parasite, or encoding a tumor antigen, whereby, upon expression of the encoded antigen in the host, the host develops an immunoprotective response against the pathogenic virus, bacterium, or parasite;

expression of the antigenic sequence(s) is/are under control of a prokaryotic promoter so that RNA encoding the antigen(s) is produced in the bacteria;

nucleic acid encoding the antigen comprises regulatory sequences that inhibit or prevent translation of encoded RNA by bacterial ribosomes, but that does not inhibit or prevent translation of the encoded RNA by eukaryotic host ribosomes, whereby translation is de-coupled from transcription in the bacteria;

the resulting bacteria infect phagocytic cells when administered to a eukaryotic subject, and deliver the nucleic acid into the phagocytic cells, wherein the RNA is translated.

The nucleic acid encoding the antigenic sequence(s) can comprise an internal ribosomal entry site (IRES) sequence, whereby host cell translation is facilitated or enhanced and bacterial translation is inhibited or prevented. The IRES can be Vascular Endothelial Growth Factor and Type 1 Collagen Inducible Protein (VCIP; see, *e.g.*, SEQ ID NO:432), and the nucleic acid encoding the antigen(s) can comprise a VCIP IRES or other IRES that inhibits bacterial translation. The IRES or the VCIP

IRES can be included in the plasmid, at a position that is 3' of the promoter and 5' of the antigen(s) coding sequence.

The pathogen can be a bacterium or a virus, or the encoded antigen can be a tumor antigen. The immunostimulatory bacteria provided herein can be vaccines to prevent or treat a viral infection or a bacterial infection, including chronic viral infections, and acute infections. The infection can be from an infection by hepatitis viruses, herpes viruses, varicella zoster virus (VZV), Epstein-Barr virus, human immunodeficiency virus (HIV), human T-cell leukemia virus (HTLV), Respiratory Syncytial Virus (RSV), measles virus, and other viruses that chronically infect subjects. The infectious agent can be Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV), or Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19).

The pathogen can be a species of *Escherichia, Staphylococcus, Pseudomonas*, or *Porphyromonas*, or the pathogen can be *P. gingivalis*, SARS-CoV, or *E. coli*.

The plasmid in these immunostimulatory bacteria can further encode an immunostimulatory protein or other adjuvant, or can encode a combination of immunostimulatory proteins or other therapeutic proteins. The immunostimulatory protein can be a STING protein, such as one that comprises a gain-of-function mutation, or one that is a chimeric STING protein. The bacteria can comprise a plasmid that encodes a combination of therapeutic products. The immunostimulatory protein(s) and/or other therapeutic proteins can be encoded on the plasmid as part of a polycistronic sequence, with the antigen under the control of a prokaryotic promoter recognized by the bacteria; or the immunostimulatory protein(s) and/or other therapeutic proteins can be encoded on the plasmid under control of a eukaryotic promoter recognized by the eukaryotic host. The prokaryotic promoter can be a bacterial promoter or a bacterial phage promoter, and the eukaryotic host can be a human.

The immunostimulatory bacteria can comprise mRNA encoding the antigen(s), and any other proteins expressed under control of a prokaryotic promoter, that are produced by culturing the bacterium *in vitro*. The immunostimulatory bacteria can comprise genome modifications whereby the bacteria lack flagella and produce

LPS with pent-acylated, and/or the bacteria can be *asd*$^-$, and/or an adenosine auxotroph, and/or *csgD*$^-$ and/or *ansB*$^-$.

The bacteria can comprise nucleic acid encoding a TLR8 agonist.

The bacteria can be *msbB*$^-$/*pagP*$^-$, and/or can lack flagella, and/or can be *asd*$^-$.

The bacteria can be a species or strain of *Escherichia*, *Listeria*, or *Salmonella*. For example, the bacteria can be *Salmonella typhimurium* strains, and the unmodified *Salmonella* strain is a wild-type strain, or is an attenuated strain. The immunostimulatory bacteria can be derived from strain AST-100 (VNP20009 or YS1646), or from strain ATCC 14028, or from a strain having all of the identifying characteristics of strain ATCC 14028.

As described herein, the immunostimulatory bacteria can contain one or more genome modification that are one or more of a deletion, insertion, disruption, and other modification in a gene, whereby the product encoded by the gene is not produced or, as produced, is inactive.

Also provided herein are pharmaceutical compositions, comprising any of the immunostimulatory bacteria described or provided herein, in a pharmaceutically acceptable vehicle. The pharmaceutical composition can be formulated as a vaccine, for example, as a liquid, a powder, or a tablet. Also provided are methods and uses of the bacteria or pharmaceutical compositions for treating or prevent (reducing the risk of developing) a disease or condition or infection or cancer, as well as uses of the bacteria for delivering RNA, such as mRNA, and methods of delivering the RNA to a subject, comprising administering the bacteria herein.

Also provided are bacteria comprising a plasmid encoding a product or products, where the product(s) is/are a therapeutic product(s), and the plasmid in the bacterium encodes the product(s) to produce mRNA that is not translated by the bacterium.

The bacteria can be attenuated, or rendered of low toxicity or non-toxic, by virtue of the modifications described herein. Exemplary of bacteria are species of *Salmonella*, such as a *Salmonella typhimurium* strain. The immunostimulatory bacteria provided herein include those that endogenously encode and express, or are modified to encode and express, a gene encoding resistance to complement killing (*rck*), such as a *Salmonella rck* gene. For example, therapeutic *E. coli* are modified to

encode *rck* so that they can be administered systemically. Also provided, as described herein, and as set forth in the claims, are delivery vehicles, cells, pharmaceutical compositions, methods, uses, and treatments of cancer, particularly in humans. Also provided are companion diagnostics and methods for selection of subjects for treatment, and methods for monitoring treatment. These are described below, and also, in the claims, which are incorporated in their entirety into this section.

**BRIEF DESCRIPTION OF THE FIGURES**

**Figures 1A-1C** depict the inserts in plasmids pATI-1.75 and pATI-1.76 (Fig. 1A and 1B, respectively). Figure 1C depicts that the Shine-Dalgarno sequence is replaced with a Kozak sequence, for translation in eukaryotic cells, such as myeloid cells.

**Figure 2** depicts the alignment of wild-type human STING (SEQ ID NO:306) and Tasmanian devil STING (SEQ ID NO:349) proteins.

**Figure 3** depicts the alignment of wild-type human STING (SEQ ID NO:306) and marmoset STING (SEQ ID NO:359) proteins.

**Figure 4** depicts the alignment of wild-type human STING (SEQ ID NO:306) and cattle STING (SEQ ID NO:360) proteins.

**Figure 5** depicts the alignment of wild-type human STING (SEQ ID NO:306) and cat STING (SEQ ID NO:356) proteins.

**Figure 6** depicts the alignment of wild-type human STING (SEQ ID NO:306) and ostrich STING (SEQ ID NO:361) proteins.

**Figure 7** depicts the alignment of wild-type human STING (SEQ ID NO:306) and crested ibis STING (SEQ ID NO:362) proteins.

**Figure 8** depicts the alignment of wild-type human STING (SEQ ID NO:306) and coelacanth STING (SEQ ID NO:363) proteins.

**Figure 9** depicts the alignment of wild-type human STING (SEQ ID NO:306) and zebrafish STING (SEQ ID NO:348) proteins.

**Figure 10** depicts the alignment of wild-type human STING (SEQ ID NO:305) and boar STING (SEQ ID NO:365) proteins.

**Figure 11** depicts the alignment of wild-type human (SEQ ID NO:305) and bat STING (SEQ ID NO:366) proteins.

**Figure 12** depicts the alignment of wild-type human (SEQ ID NO:305) and manatee STING (SEQ ID NO:367) proteins.

**Figure 13** depicts the alignment of wild-type human (SEQ ID NO:305) and ghost shark STING (SEQ ID NO:368) proteins.

**Figure 14** depicts the alignment of wild-type human (SEQ ID NO:305) and mouse STING (SEQ ID NO:369) proteins.

## DETAILED DESCRIPTION

### OUTLINE

**A. DEFINITIONS**

**B. OVERVIEW OF IMMUNOSTIMULATORY BACTERIA FOR CANCER THERAPY**

**1. Bacterial Cancer Immunotherapy**

**2. Prior Therapies that Target the Tumor Microenvironment**

**a. Limitations of Autologous T-Cell Therapies**

**b. Viral Vaccine Platforms**

**c. Bacterial Cancer Therapies**

**i. *Listeria***

**ii. *Salmonella* Species**

**iii. VNP20009**

**iv. Wild-Type Strains**

**3. Limitations of Existing Bacterial Cancer Immunotherapies**

**C. MODIFICATIONS AND ENHANCEMENTS OF IMMUNOSTIMULATORY BACTERIA TO INCREASE THERAPEUTIC INDEX AND TO INCREASE ACCUMULATION IN TUMOR-RESIDENT MYELOID CELLS**

**1. Deletions in Genes in the LPS Biosynthetic Pathway**

**a. *msbB* Deletion**

**b. *pagP* Deletion or Inactivation**

**2. Nutrient Auxotrophy**

**a. *purI* Deletion/Disruption**

**b. Adenosine Auxotrophy**

**c. Thymidine Auxotrophy**

**3. Plasmid Maintenance and Delivery**

**a. *asd* Deletion**

**b. *endA* Deletion/Disruption**

**4. Flagellin Knockout Strains**

5. Engineering Bacteria to Promote Adaptive Immunity and Enhance T-Cell Function

L-Asparaginase II (*ansB*) Deletion/Disruption

6. Deletions/Disruptions in *Salmonella* Genes Required to Produce Curli Fimbriae

*csgD* Deletion

7. Improving Resistance to Complement

*Rck* Expression

8. Deletions of Genes Required for Lipoprotein Expression in *Salmonella* and Other Gram-Negative Bacteria

9. Robust Immunostimulatory Bacteria Whose Genomes are Optimized for Anti-Tumor Therapy, and that Encode Therapeutic Products, Including a Plurality Thereof

10. Vaccines and Bacteria that Deliver RNA, including mRNA and other Forms of RNA, for Expression in a Eukaryotic Host

11. Bacterial Vaccines Against Particular Antigens, Including from Pathogens, and also from Tumors, for use as Anti-Pathogen Treatments and Vaccines, and For Anti-Cancer Treatment and/or Prevention

12. Conversion of M2 Phenotype Macrophages into M1 and M1-Like Phenotype Macrophages

D. IMMUNOSTIMULATORY BACTERIA WITH ENHANCED THERAPEUTIC INDEX ENCODING GENETIC PAYLOADS THAT STIMULATE THE IMMUNE RESPONSE IN THE TUMOR MICROENVIRONMENT

1. Immunostimulatory Proteins

a. Cytokines and Chemokines

b. Co-Stimulatory Molecules

2. Constitutively Active Proteins that Stimulate the Immune Response and/or Type I IFN, Non-Human STING Proteins, STING Chimeras, and Modified Forms

a. Constitutive STING Expression and Gain-of-Function Mutations

b. Constitutive IRF3 Expression and Gain-of-Function Mutations

c. Non-Human STING Proteins, and Variants Thereof with Increased or Constitutive Activity, and STING Chimeras, and Variants Thereof with Increased or Constitutive Activity

d. Other Gene Products that Act as Cytosolic DNA/RNA Sensors and Constitutive Variants Thereof

i. RIG-I

ii. MDA5/IFIH1

iii. IRF7

e. Other Type I IFN Regulatory Proteins

3. Antibodies and Antibody Fragments

a. TGF-β

b. Bispecific scFvs and T-Cell Engagers

c. Anti-PD-1, Anti-PD-L1 and Anti-CTLA-4 Antibodies

i. Anti-PD-1/Anti-PD-L1 Antibodies

ii. Anti-CTLA-4 Antibodies

d. Additional Exemplary Checkpoint Targets

4. Combinations of Immunomodulatory Proteins can have Synergistic Effects and/or Complementary Effects

5. Molecules that Activate Prodrugs

6. Immunostimulatory Bacteria that Deliver Combination Therapies

E. IMMUNOSTIMULATORY BACTERIA AS ANTIVIRAL THERAPEUTICS AND AS THERAPEUTICS AGAINST OTHER INFECTIOUS PATHOGENS

F. CONSTRUCTING EXEMPLARY PLASMIDS ENCODING THERAPEUTIC PRODUCTS FOR BACTERIAL DELIVERY

1. Constitutive Promoters for Heterologous Expression of Proteins

2. Multiple Therapeutic Product Expression Cassettes

a. Single Promoter Constructs

b. Dual/Multiple Promoter Constructs

3. Regulatory Elements

a. Post-Transcriptional Regulatory Elements

b. Polyadenylation Signal Sequences and Terminators

c. Enhancers

d. Secretion Signals

e. Improving Bacterial Fitness

4. Origin of Replication and Plasmid Copy Number

5. CpG Motifs and CpG Islands

6. Plasmid Maintenance/Selection Components

7. DNA Nuclear Targeting Sequences

G. EXEMPLARY BACTERIAL STRAINS AND MECHANISM OF ACTION FOR USE AS VACCINES AND THERAPEUTICS

1. Exemplary Immunostimulatory Bacteria — *In Situ* Cancer Vaccination Mechanism of Action (MOA)

2. Exemplary Immunostimulatory Bacteria for Peripheral Cancer Vaccination and Mechanism of Action

3. Exemplary Immunostimulatory Bacteria for Pathogen Vaccination and MOA

H. PHARMACEUTICAL PRODUCTION, COMPOSITIONS, AND FORMULATIONS

1. Manufacturing

a. Cell Bank Manufacturing

b. Drug Substance Manufacturing

c. Drug Product Manufacturing

2. Compositions

3. Formulations

a. Liquids, Injectables, Emulsions

b. Dried Thermostable Formulations

4. Compositions for Other Routes of Administration

5. Dosages and Administration

6. Packaging and Articles of Manufacture

I. METHODS OF TREATMENT AND USES

1. Diagnostics for Patient Selection for Treatment and for Monitoring Treatment

a. Patient Selection

b. Diagnostics to Assess or Detect Activity of the Immunostimulatory Bacteria are Indicative of the Effectiveness of Treatment

2. Tumors

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4. Monitoring

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## A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and

publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, "therapeutic bacteria" are bacteria that effect therapy, such as anti-cancer or anti-tumor therapy, when administered to a subject, such as a human.

As used herein, "immunostimulatory bacteria" are therapeutic bacteria that, when introduced into a subject, accumulate in immunoprivileged tissues and cells, such as tumors, the tumor microenvironment and tumor-resident immune cells, and replicate and/or express products that are immunostimulatory or that result in immunostimulation. For example, the immunostimulatory bacteria are attenuated in the host by virtue of reduced toxicity or pathogenicity and/or by virtue of encoded products that reduce toxicity or pathogenicity, as the immunostimulatory bacteria cannot replicate and/or express products (or have reduced replication/product expression), except primarily in immunoprivileged environments, such as the tumor microenvironment (TME). Immunostimulatory bacteria provided herein are modified to encode a product or products, and/or to exhibit a trait or property that renders them immunostimulatory. The immunostimulatory bacteria also include genome modifications so that an endogenous product or products is/are not expressed. The bacteria can be said to be deleted in such product(s). Those of skill in the art recognize that genes can be inactivated by deletions, disruptions, including transposition or insertion of transposons, insertions, and any other changes that eliminate the gene product. This can be achieved by insertions, deletions, and/or disruptions, including transpositions or inclusion of transposons. Examples of genes that are inactivated include, for example, *msbB*, *pagP*, *ansB*, gene(s) encoding curli fimbriae, genes encoding flagella whereby the bacterium lacks flagella, and other modifications described herein and/or known to those of skill in the art. Those of skill in the art also understand that corresponding genes in various bacterial species may

have different designations. The encoded products, properties and traits in the immunostimulatory bacteria, include, but are not limited to, for example, at least one of: an immunostimulatory protein, such as a cytokine, chemokine, or co-stimulatory molecule; a cytosolic DNA/RNA sensor or gain-of-function or constitutively active variant thereof (*e.g.*, STING, IRF3, IRF7, MDA5, RIG-I); RNAi, such as siRNA (shRNA and microRNA), or CRISPR, that targets, disrupts, or inhibits an immune checkpoint, such as, for example, TREX1, PD-1, CTLA-4 and/or PD-L1; antibodies and fragments thereof, such as an anti-immune checkpoint antibody, an anti-IL-6 antibody, an anti-VEGF antibody, or a TGF-β inhibitory antibody; other antibody constructs, such as bi-specific T-cell engagers (BiTE® antibodies); soluble TGF-β receptors that act as decoys for binding TGF-β, or TGF-β antagonizing polypeptides; and IL-6 binding decoy receptors. Immunostimulatory bacteria also can include a modification that renders the bacteria auxotrophic for a metabolite that is immunosuppressive or that is in an immunosuppressive pathway, such as adenosine.

As used herein, the strain designations VNP20009 (see, *e.g.*, International PCT Application Publication No. WO 99/13053, see, also U.S. Patent No. 6,863,894), YS1646, and 41.2.9 are used interchangeably, and each refers to the strain deposited with the American Type Culture Collection (ATCC) and assigned Accession No. 202165. VNP20009 is a modified attenuated strain of *Salmonella typhimurium*, which contains deletions or other modifications in *msbB* and *purI*, and was generated from wild-type *S. typhimurium* strain ATCC #14028.

As used herein, the strain designations YS1456 and 8.7 are used interchangeably and each refer to the strain deposited with the American Type Culture Collection (ATCC) and assigned Accession No. 202164 (see, U.S. Patent No. 6,863,894).

As used herein, recitation that a bacterium is "derived from" a particular strain means that such strain can serve as a starting material and can be modified to result in the particular bacterium.

As used herein, an "expression cassette" refers to a nucleic acid construct that includes regulatory sequences for gene expression, operatively linked to nucleic acid encoding open reading frames (ORFs) that encode payloads, such as therapeutic products, or other proteins.

As used herein, 2A peptides are 18–22 amino-acid (aa)-long viral oligopeptides that mediate cleavage of polypeptides during translation in eukaryotic cells. The designation "2A" refers to a specific region of the viral genome, and different viral 2As have generally been named after the virus they were derived from. Exemplary of these are F2A (foot-and-mouth disease virus 2A), E2A (equine rhinitis A virus), P2A (porcine teschovirus-1 2A), and T2A (Thosea asigna virus 2A). See, *e.g.*, Liu *et al.* (2017) *Scientific Reports* 7:2193, Fig. 1, for encoding sequences. See, also, SEQ ID NOs:327-330. These peptides generally share a core sequence motif of DxExNPGP, and occur in a large number of viral families. They help break apart polyproteins by causing the ribosome to fail at making a peptide bond. The 2A peptides provide for multicistronic/polycistronic vectors, in which a plurality of proteins are expressed from a single open reading frame (ORF). For purposes herein, the 2A peptides include those that are naturally occurring, and any modified forms thereof, such as any having at least 97%, 98%, or 99% sequence identity with any naturally-occurring 2A peptide, including those disclosed herein, that result in single polypeptides being transcribed and translated from a transcript comprising a plurality (2 or more) of open reading frames.

As used herein, an "interferonopathy" refers to a disorder associated with an upregulation of interferon by virtue of a mutation in a gene product involved in a pathway that regulates or induces expression of interferon. The activity of the products normally is regulated by a mediator, such as cytosolic DNA or RNA or nucleotides; when the protein product is mutated, the activity is constitutive. Type I interferonopathies include a spectrum of conditions, including the severe forms of Aicardi-Goutières Syndrome (AGS), and the milder Familial Chilblain Lupus (FCL). Nucleic acid molecules encoding mutated products with these properties can be produced *in vitro*, such as by selecting for mutations that result in a gain-of-function in the product, compared to the product of an allele that has normal activity, or has further gain-of-function compared to the disease-associated gain-of-function mutants described herein.

As used herein, a "gain-of-function mutation" is one that increases the activity of a protein compared to the same protein that does not have the mutation. For example, if the protein is a receptor, it will have increased affinity for a ligand; if it is

an enzyme, it will have increased activity, including constitutive activity. In particular, with respect to products, such as STING, IRF3, IRF7, MDA5, RIG-I, a constitutively active product is one that is active in the absence of a its activating ligands, such as cGAS for STING, and/or in the absence of cytosolic nucleic acids, such as DNA, RNA, nucleotides, dinucleotides, cyclic nucleotides and/or cyclic dinucleotides or other nucleic acid molecules, that lead to production of type I interferon. These nucleic acid molecules in the cytosol occur from viral or bacterial infection and/or radiation or other such exposure, leading to activation of an immune response in a host against such pathogen.

As used herein, an "origin of replication" is a sequence of DNA at which replication is initiated on a chromosome, or a plasmid, or in a virus. For small DNA, including bacterial plasmids and small viruses, a single origin is sufficient.

The origin of replication determines the vector copy number, which depends upon the selected origin of replication. For example, if the expression vector is derived from the low-copy-number plasmid pBR322, the copy number is between about 15-20 copies/cell, and if derived from the high-copy-number plasmid pUC, it can be 500-700 copies/cell. As used herein, medium copy number of a plasmid in cells is about or is 150 or less than 150, and low copy number is 5-30, such as 20 or less than 20. Low to medium copy number is less than 150 copies/cell. High copy number is greater than 150 copies/cell.

As used herein, a "CpG motif" is a pattern of bases that includes an unmethylated central CpG ("p" refers to the phosphodiester link between consecutive C and G nucleotides), surrounded by at least one base flanking (on the 3' and the 5' side of) the central CpG. A CpG oligodeoxynucleotide is an oligodeoxynucleotide that is at least about ten nucleotides in length and includes an unmethylated CpG. At least the C of the 5' CG 3' is unmethylated.

As used herein, a "RIG-I binding sequence" refers to a 5'triphosphate (5'ppp) structure directly, or that which is synthesized by RNA pol III from a poly(dA-dT) sequence, which, by virtue of interaction with RIG-I, can activate type I IFN via the RIG-I pathway. The RNA includes at least four A ribonucleotides (A-A-A-A); it can contain 4, 5, 6, 7, 8, 9, 10, or more. The RIG-I binding sequence is introduced into a plasmid in the bacterium for transcription into the polyA.

As used herein, "cytokines" are a broad and loose category of small proteins (~5–20 kDa) that are important in cell signaling. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors. Cytokines are cell signaling molecules that aid cell to cell communication in immune responses, and stimulate the movement of cells towards sites of inflammation, infection and trauma.

As used herein, "chemokines" refer to chemoattractant (chemotactic) cytokines that bind to chemokine receptors and include proteins isolated from natural sources as well as those made synthetically, as by recombinant means or by chemical synthesis. Exemplary chemokines include, but are not limited to, IL-8, IL-10, GCP-2, GRO-α, GRO-β, GRO-γ, ENA-78, PBP, CTAP III, NAP-2, LAPF-4, MIG (CXCL9), CXCL10 (IP-10), CXCL11, PF4, SDF-1α, SDF-1β, SDF-2, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, MIP-1α (CCL3), MIP-1β (CCL4), MIP-1γ (CCL9), MIP-2, MIP-2α, MIP-3α, MIP-3β, MIP-4, MIP-5, MDC, HCC-1, ALP, lungkine, Tim-1, eotaxin-1, eotaxin-2, I-309, SCYA17, TRAC, RANTES (CCL5), DC-CK-1, lymphotactin, and fractalkine, and others known to those of skill in the art. Chemokines are involved in the migration of immune cells to sites of inflammation, as well as in the maturation of immune cells, and in the generation of adaptive immune responses.

As used herein, an "immunostimulatory protein" is a protein that exhibits or promotes an anti-tumor immune response in the tumor microenvironment. Exemplary of such proteins are cytokines, chemokines, and co-stimulatory molecules, such as, but not limited to, IFN-α, IFN-β, GM-CSF, IL-2, IL-7, IL-12, IL-15, IL-18, IL-21, IL-23, IL-12p70 (IL-12p40 + IL-12p35), IL-15/IL-15R alpha chain complex (also referred to herein as IL-15/IL-15Rα, IL-15Rα-IL-15sc, IL-15 complex, and other variations, set forth herein), IL-36 gamma, IL-2 that has attenuated binding to IL-2Ra, IL-2 that is modified so that it does not bind to IL-2Ra, CXCL9, CXCL10 (IP-10), CXCL11, CCL3, CCL4, CCL5, molecules involved in the potential recruitment and/or persistence of T-cells, CD40, CD40 ligand (CD40L), OX40, OX40 ligand (OX40L), 4-1BB, 4-1BB ligand (4-1BBL), 4-1BBL with a deleted cytoplasmic domain (1BBLΔcyt), or with a partially deleted (truncated) cytoplasmic domain, members of the B7-CD28 family, and members of the tumor necrosis factor receptor (TNFR) superfamily.

Among the immunostimulatory proteins are truncated co-stimulatory molecules, such as, for example, 4-1BBL, CD80, CD86, CD27L, B7RP1 and OX40L, each with a full or partial cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC). These truncated gene products, such as those with deletions or partial deletions of the cytoplasmic domain, are truncated such that they are capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, but are unable to counter-regulatory signal to the APC, due to a deleted or truncated cytoplasmic domain.

As used herein, a "cytoplasmic domain deletion" is a deletion in all, or a portion of, the amino acid residues that comprise the cytoplasmic, or intracellular, domain of the protein, where the deletion is sufficient to effect constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is sufficient to inhibit counter-regulatory signaling to the APC. For example, the cytoplasmic domain of human 4-1BBL (also known as TNFSF9) comprises amino acid residues 1-28 of SEQ ID NO:342. The cytoplasmic domain of human CD80 comprises amino acid residues 264-288 of the protein; the cytoplasmic domain of human CD86 comprises amino acid residues 269-329 of the protein; the cytoplasmic domain of human CD27L (also known as CD70) comprises amino acid residues 1-17 of the protein; the cytoplasmic domain of human B7RP1 (also known as ICOSLG or ICOS ligand) comprises amino acid residues 278-302 of the protein; and the cytoplasmic domain of human OX40L (also known as TNFSF4 or CD252) comprises amino acid residues 1-23 of the protein.

As used herein, a "decoy receptor" is a receptor that can specifically bind to specific growth factors or cytokines efficiently, but is not structurally able to signal or activate the intended receptor complex. The decoy receptor acts as an inhibitor by binding to a ligand and preventing it from binding to its cognate receptor.

For example, TGF-β family receptors include the cell-surface serine/threonine kinase receptors type I (TβRI or TGFβR1) and type II (TβRII or TGFβR2), which form heteromeric complexes in the presence of dimerized ligands, as well as the type III receptor betaglycan (TβRIII or TGFβR3). Soluble decoy receptors for TGF-β, which prevent the binding of TGF-β to its receptors, include the soluble extracellular domains (the TGF-β binding regions) of TβRI, TβRII, or TβRIII (βglycan), which can

be fused with other molecules, such as an Fc domain. Additionally, BAMBI (bone morphogenetic protein (BMP) and activin membrane-bound inhibitor) is structurally related to type I receptors and acts as a decoy that inhibits receptor activation. A dominant negative TGFβR2 (DN-TGFβR2), which comprises the extracellular domain of TGFβR2 and the transmembrane region, but which lacks the cytoplasmic domain required for signaling, also can be used as a TGF-β decoy receptor (see, *e.g.*, International Application Publication No. WO 2018/138003).

As used herein, a co-stimulatory molecule agonist is a molecule that, upon binding to the co-stimulatory molecule, activates it or increases its activity. For example, the agonist can be an agonist antibody. CD40 agonist antibodies include, for example, CP-870,893, dacetuzumab, ADC-1013 (mitazalimab), and Chi Lob 7/4.

As used herein, a cytosolic DNA/RNA sensor pathway is one that is initiated by the presence of DNA, RNA, nucleotides, dinucleotides, cyclic nucleotides and/or cyclic dinucleotides or other nucleic acid molecules, that leads to production of type I interferon. The nucleic acid molecules in the cytosol occur from viral or bacterial or radiation or other such exposure, leading to activation of an immune response in a host.

As used herein, a "type I interferon pathway protein" is a protein that induces an innate immune response, such as the induction of type I interferon.

As used herein, a "cytosolic DNA/RNA sensor," is a protein that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of an immune response mediator, such as type I interferon. A "cytosolic DNA/RNA sensor," includes type I interferon pathway proteins. For example, as described herein and known to those of skill in the art, cytosolic DNA is sensed by cGAS, leading to the production of cGAMP and subsequent STING/TBK1/IRF3 signaling, and type I IFN production. Bacterial cyclic dinucleotides (CDNs, such as bacterial cyclic di-AMP) also activate STING. Hence, STING is an immunomodulatory protein that induces type I interferon. 5'- triphosphate RNA and double stranded RNA are sensed by RIG-I and either MDA-5 alone, or MDA-5/LGP2. This leads to polymerization of mitochondrial MAVS (mitochondrial antiviral-signaling protein), and also activates TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3). The proteins in such pathways are immunostimulatory proteins, and lead to the expression of innate

immune response mediators, such as type I interferon. The immunomodulatory proteins in the DNA/RNA sensor pathways can be modified so that they have increased activity, or act constitutively in the absence of cytosolic nucleic acids and/or activating/stimulating ligands, to lead to the immune response, such as the expression of type I interferon.

As used herein, the "carboxy-terminal tail" or "C-terminal tail" (CTT) of the innate immune protein STING refers to the C-terminal portion of a STING protein that, in a wild-type STING protein, is tethered to the cGAMP-binding domain by a flexible linker region. The CTT includes an IRF3 binding site, a TBK1 binding site, and a TRAF6 binding site. STING promotes the induction of interferon beta (IFN-β) production via the phosphorylation of the STING protein C-terminal tail (CTT) by TANK-binding kinase 1 (TBK1). The interaction between STING and TBK1 is mediated by an evolutionarily conserved stretch of eight amino-acid residues in the carboxy-terminal tail (CTT) of STING. TRAF6 catalyzes the formation of K63-linked ubiquitin chains on STING, leading to the activation of the transcription factor NF-κB and the induction of an alternative STING-dependent gene expression program. Deletion or disruption of the TRAF6 binding site in the CTT can reduce activation of NF-κB signaling. Substitution of the human STING CTT (or portions thereof), with the CTT (or corresponding portion thereof) from the STING protein of a species with low NF-κB activation, can decrease the NF-κB activation by the resulting modified human STING protein. The STING CTT is an unstructured stretch of ~40 amino acids that contains sequence motifs required for STING phosphorylation and recruitment of IRF3 (see, de Oliveira Mann *et al.* (2019) *Cell Reports 27*:1165-1175). Human STING residue S366 has been identified as a primary TBK1 phosphorylation site that is part of an LxIS motif shared among innate immune adaptor proteins that activate interferon signaling (see, de Oliveira Mann *et al.* (2019) *Cell Reports 27*:1165-1175). The human STING CTT contains a second PxPLR motif that includes the residue L374, which is required for TBK1 binding; the LxIS and PxPLR sequences are conserved among vertebrate STING alleles (see, de Oliveira Mann *et al.* (2019) *Cell Reports 27*:1165-1175). Exemplary STING CTT sequences, and the IRF3, TBK1, and TRAF6 binding sites, are set forth in the following table:

| Species | C-Terminal Tail (CTT) Sequence | SEQ ID NO. | IRF3 Binding Site | TBK1 Binding Site | TRAF6 Binding Site |
|---|---|---|---|---|---|
| Human | EKEEVTVGSLKTSAVPSTSTMS QEPELLISGMEKPLPLRTDFS | 370 | PELLIS | PLPLRT | DFS |
| Tasmanian devil | RQEEFAIGPKRAMTVTTSSTLS QEPQLLISGMEQPLSLRTDGF | 371 | PQLLIS | PLSLRT | DGF |
| Marmoset | EEEEVTVGSLKTSEVPSTSTMS QEPELLISGMEKPLPLRSDLF | 372 | PELLIS | PLPLRS | DLF |
| Cattle | EREVTMGSTETSVMPGSSVLS QEPELLISGLEKPLPLRSDVF | 373 | PELLIS | PLPLRS | DVF |
| Cat | EREVTVGSVGTSMVRNPSVLS QEPNLLISGMEQPLPLRTDVF | 374 | PNLLIS | PLPLRT | DVF |
| Ostrich | RQEEYTVCDGTLCSTDLSLQIS ESDLPQPLRSDCL | 375 | LSLQIS | PQPLRS | DCL |
| Boar | EREVTMGSAETSVVPTSSTLSQ EPELLISGMEQPLPLRSDIF | 376 | PELLIS | PLPLRS | DIF |
| Bat | EKEEVTVGTVGTYEAPGSSTL HQEPELLISGMDQPLPLRTDIF | 377 | PELLIS | PLPLRT | DIF |
| Manatee | EREEVTVGSVGTSVVPSPSSPS TSSLSQEPKLLISGMEQPLPLRT DVF | 378 | PKLLIS | PLPLRT | DVF |
| Crested ibis | CHEEYTVYEGNQPHNPSTTLH STELNLQISESDLPQPLRSDCF | 379 | LNLQIS | PQPLRS | DCF |
| Coelacanth (variant 1) | QKEEYFMSEQTQPNSSSTSCLS TEPQLMISDTDAPHTLKRQVC | 380 | PQLMIS | PHTLKR | QVC |
| Coelacanth (variant 2) | QKEEYFMSEQTQPNSSSTSCLS TEPQLMISDTDAPHTLKSGF | 381 | PQLMIS | PHTLKS | GF |
| Zebrafish | DGEIFMDPTNEVHPVPEEGPV GNCNGALQATFHEEPMSDEPT LMFSRPQSLRSEPVETTDYFNP SSAMKQN | 382 | PTLMFS | PQSLRS | EPVETTDY |
| Ghost shark | LTEYPVAEPSNANETDCMSSE PHLMISDDPKPLRSYCP | 383 | PHLMIS | PKPLRS | YCP |
| Mouse | EKEEVTMNAPMTSVAPPPSVL SQEPRLLISGMDQPLPLRTDLI | 384 | PRLLIS | PLPLRT | DLI |

As used herein, a bacterium that is modified so that it "induces less cell death in tumor-resident immune cells" or "induces less cell death in immune cells" is one that is less toxic than the bacterium without the modification, or one that has reduced virulence compared to the bacterium without the modification. Exemplary of such modifications are those that eliminate pyroptosis in phagocytic cells and that alter lipopolysaccharide (LPS) profiles on the bacterium. These modifications include disruption of or deletion of flagellin genes, *pagP*, or one or more components of the SPI-1 pathway, such as *hilA*, rod protein (*e.g.*, *prgJ*), needle protein (*e.g.*, *prgI*), and QseC.

As used herein, a bacterium that is "modified so that it preferentially infects tumor-resident immune cells" or "modified so that it preferentially infects immune

cells" has a modification in its genome that reduces its ability to infect cells other than immune cells. Exemplary of such modifications are modifications that disrupt the type 3 secretion system or type 4 secretion system or other genes or systems that affect the ability of a bacterium to invade a non-immune cell. For example, modifications include disruption/deletion of an SPI-1 component, which is needed for infection of cells, such as epithelial cells, but does not affect infection of immune cells, such as phagocytic cells, by *Salmonella*.

As used herein, a "modification" is in reference to modification of a sequence of amino acids of a polypeptide, or a sequence of nucleotides in a nucleic acid molecule, and includes deletions, insertions, and replacements of amino acids or nucleotides, respectively. Methods of modifying a polypeptide are routine to those of skill in the art, such as by using recombinant DNA methodologies.

As used herein, a modification to a bacterial genome, or to a plasmid, or to a gene, includes deletions, replacements, and insertions of nucleic acid.

As used herein, RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules to inhibit translation, and thereby expression, of a targeted gene.

As used herein, RNA molecules that act via RNAi are referred to as inhibitory by virtue of their silencing of the expression of a targeted gene. Silencing expression means that expression of the targeted gene is reduced, or suppressed, or inhibited.

As used herein, gene silencing via RNAi is said to inhibit, suppress, disrupt, or silence expression of a targeted gene. A targeted gene contains sequences of nucleotides that correspond to the sequences in the inhibitory RNA, whereby the inhibitory RNA silences expression of target mRNA.

As used herein, inhibiting, suppressing, disrupting, or silencing a targeted gene, refers to processes that alter expression, such as translation, of the targeted gene, whereby activity or expression of the product encoded by the targeted gene is reduced. Reduction, includes a complete knock-out or a partial knockout, whereby, with reference to the immunostimulatory bacteria provided herein and administration herein, treatment is effected.

As used herein, small interfering RNAs (siRNAs) are small pieces of double-stranded (ds) RNA, usually about 21 nucleotides long, with 3' overhangs (2

nucleotides) at each end that can be used to "interfere" with the translation of proteins by binding to and promoting the degradation of messenger RNA (mRNA) at specific sequences. In doing so, siRNAs prevent the production of specific proteins based on the nucleotide sequences of their corresponding mRNAs. The process is called RNA interference (RNAi), and also is referred to as siRNA silencing, or siRNA knockdown.

As used herein, a short-hairpin RNA or small-hairpin RNA (shRNA) is an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi). Expression of shRNA in cells is typically accomplished by delivery of plasmids, or through viral or bacterial vectors.

As used herein, a tumor microenvironment (TME) is the cellular environment in which the tumor exists, including surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix (ECM). Conditions that exist include, but are not limited to, increased vascularization, hypoxia, low pH, increased lactate concentration, increased pyruvate concentration, increased interstitial fluid pressure, and altered metabolites or metabolism, such as higher levels of adenosine, which are indicative of a tumor.

As used herein, an immune desert tumor or immune-excluded tumor is a tumor devoid of tumor infiltrating T-cells. Immune desert tumors are solid tumors where minimal effector immune cells infiltrate the tumor and there is a lack of immune response present in the tumor. Desert tumors are devoid tumor-infiltrating lymphocytes, CD8+ T-cells are absent from the tumor, including the parenchyma, stroma, and tumor periphery.

As used herein, "bactofection" refers to the bacteria-mediated transfer of genes or plasmid DNA into eukaryotic cells, such as mammalian cells.

As used herein, human type I interferons (IFNs) are a subgroup of interferon proteins that regulate the activity of the immune system. All type I IFNs bind to a specific cell surface receptor complex, such as the IFN-α receptor. Type I interferons include IFN-α and IFN-β, among others. Myeloid cells are the primary producers of IFN-α and IFN-β, which have antiviral activity that is involved mainly in innate immune responses. Two types of IFN-β are IFN-β1 (IFNB1) and IFN-β3 (IFNB3).

As used herein, "M1 macrophage phenotype" and "M2 macrophage phenotype" refer to the two broad groups into which macrophage phenotypes are divided: M1 (classically activated macrophages) and M2 (alternatively activated macrophages). The role of M1 macrophages is to secrete pro-inflammatory cytokines and chemokines, and to present antigens, so that they participate in the positive immune response, and function as an immune monitor. The main pro-inflammatory cytokines they produce are IL-6, IL-12, and TNF-alpha. M2 macrophages primarily secrete arginase-I, IL-10, TGF-β, and other anti-inflammatory cytokines, which have the function of reducing inflammation, and contributing to tumor growth and immunosuppressive function. A macrophage with an M1-like phenotype secretes pro-inflammatory cytokines, and does not have the immunosuppressive activity(ies) of an M2 macrophage. Conversion of an M2 macrophage into a macrophage with an M1 or M1-like phenotype converts an M2 macrophage into one that is not immunosuppressive, but participates in an anti-tumor response. An M2 macrophage that is converted into a macrophage with an M1 or M1-like phenotype exhibits the secretion/expression of more pro-inflammatory cytokines/chemokines and receptors, such as CD80 and CCR7, and chemokines, such as IFNγ and CXCL10. M1 phenotypic markers include, but are not limited to, one or more of CD80, CD86, CD64, CD16, and CD32. The expression of nitric oxide synthase (iNOS) in M1 macrophages also can serve as a phenotypic marker. CD163 and CD206 are major markers for the identification of M2 macrophages. Other surface markers for M2-type cells also include CD68. A reduction or elimination of any of the M2 markers, and an increase in cytokines/chemokines that are indicative of M1 macrophages, reflect a conversion from an M2 phenotype into an M1 or M1-like phenotype. The sections below, and the working examples regarding M2 to M1-like or M1 phenotype conversion, describe exemplary cytokine profiles and markers that are induced.

As used herein, recitation that a nucleic acid or encoded RNA targets a gene means that it inhibits or suppresses or silences expression of the gene by any mechanism. Generally, such nucleic acid includes at least a portion complementary to the targeted gene, where the portion is sufficient to form a hybrid with the complementary portion.

As used herein, "deletion," when referring to a nucleic acid or polypeptide sequence, refers to the deletion of one or more nucleotides or amino acids compared to a sequence, such as a target polynucleotide, or polypeptide, or a native or wild-type sequence.

As used herein, "insertion," when referring to a nucleic acid or amino acid sequence, describes the inclusion of one or more additional nucleotides or amino acids, within a target, native, wild-type or other related sequence. Thus, a nucleic acid molecule that contains one or more insertions compared to a wild-type sequence, contains one or more additional nucleotides within the linear length of the sequence.

As used herein, "additions" to nucleic acid and amino acid sequences describe addition of nucleotides or amino acids onto either termini compared to another sequence.

As used herein, "substitution" or "replacement" refers to the replacing of one or more nucleotides or amino acids in a native, target, wild-type or other nucleic acid or polypeptide sequence with an alternative nucleotide or amino acid, without changing the length (as described in numbers of nucleotides or residues) of the molecule. Thus, one or more substitutions in a molecule does not change the number of nucleotides or amino acid residues of the molecule. Amino acid replacements compared to a particular polypeptide can be expressed in terms of the number of the amino acid residue along the length of the polypeptide sequence.

As used herein, "at a position corresponding to," or recitation that nucleotides or amino acid positions "correspond to" nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence Listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm, such as the GAP algorithm. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides. In general, to identify corresponding positions, the sequences of amino acids are aligned so that the highest order match is obtained (see, *e.g.*, *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G.,

eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carrillo *et al.* (1988) *SIAM J. Applied Math* 48:1073).

As used herein, alignment of a sequence refers to the use of homology to align two or more sequences of nucleotides or amino acids. Typically, two or more sequences that are related by 50% or more identity are aligned. An aligned set of sequences refers to 2 or more sequences that are aligned at corresponding positions and can include aligning sequences derived from RNAs, such as ESTs and other cDNAs, aligned with a genomic DNA sequence. Related or variant polypeptides or nucleic acid molecules can be aligned by any method known to those of skill in the art. Such methods typically maximize matches, and include methods, such as using manual alignments, and by using the numerous alignment programs available (*e.g.*, BLASTP) and others known to those of skill in the art. By aligning the sequences of polypeptides or nucleic acids, one skilled in the art can identify analogous portions or positions, using conserved and identical amino acid residues as guides. Further, one skilled in the art also can employ conserved amino acid or nucleotide residues as guides to find corresponding amino acid or nucleotide residues between and among human and non-human sequences. Corresponding positions also can be based on structural alignments, for example by using computer simulated alignments of protein structure. In other instances, corresponding regions can be identified. One skilled in the art also can employ conserved amino acid residues as guides to find corresponding amino acid residues between and among human and non-human sequences.

As used herein, a “property” of a polypeptide, such as an antibody, refers to any property exhibited by a polypeptide, including, but not limited to, binding specificity, structural configuration or conformation, protein stability, resistance to proteolysis, conformational stability, thermal tolerance, and tolerance to pH conditions. Changes in properties can alter an “activity” of the polypeptide. For example, a change in the binding specificity of the antibody polypeptide can alter the ability to bind an antigen, and/or various binding activities, such as affinity or avidity, or *in vivo* activities of the polypeptide.

As used herein, an "activity" or a "functional activity" of a polypeptide, such as an antibody, refers to any activity exhibited by the polypeptide. Such activities can be empirically determined. Exemplary activities include, but are not limited to, the ability to interact with a biomolecule, for example, through antigen-binding, DNA binding, ligand binding, or dimerization, or enzymatic activity, for example, kinase activity, or proteolytic activity. For an antibody (including antibody fragments), activities include, but are not limited to, the ability to specifically bind a particular antigen, affinity of antigen-binding (*e.g.*, high or low affinity), avidity of antigen-binding (*e.g.*, high or low avidity), on-rate, off-rate, effector functions, such as the ability to promote antigen neutralization or clearance, virus neutralization, and *in vivo* activities, such as the ability to prevent infection or invasion of a pathogen, or to promote clearance, or to penetrate a particular tissue or fluid or cell in the body. Activity can be assessed *in vitro* or *in vivo* using recognized assays, such as ELISA, flow cytometry, surface plasmon resonance or equivalent assays to measure on-rate or off-rate, immunohistochemistry and immunofluorescence histology and microscopy, cell-based assays, and binding assays (*e.g.*, panning assays).

As used herein, "bind," "bound," or grammatical variations thereof, refers to the participation of a molecule in any attractive interaction with another molecule, resulting in a stable association in which the two molecules are in close proximity to one another. Binding includes, but is not limited to, non-covalent bonds, covalent bonds (such as reversible and irreversible covalent bonds), and includes interactions between molecules such as, but not limited to, proteins, nucleic acids, carbohydrates, lipids, and small molecules, such as chemical compounds, including drugs.

As used herein, "antibody" refers to immunoglobulins and immunoglobulin fragments, whether natural, or partially or wholly synthetically, such as recombinantly produced, including any fragment thereof containing at least a portion of the variable heavy chain and light region of the immunoglobulin molecule that is sufficient to form an antigen-binding site and, when assembled, to specifically bind an antigen. Hence, an antibody includes any protein having a binding domain that is homologous or substantially homologous to an immunoglobulin antigen-binding domain (antibody combining site). For example, an antibody refers to an antibody that contains two heavy chains (which can be denoted H and H') and two light chains (which can be

denoted L and L'), where each heavy chain can be a full-length immunoglobulin heavy chain or a portion thereof sufficient to form an antigen-binding site (*e.g.*, heavy chains include, but are not limited to, $V_H$ chains, $V_H$-$C_H1$ chains and $V_H$-$C_H1$-$C_H2$-$C_H3$ chains), and each light chain can be a full-length light chain or a portion thereof sufficient to form an antigen-binding site (*e.g.*, light chains include, but are not limited to, $V_L$ chains and $V_L$-$C_L$ chains). Each heavy chain (H and H') pairs with one light chain (L and L', respectively). Typically, antibodies minimally include all or at least a portion of the variable heavy ($V_H$) chain and/or the variable light ($V_L$) chain. The antibody also can include all or a portion of the constant region.

For purposes herein, the term antibody includes full-length antibodies and portions thereof including antibody fragments, such as anti-CTLA-4 antibody fragments. Antibody fragments, include, but are not limited to, Fab fragments, Fab' fragments, F(ab')$_2$ fragments, Fv fragments, disulfide-linked Fvs (dsFvs), Fd fragments, Fd' fragments, single-chain Fvs (scFvs), single-chain Fabs (scFabs), diabodies, anti-idiotypic (anti-Id) antibodies, or antigen-binding fragments of any of the above. Antibody also includes synthetic antibodies, recombinantly produced antibodies, multi-specific antibodies (*e.g.*, bispecific antibodies), human antibodies, non-human antibodies, humanized antibodies, chimeric antibodies, and intrabodies. Antibodies provided herein include members of any immunoglobulin class (*e.g.*, IgG, IgM, IgD, IgE, IgA and IgY), any subclass (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), or sub-subclass (*e.g.*, IgG2a and IgG2b). Antibodies for human therapy generally are human antibodies or are humanized.

As used herein, "antibody fragment(s)" refers to (i) monovalent and monospecific antibody derivatives that contain the variable heavy and/or light chains, or functional fragments of an antibody, and that lack an Fc part; and (ii) BiTE® antibodies (such as tandem scFvs), dual-affinity re-targeting antibodies (DARTs), other dimeric and multimeric antibodies, diabodies, and single-chain diabodies (scDBs). Thus, an antibody fragment includes, for example, a/an: Fab, Fab', scFab, scFv, Fv fragment, nanobody (see, *e.g.*, antibodies derived from *Camelus bactriamus*, *Camelus dromedarius*, or *Lama paccos*) (see, *e.g.*, U.S. Pat. No. 5,759,808; and Stijlemans *et al.* (2004) *J. Biol. Chem.* 279:1256-1261), $V_{HH}$, single-domain antibody (dAb or sdAb), minimal recognition unit, single-chain diabody (scDb), BiTE®

antibody, and DART antibody, and other antibody constructs that bind to antigens. Typically, the recited antibody fragments have a molecular weight below 60 kDa.

As used herein, "nucleic acid" refers to at least two linked nucleotides or nucleotide derivatives, including a deoxyribonucleic acid (DNA) and a ribonucleic acid (RNA), joined together, typically by phosphodiester linkages. Also included in the term "nucleic acid" are analogs of nucleic acids, such as peptide nucleic acid (PNA), phosphorothioate DNA, and other such analogs and derivatives, or combinations thereof. Nucleic acids also include DNA and RNA derivatives containing, for example, a nucleotide analog or a "backbone" bond other than a phosphodiester bond, for example, a phosphotriester bond, a phosphoramidate bond, a phosphorothioate bond, a thioester bond, or a peptide bond (peptide nucleic acid). The term also includes equivalents, derivatives, variants, and analogs of either RNA or DNA made from nucleotide analogs, and single-stranded (sense or antisense) and double-stranded nucleic acids. Deoxyribonucleotides include deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine. For RNA, the uracil base is uridine.

As used herein, an isolated nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. An "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Exemplary isolated nucleic acid molecules provided herein include isolated nucleic acid molecules encoding an antibody or antigen-binding fragments provided herein.

As used herein, "operably linked" or "operatively linked," with reference to nucleic acid sequences, regions, elements, or domains, means that the nucleic acid regions are functionally related to each other. It refers to a juxtaposition whereby the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter effects or affects its transcription or expression. For example, a nucleic acid encoding a leader peptide can be operably linked to a nucleic acid encoding a polypeptide, whereby the nucleic acids can be transcribed and translated to express a

functional fusion protein, wherein the leader peptide effects secretion of the fusion polypeptide. In some instances, the nucleic acid encoding a first polypeptide (*e.g.*, a leader peptide) is operably linked to a nucleic acid encoding a second polypeptide, and the nucleic acids are transcribed as a single mRNA transcript, but translation of the mRNA transcript can result in one of two polypeptides being expressed. For example, an amber stop codon can be located between the nucleic acid encoding the first polypeptide and the nucleic acid encoding the second polypeptide, such that, when introduced into a partial amber suppressor cell, the resulting single mRNA transcript can be translated to produce either a fusion protein containing the first and second polypeptides, or can be translated to produce only the first polypeptide. In another example, a promoter can be operably linked to nucleic acid encoding a polypeptide, whereby the promoter regulates or mediates the transcription of the nucleic acid.

As used herein, "synthetic," with reference to, for example, a synthetic nucleic acid molecule, or a synthetic gene, or a synthetic peptide, refers to a nucleic acid molecule, or a gene, or a polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

As used herein, the residues of naturally occurring α-amino acids are the residues of those 20 α-amino acids found in nature which are incorporated into a protein by the specific recognition of the charged tRNA molecule with its cognate mRNA codon in humans.

As used herein, a "polypeptide" refers to two or more amino acids covalently joined. The terms "polypeptide" and "protein" are used interchangeably herein.

As used herein, a "peptide" refers to a polypeptide that is from 2 to about or 40 amino acids in length.

As used herein, an "amino acid" is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids contained in the antibodies and immunostimulatory proteins provided herein, include the twenty naturally-occurring amino acids (see Table below), non-natural amino acids, and amino acid analogs (*e.g.*, amino acids wherein the α-carbon has a side chain). As used herein, the amino acids, which occur in the various amino acid sequences of polypeptides appearing herein, are identified

according to their well-known, three-letter or one-letter abbreviations (see Table below). The nucleotides, which occur in the various nucleic acid molecules and fragments, are designated with the standard single-letter designations used routinely in the art.

As used herein, "amino acid residue" refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are generally in the "L" isomeric form. Residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. $NH_2$ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in *J. Biol. Chem.*, 243:3557-59 (1968) and adopted at 37 C.F.R. §§ 1.821-1.822, abbreviations for amino acid residues are shown in the following Table:

**Table of Correspondence**

| SYMBOL | | |
|---|---|---|
| **1-Letter** | **3-Letter** | **AMINO ACID** |
| Y | Tyr | Tyrosine |
| G | Gly | Glycine |
| F | Phe | Phenylalanine |
| M | Met | Methionine |
| A | Ala | Alanine |
| S | Ser | Serine |
| I | Ile | Isoleucine |
| L | Leu | Leucine |
| T | Thr | Threonine |
| V | Val | Valine |
| P | Pro | Proline |
| K | Lys | Lysine |
| H | His | Histidine |
| Q | Gln | Glutamine |
| E | Glu | Glutamic acid |
| Z | Glx | Glutamic Acid and/or Glutamine |
| W | Trp | Tryptophan |
| R | Arg | Arginine |
| D | Asp | Aspartic acid |
| N | Asn | Asparagine |
| B | Asx | Aspartic Acid and/or Asparagine |
| C | Cys | Cysteine |
| X | Xaa | Unknown or other |

All sequences of amino acid residues represented herein by a formula have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. The phrase "amino acid residue" is defined to include the amino acids listed in the above Table of Correspondence, as well as modified, non-natural, and unusual amino acids. A dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues, or to an amino-terminal group such as $NH_2$, or to a carboxyl-terminal group such as COOH.

In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in the art and generally can be made without altering a biological activity of a resulting molecule. Those of skill in the art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al*., *Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p. 224).

Such substitutions can be made in accordance with the exemplary substitutions set forth in the following Table:

**Exemplary Conservative Amino Acid Substitutions**

| Original Residue | Exemplary Conservative Substitution(s) |
|---|---|
| Ala (A) | Gly; Ser |
| Arg (R) | Lys |
| Asn (N) | Gln; His |
| Cys (C) | Ser |
| Gln (Q) | Asn |
| Glu (E) | Asp |
| Gly (G) | Ala; Pro |
| His (H) | Asn; Gln |
| Ile (I) | Leu; Val |
| Leu (L) | Ile; Val |
| Lys (K) | Arg; Gln; Glu |
| Met (M) | Leu; Tyr; Ile |
| Phe (F) | Met; Leu; Tyr |
| Ser (S) | Thr |
| Thr (T) | Ser |
| Trp (W) | Tyr |
| Tyr (Y) | Trp; Phe |
| Val (V) | Ile; Leu |

Other substitutions also are permissible and can be determined empirically or in accord with other known conservative or non-conservative substitutions.

As used herein, "naturally occurring amino acids" refer to the 20 L-amino acids that occur in polypeptides.

As used herein, the term "non-natural amino acid" refers to an organic compound that has a structure similar to a natural amino acid, but that has been modified structurally to mimic the structure and reactivity of a natural amino acid. Non-naturally occurring amino acids thus include, for example, amino acids or analogs of amino acids other than the 20 naturally occurring amino acids and include, but are not limited to, the D-stereoisomers of amino acids. Exemplary non-natural amino acids are known to those of skill in the art, and include, but are not limited to, 2-Aminoadipic acid (Aad), 3-Aminoadipic acid (bAad), β-alanine/β-Amino-propionic acid (Bala), 2-Aminobutyric acid (Abu), 4-Aminobutyric acid/piperidinic acid (4Abu), 6-Aminocaproic acid (Acp), 2-Aminoheptanoic acid (Ahe), 2-Aminoisobutyric acid (Aib), 3-Aminoisobutyric acid (Baib), 2-Aminopimelic acid (Apm), 2,4-Diaminobutyric acid (Dbu), Desmosine (Des), 2,2'-Diaminopimelic acid (Dpm), 2,3-Diaminopropionic acid (Dpr), N-Ethylglycine (EtGly), N-Ethylasparagine (EtAsn), Hydroxylysine (Hyl), allo-Hydroxylysine (Ahyl), 3-Hydroxyproline (3Hyp), 4-Hydroxyproline (4Hyp), Isodesmosine (Ide), allo-Isoleucine (Aile), N-Methylglycine, sarcosine (MeGly), N-Methylisoleucine (MeIle), 6-N-Methyllysine (MeLys), N-Methylvaline (MeVal), Norvaline (Nva), Norleucine (Nle), and Ornithine (Orn).

As used herein, a DNA construct is a single-stranded or double-stranded, linear or circular DNA molecule that contains segments of DNA combined and juxtaposed in a manner not found in nature. DNA constructs exist as a result of human manipulation, and include clones and other copies of manipulated molecules.

As used herein, a DNA segment is a portion of a larger DNA molecule having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, which, when read from the 5' to 3' direction, encodes the sequence of amino acids of the specified polypeptide.

As used herein, the term polynucleotide means a single- or double-stranded polymer of deoxyribonucleotides or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and can be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated "nt"), or base pairs (abbreviated "bp"). The term nucleotides is used for single- and double-stranded molecules where the context permits. When the term is applied to double-stranded molecules, it is used to denote overall length and will be understood to be equivalent to the term base pairs. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide can differ slightly in length and that the ends thereof can be staggered; thus, all nucleotides within a double-stranded polynucleotide molecule cannot be paired. Such unpaired ends will, in general, not exceed 20 nucleotides in length.

As used herein, production by recombinant methods refers to the use of the well-known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, "heterologous nucleic acid" is nucleic acid that encodes products (*i.e.*, RNA and/or proteins) that are not normally produced *in vivo* by the cell in which it is expressed, or nucleic acid that is in a locus in which it does not normally occur, or that mediates or encodes mediators that alter expression of endogenous nucleic acid, such as DNA, by affecting transcription, translation, or other regulatable biochemical processes. Heterologous nucleic acid, such as DNA, also is referred to as foreign nucleic acid. Any nucleic acid, such as DNA, that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which it is expressed, is herein encompassed by heterologous nucleic acid; heterologous nucleic acid includes exogenously added nucleic acid that is also expressed endogenously. Heterologous nucleic acid is generally not endogenous to the cell into which it is introduced, but has been obtained from another cell, or prepared synthetically, or is introduced into a genomic locus in which it does not occur naturally, or its expression is under the control of regulatory sequences or a sequence that differs from the natural regulatory sequence or sequences.

Examples of heterologous nucleic acid herein include, but are not limited to, nucleic acid that encodes a protein in a DNA/RNA sensor pathway or a gain-of-function or constitutively active variant thereof, or an immunostimulatory protein, such as a cytokine, chemokine or co-stimulatory molecule, that confers or contributes to anti-tumor immunity in the tumor microenvironment. Other products, such as antibodies and fragments thereof, BiTEs®, decoy receptors, antagonizing polypeptides and RNAi, that confer or contribute to anti-tumor immunity in the tumor microenvironment, also are included. In the immunostimulatory bacteria, the heterologous nucleic acid generally is encoded on the introduced plasmid, but it can be introduced into the genome of the bacterium, such as a promoter that alters expression of a bacterial product. Heterologous nucleic acid, such as DNA, includes nucleic acid that can, in some manner, mediate expression of DNA that encodes a therapeutic product, or it can encode a product, such as a peptide or RNA, that in some manner mediates, directly or indirectly, expression of a therapeutic product.

As used herein, cell therapy involves the delivery of cells to a subject to treat a disease or condition. The cells, which can be allogeneic or autologous to the subject, are modified *ex vivo*, such as by infection of cells with immunostimulatory bacteria provided herein, so that they deliver or express products when introduced to a subject.

As used herein, genetic therapy involves the transfer of heterologous nucleic acid, such as DNA, into certain cells, such as target cells, of a mammal, particularly a human, with a disorder or condition for which such therapy is sought. The nucleic acid, such as DNA, is introduced into the selected target cells in a manner such that the heterologous nucleic acid, such as DNA, is expressed, and a therapeutic product(s) encoded thereby is (are) produced. Genetic therapy can also be used to deliver nucleic acid encoding a gene product that replaces a defective gene or supplements a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid can encode a therapeutic compound, such as a growth factor or inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor thereof, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous nucleic acid, such as DNA, encoding the therapeutic product, can be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter

the product or expression thereof. Genetic therapy can also involve delivery of an inhibitor or repressor or other modulator of gene expression.

As used herein, "expression" refers to the process by which polypeptides are produced by transcription and translation of polynucleotides. The level of expression of a polypeptide can be assessed using any method known in art, including, for example, methods of determining the amount of the polypeptide produced from the host cell. Such methods can include, but are not limited to, quantitation of the polypeptide in the cell lysate by ELISA, Coomassie blue staining following gel electrophoresis, Lowry protein assay, and Bradford protein assay.

As used herein, a "host cell" is a cell that is used to receive, maintain, reproduce and/or amplify a vector. A host cell also can be used to express the polypeptide encoded by the vector. The nucleic acid contained in the vector is replicated when the host cell divides, thereby amplifying the nucleic acid.

As used herein, a "vector" is a replicable nucleic acid from which one or more heterologous proteins can be expressed when the vector is transformed into an appropriate host cell. Reference to a vector includes those vectors into which a nucleic acid encoding a polypeptide or fragment thereof can be introduced, typically by restriction digest and ligation. Reference to a vector also includes those vectors that contain nucleic acid encoding a polypeptide, such as a modified anti-CTLA-4 antibody. The vector is used to introduce the nucleic acid encoding the polypeptide into the host cell for amplification of the nucleic acid, or for expression/display of the polypeptide encoded by the nucleic acid. The vectors typically remain episomal, but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well-known to those of skill in the art. A vector also includes "virus vectors" or "viral vectors." Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

As used herein, an "expression vector" includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional

segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well-known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, "primary sequence" refers to the sequence of amino acid residues in a polypeptide, or the sequence of nucleotides in a nucleic acid molecule.

As used herein, "sequence identity" refers to the number of identical or similar amino acids or nucleotide bases in a comparison between a test and a reference polypeptide or polynucleotide. Sequence identity can be determined by sequence alignment of nucleic acid or protein sequences to identify regions of similarity or identity. For purposes herein, sequence identity is generally determined by alignment to identify identical residues. The alignment can be local or global. Matches, mismatches and gaps can be identified between compared sequences. Gaps are null amino acids or nucleotides inserted between the residues of aligned sequences so that identical or similar characters are aligned. Generally, there can be internal and terminal gaps. When using gap penalties, sequence identity can be determined with no penalty for end gaps (*e.g.*, terminal gaps are not penalized). Alternatively, sequence identity can be determined without taking into account gaps, as the number of identical positions/length of the total aligned sequence x 100.

As used herein, a "global alignment" is an alignment that aligns two sequences from beginning to end, aligning each letter in each sequence only once. An alignment is produced, regardless of whether or not there is similarity or identity between the sequences. For example, 50% sequence identity based on "global alignment" means that in an alignment of the full sequence of two compared sequences each of 100 nucleotides in length, 50% of the residues are the same. It is understood that global alignment also can be used in determining sequence identity even when the length of

the aligned sequences is not the same. The differences in the terminal ends of the sequences will be taken into account in determining sequence identity, unless the "no penalty for end gaps" is selected. Generally, a global alignment is used on sequences that share significant similarity over most of their length. Exemplary algorithms for performing global alignment include the Needleman-Wunsch algorithm (Needleman *et al.* (1970) *J. Mol. Biol.* 48:443-453). Exemplary programs for performing global alignment are publicly available and include the Global Sequence Alignment Tool available at the National Center for Biotechnology Information (NCBI) website (ncbi.nlm.nih.gov/), and the program available at deepc2.psi.iastate.edu/aat/align/align.html.

As used herein, a "local alignment" is an alignment that aligns two sequences, but only aligns those portions of the sequences that share similarity or identity. Hence, a local alignment determines if sub-segments of one sequence are present in another sequence. If there is no similarity, no alignment will be returned. Local alignment algorithms include BLAST, or the Smith-Waterman algorithm (*Adv. Appl. Math.* 2:482 (1981)). For example, 50% sequence identity based on "local alignment" means that in an alignment of the full sequence of two compared sequences of any length, a region of similarity or identity of 100 nucleotides in length has 50% of the residues that are the same in the region of similarity or identity.

For purposes herein, sequence identity can be determined by standard alignment algorithm programs used with default gap penalties established by each supplier. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986) *Nucl. Acids Res.* 14:6745-6763, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Whether any two nucleic acid molecules have nucleotide sequences, or any two polypeptides have amino acid sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% "identical," or other similar variations reciting a percent identity, can be determined using known computer algorithms based on local or global alignment (see, *e.g.*,

wikipedia.org/wiki/Sequence_alignment_software, providing links to dozens of known and publicly available alignment databases and programs). Generally, for purposes herein sequence identity is determined using computer algorithms based on global alignment, such as the Needleman-Wunsch Global Sequence Alignment tool available from NCBI/BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&Page_TYPE=BlastHome); LAlign (William Pearson implementing the Huang and Miller algorithm (*Adv. Appl. Math.* (1991) 12:337-357)); and the program from Xiaoqui Huang available at deepc2.psi.iastate.edu/aat/align/align.html. Typically, the full-length sequence of each of the compared polypeptides or nucleotides is aligned across the full-length of each sequence in a global alignment. Local alignment also can be used when the sequences being compared are substantially the same length.

Therefore, as used herein, the term "identity" represents a comparison or alignment between a test and a reference polypeptide or polynucleotide. In one non-limiting example, "at least 90% identical to" refers to percent identities from 90% to 100% relative to the reference polypeptide or polynucleotide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide or polynucleotide length of 100 amino acids or nucleotides are compared, no more than 10% (*i.e.*, 10 out of 100) of amino acids or nucleotides in the test polypeptide or polynucleotide differ from those of the reference polypeptide or polynucleotide. Similar comparisons can be made between a test and reference polynucleotide. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence, or they can be clustered in one or more locations of varying length up to the maximum allowable, *e.g.*, 10/100, amino acid differences (approximately 90% identity). Differences also can be due to deletions or truncations of amino acid residues. Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. Depending on the length of the compared sequences, at the level of homologies or identities above about 85-90%, the result can be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

As used herein, a "disease or disorder" refers to a pathological condition in an organism resulting from a cause or condition, including, but not limited to, infections,

acquired conditions, and genetic conditions, and that is characterized by identifiable symptoms.

As used herein, "treating" a subject with a disease or condition means that the subject's symptoms are partially or totally alleviated, or remain static following treatment.

As used herein, "treatment" refers to any effects that ameliorate symptoms of a disease or disorder. Treatment encompasses prophylaxis, therapy and/or cure. Treatment also encompasses any pharmaceutical use of any immunostimulatory bacterium or composition provided herein.

As used herein, "prophylaxis" refers to prevention of a potential disease and/or a prevention of worsening of symptoms or of progression of a disease.

As used herein, "prevention" or prophylaxis, and grammatically equivalent forms thereof, refers to methods in which the risk or probability of developing a disease or condition is reduced.

As used herein, a "pharmaceutically effective agent" includes any therapeutic agent or bioactive agent, including, but not limited to, for example, anesthetics, vasoconstrictors, dispersing agents, and conventional therapeutic drugs, including small molecule drugs and therapeutic proteins.

As used herein, a "therapeutic effect" means an effect resulting from treatment of a subject that alters, typically improves or ameliorates, the symptoms of a disease or condition, or that cures a disease or condition.

As used herein, a "therapeutically effective amount" or a "therapeutically effective dose" refers to the quantity of an agent, compound, material, or composition containing a compound that is at least sufficient to produce a therapeutic effect following administration to a subject. Hence, it is the quantity necessary for preventing, curing, ameliorating, arresting, or partially arresting, a symptom of a disease or disorder.

As used herein, "therapeutic efficacy" refers to the ability of an agent, compound, material, or composition containing a compound to produce a therapeutic effect in a subject to whom the agent, compound, material, or composition containing a compound has been administered.

As used herein, a "prophylactically effective amount" or a "prophylactically effective dose" refers to the quantity of an agent, compound, material, or composition containing a compound, that when administered to a subject, will have the intended prophylactic effect, *e.g.*, preventing or delaying the onset or reoccurrence, of disease or symptoms, reducing the likelihood of the onset or reoccurrence, of disease or symptoms, or reducing the incidence of viral infection. The full prophylactic effect does not necessarily occur by administration of one dose, and can occur only after administration of a series of doses. Thus, a prophylactically effective amount can be administered in one or more administrations.

As used herein, amelioration of the symptoms of a particular disease or disorder by a treatment, such as by administration of a pharmaceutical composition or other therapeutic, refers to any lessening, whether permanent or temporary, lasting or transient, of the symptoms, that can be attributed to or associated with administration of the composition or therapeutic.

As used herein, an "anti-cancer agent" or "an anti-cancer therapeutic" refers to any agent or therapeutic that is destructive or toxic, either directly or indirectly, to malignant cells and tissues. For example, anti-cancer agents include agents that kill cancer cells or otherwise inhibit or impair the growth of tumors or cancer cells. Exemplary anti-cancer agents are chemotherapeutic agents, and immunotherapeutic agents.

As used herein "therapeutic activity" refers to the *in vivo* activity of a therapeutic product, such as a polypeptide, a nucleic acid molecule, and other therapeutic molecules. Generally, the therapeutic activity is the activity that is associated with treatment of a disease or condition.

As used herein, the term "subject" refers to an animal, including a mammal, such as a human being.

As used herein, a patient refers to a human subject.

As used herein, "animal" includes any animal, such as, but not limited to, primates, including humans, gorillas and monkeys; rodents, such as mice and rats; fowl, such as chickens; ruminants, such as goats, cows, deer, and sheep; and pigs and other animals. Non-human animals exclude humans as the contemplated animal. The

polypeptides provided herein are from any source, animal, plant, prokaryotic and fungal. Most polypeptides are of animal origin, including mammalian origin.

As used herein, a "composition" refers to any mixture. It can be a solution, suspension, liquid, powder, paste, aqueous, non-aqueous, or any combination thereof.

As used herein, a "combination" refers to any association between or among two or more items. The combination can be two or more separate items, such as two compositions or two collections, a mixture thereof, such as a single mixture of the two or more items, or any variation thereof. The elements of a combination are generally functionally associated or related.

As used herein, "combination therapy" refers to administration of two or more different therapeutics. The different therapeutic agents can be provided and administered separately, sequentially, intermittently, or can be provided in a single composition.

As used herein, a "kit" is a packaged combination that optionally includes other elements, such as additional reagents and instructions for use of the combination or elements thereof, for a purpose including, but not limited to, activation, administration, diagnosis, and assessment of a biological activity or property.

As used herein, a "unit dose form" refers to physically discrete units suitable for human and animal subjects and packaged individually, as is known in the art.

As used herein, a "single dosage formulation" refers to a formulation for direct administration.

As used herein, a "multi-dose formulation" refers to a formulation that contains multiple doses of a therapeutic agent and that can be directly administered to provide several single doses of the therapeutic agent. The doses can be administered over the course of minutes, hours, weeks, days, or months. Multi-dose formulations can allow dose adjustment, dose-pooling and/or dose-splitting. Because multi-dose formulations are used over time, they generally contain one or more preservatives to prevent microbial growth.

As used herein, an "article of manufacture" is a product that is made and sold. As used throughout this application, the term is intended to encompass any of the compositions provided herein contained in articles of packaging.

As used herein, a "fluid" refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams, and other such compositions.

As used herein, an isolated or purified polypeptide or protein (*e.g.*, an isolated antibody or antigen-binding fragment thereof) or a biologically-active portion thereof (*e.g.*, an isolated antigen-binding fragment), is substantially free of cellular material or other contaminating proteins from the cell or tissue from which the polypeptide or protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), that are used by those of skill in the art to assess such purity, or are sufficiently pure such that further purification does not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, can be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound. As used herein, a "cellular extract" or "lysate" refers to a preparation or fraction which is made from a lysed or disrupted cell.

As used herein, "persistent viral infections" are those in which the virus is not cleared, but remains in specific cells of infected individuals. Persistent infections involve stages of silent and productive infection without rapidly killing or even producing excessive damage of the host cells. There are three types of overlapping persistent virus-host interactions that may be defined as latent, chronic, and slow infections. Diseases caused by persistent viral infections include acquired immunodeficiency syndrome (AIDS), AIDS-related complexes, chronic hepatitis, subacute sclerosing panencephalitis (chronic measles encephalitis), chronic papovavirus encephalitis (progressive multifocal leukoencephalopathy), spongiform encephalopathies (caused by prions), several herpesvirus-induced diseases, and some neoplasias. Viruses that cause these and other infections include, for example, herpesviruses, varicella-zoster virus (VZV), measles virus, human T-cell leukemia

viruses (HTLVs), human immunodeficiency virus (HIV), human papovaviruses, human parvoviruses, human papillomaviruses, hepatitis viruses, adenoviruses, and parvoviruses.

As used herein, a "control" refers to a sample that is substantially identical to the test sample, except that it is not treated with a test parameter, or, if it is a plasma sample, it can be from a normal volunteer not affected with the condition of interest. A control also can be an internal control.

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a polypeptide, comprising "an immunoglobulin domain" includes polypeptides with one or a plurality of immunoglobulin domains.

As used herein, the term "or" is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive.

As used herein, ranges and amounts can be expressed as "about" a particular value or range. "About" also includes the exact amount. Hence, "about 5 amino acids" means "about 5 amino acids" and also "5 amino acids."

As used herein, "optional" or "optionally" means that the subsequently described event or circumstance does or does not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally variant portion means that the portion is variant or non-variant.

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, *Biochem.* (1972) 11(9):1726-1732).

For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow.

## B. OVERVIEW OF IMMUNOSTIMULATORY BACTERIA FOR THERAPY

The recognition that bacteria have anti-cancer activity goes back to the 1800s, when several physicians observed the regression of tumors in patients infected with *Streptococcus pyogenes*. William Coley began the first study utilizing bacteria for the

treatment of end-stage cancers, and developed a vaccine composed of *S. pyogenes* and *Serratia marcescens*, which was successfully used to treat a variety of cancers, including sarcomas, carcinomas, lymphomas and melanomas. Since then, a number of bacterial species, including *Clostridium*, *Mycobacterium*, *Bifidobacterium*, *Listeria monocytogenes and Escherichia*, have been studied as sources of anti-cancer vaccines (See, *e.g.*, International PCT Application Publication Nos. WO 1999/013053 and WO 2001/025399; Bermudes *et al.* (2002) *Curr. Opin. Drug Discov. Devel.* 5:194-199; Patyar *et al.* (2010) *Journal of Biomedical Science* 17:21; and Pawlek *et al.* (2003) *Lancet Oncol.* 4:548-556).

As a therapeutic platform, bacteria have several advantages over other therapies such as oncolytic viruses. Some bacterial species can be engineered to be orally and systemically (intravenously; IV) administered, they propagate readily *in vitro* and *in vivo*, and they can be stored and transported in a lyophilized state. Bacterial chromosomes readily can be manipulated as they lack exons, and the complete genomes for numerous strains have been fully characterized (Felgner *et al.* (2016) *mBio* 7(5):e01220-16). Many types of bacteria are cheaper and easier to produce than viruses, and proper delivery of engineered bacteria can be favorable over viral delivery because they do not permanently integrate into host cell genomes, they preferentially infect myeloid cells over epithelial cells, and they can be rapidly eliminated by antibiotics if necessary, rendering them safe.

Provided herein are immunostimulatory bacteria that are modified to exploit these advantageous properties. The bacteria provided herein are modified so that they infect and accumulate in the tumor microenvironment, particularly in tumor-resident immune cells (myeloid cells), such as tumor-associated macrophages (TAMs), dendritic cells (DCs), and myeloid-derived suppressor cells (MDSCs), and also are designed to express and deliver high levels of therapeutic proteins and combinations, particularly complementary combinations, thereof. As described herein, the immunostimulatory bacteria provided herein can be used as vaccines to prevent and/or treat cancers and also as vaccines against pathogens, including bacterial, viral, parasitic, and other pathogens. These uses rely on the ability of the immunostimulatory bacteria provided herein to accumulate in tumor-resident macrophage, and also, when administered in directly, such as by intramuscular or

inhalation, to accumulate in phagocytic cell, to promote durable immune responses and immunity. The properties of the bacteria that are for treatment of cancer also are advantageous for their use as vaccines. These properties derive from genome modifications that reduce TLR2 and TLR4 and 5 responses, and other modifications, such as auxotrophies that permit growth *in vitro*, but reduce or eliminate growth *in vivo*, and also auxotrophies that can reduce immunosuppressive effects of accumulated nutrients in the tumor microenvironment, such as adenosine, and those genome modifications that eliminate immunosuppressive effects of bacterial enzymes, such as asparaginase, which can inactivate T-cells.

The immunostimulatory bacteria provided herein have advantageous properties that are superior to existing bacterial therapies, and also cell therapies, oncolytic virus therapies, and prior bacterial therapies. The immunostimulatory bacteria provided herein, while they can be administered by any suitable route, are suitable for systemic, such as intravenous, administration. As shown and described herein, the immunostimulatory bacteria provided herein can target major immune pathways.

Provided are immunostimulatory bacteria that can be used or adapted as an anti-cancer therapeutics as well as an anti-cancer vaccines and pathogen vaccines, and also RNA delivery vehicles. The particular use can be selected based on the particular genome modifications and payloads. Provided are immunostimulatory bacteria that delivers a genetic payload encoding one or more therapeutic products, including, for example, a truncated co-stimulatory molecule (receptor or ligand; *e.g.*, 4-1BBL, CD80/CD86, CD27L, B7RP1, OX40L) with a complete or partial cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC), where the truncated gene product is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC, due to a deleted or truncated cytoplasmic domain. The immunostimulatory bacteria can encode a plurality of products include those that constitutively induce type I interferon (IFN) and those that stimulate anti-viral type of immune responses, such as IL-15, particularly IL-15 provided a IL-15/IL-15R alpha chain complex (IL-15 complex), and engineered STING proteins that constitutively

induce type I IFN, and also be modified to have reduced NF-κB signaling to eliminate or reduce undesirable inflammatory responses.

The immunostimulatory bacteria can encode and express one or more of IL-2, IL-7, IL-12p70 (IL-12p40 + IL-12p35), IL-12, IL-15, IL-15/IL-15Rα chain complex, IL-18, IL-21, IL-23, IL-36γ, interferon-α, interferon-β, IL-2 that has attenuated binding to IL-2Ra, IL-2 that is modified so that it does not bind to IL-2Ra, CXCL9, CXCL10, CXCL11, CCL3, CCL4, CCL5, cytosolic DNA/RNA sensors or type I IFN pathway proteins, such as gain-of-function of constitutively active STING, IRF3, IRF7, MDA5, or RIG-I variants (that induce Type I IFN), inhibitors of TGF-beta, such as TGF-β inhibitory antibodies, TGF-beta polypeptide antagonists, and TGF-beta binding decoy receptors, antibodies and fragments thereof, such as those targeting immune checkpoints and other anti-cancer targets such as VEGF and IL-6, co-stimulatory receptors/molecules, such as 4-1BBL, including 4-1BBL with the cytoplasmic domain deleted or truncated or otherwise eliminated, and others. The immunostimulatory bacteria also can encode and express a truncated co-stimulatory molecule (*e.g.*, 4-1BBL, CD80/CD86, CD27L, B7RP1, OX40L) with a complete or partial cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC), where the truncated gene product is capable of constitutive immuno-stimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC, due to a deleted or truncated cytoplasmic domain. Other encoded therapeutic products include those referred to as bispecific T-cell engagers (commercially available under the trademark BiTEs®), such as DLL3 X CD3 engagers, exemplified herein.

Combinations of such therapeutic products and agents can be expressed in a single therapeutic composition. By virtue of the modifications of the bacterial genome, the immunostimulatory bacteria exhibit tumor-specific localization and enrichment, and provide intravenous (IV) administration for activation of anti-tumor immune pathways that are otherwise toxic if systemically activated.

The immunostimulatory bacteria provided herein are genetically designed to be safe and to target tumors, the tumor microenvironment, and/or tumor-resident immune cells, and also to phagocytic cells when administered as vaccines, such as by direct administration. The immunostimulatory bacteria provided herein include a

combination of genomic modifications and other modifications, as well as encoded therapeutic products, that function in concert to provide immunostimulatory bacteria that accumulate in tumor-resident immune cells, and that persist sufficiently long enough to deliver therapeutic products, particularly combinations that induce or promote anti-cancer immune stimulation in tumors and the tumor microenvironment, without toxic side-effects, or with limited toxic side-effects. When delivered systemically, such as intravenously (IV), the immunostimulatory bacteria enrich in tumors, including in metastatic lesions; they provide efficient genetic transfer of immune payloads, specifically to tumor-resident myeloid cells, including tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and dendritic cells (DCs); they induce powerful, local immune responses, destroying tumors and vaccinating against future recurrence; and, when therapy is finished, they are naturally eliminated, such as by phagocytosis and destruction by the infected cells, or they can be destroyed rapidly by a course of antibiotics.

The immunostimulatory bacteria provided herein exhibit preferential accumulation in the tumor microenvironment and/or in tumor-resident immune cells due to a designed purine/adenosine auxotrophy, and exhibit an inability to replicate inside of phagocytic cells. Immunostimulatory bacteria that avoid inactivation by serum complement allow for the delivery of a variety of immunotherapeutic agents and therapeutic products at high concentrations, directly within the tumor microenvironment, while minimizing toxicity to normal tissues, and are provided herein.

For example, as described in more detail, such as in section C.9., the immunostimulatory bacteria provided herein include one or more modifications of the genome that render them *msbB*$^-$/*pagP*$^-$, which alters the lipid A in LPS, resulting in penta-acylation (wild-type lipid A has 6-7 fatty acid chains), reducing the TLR4 affinity; are adenosine/adenine auxotrophs, such as *purI*$^-$; are asparaginase II$^-$ (*ansB*$^-$), which improves T-cell quality; are lacking in flagella (flagellin deficient), are deficient in genes/products that produce curli fimbriae, such as *csgD*$^-$, which, among other properties, removes curli fimbriae; and include other optional genomic modifications, such as insertions, deletions, disruptions, and any other modification, so that the encoded product(s) is(are) not produced in active form, as discussed in

detail herein. The immunostimulatory bacteria include a plasmid that encodes one or more therapeutic products, particularly anti-cancer products, under control of a eukaryotic promoter. These same modifications and properties render them useful as vaccines and as delivery vehicles for RNA, for use as cancer therapeutics and cancer vaccines, and pathogen vaccines, particularly when delivered by direct administration, such as by inhalation, and IM, and other direct routes for delivery of payloads to phagocytic cells.

The immunostimulatory bacteria provided herein include genome modifications, such as deletions, disruptions, and other alterations that result in inactive encoded product(s), such as changing the orientation of all or part of the gene, so that functional gene product(s) is/are not expressed. Among the immunostimulatory bacteria provided are those that are modified so that the resulting bacteria are *msbB*$^-$/*purI*$^-$. In some embodiments, the bacteria are *msbB*$^-$ and *purI*$^-$, whereby the full length of at least the coding portion of the *msbB* and/or *purI* genes are/is deleted. The genome of the bacteria also can be modified so that the bacteria lack flagella. This is effected in bacteria that normally express flagella. In such bacteria, for example the *fliC* and *fljB* genes in *Salmonella*, or equivalent genes in other species to *fliC* and *fljB*, can be deleted or otherwise modified so that functional gene product is not expressed. The bacteria also can be modified so that they are adenosine auxotrophs, and/or are *msbB*$^-$/*pagP*$^-$. Also provided are immunostimulatory bacteria and pharmaceutical compositions containing them, where the bacteria do not express L-asparaginase II, whereby the bacteria are *ansB*$^-$. Elimination of the encoded asparaginase activity improves or retains T-cell viability/activity. Therapeutic bacteria, such as inactivated or attenuated bacteria that are used as vaccines, can be improved by modifying the genome to eliminate asparaginase activity. Exemplary of such vaccines is the Bacillus Calmette-Guérin (BCG) vaccine and related vaccines, used to immunize against tuberculosis. The BCG vaccine is known to have variable effectiveness; eliminating the asparaginase can improve the effectiveness of such vaccine because the endogenouse bacterial asparaginase inhibits or reduces T-cell activity.

The immunostimulatory bacteria provided herein, that deliver therapeutic products (such as constitutively active STING variants and other immunomodulatory

proteins and products), to the tumor-resident myeloid cells promote adaptive immunity and enhance T-cell function. The immunostimulatory bacteria lead to a complete remodeling of the immunosuppressive tumor microenvironment, towards an adaptive anti-tumor phenotype, and away from a bacterial phenotype, which is characterized by the promotion of innate immunity and the suppression of adaptive immunity. As described herein, these properties and payloads also can be exploited for use of the bacteria as vaccines and RNA delivery platforms.

Immunostimulatory bacteria provided herein can exhibit significantly more, such as at least about 100,000-fold greater, tumor infiltration and enrichment, compared to unmodified bacteria, or compared to strain VNP20009. For example, the bacteria provided herein contain genome modifications whereby they infect macrophage in tumors (and other phagocytic cells when administered as vaccines) to deliver their payload, such as the combination of engineered STING (see discussion throughout regarding the various engineered STING proteins, such as the human STING with gain-of-function mutations, such as N154S/R284G, to render induction of type I IFN constitutive, and the CTT from a non-human STING that has lower NF-κB signaling activity, such as the CTT from Tasmanian devil, compared to human STING, and IL-15 in various forms, particularly IL-15/IL-15R alpha chain complex (IL-15 complex). It is shown herein that this combination has synergistic effects, and results in high degree of T-cell, including CD4+ and CD8+ cells, infiltration of tumors. This combination is comprehensive immunotherapy by virtue of acting at numerous therapeutic intervention points. The immunostimulatory bacteria are consumed by tumor-activated macrophages (TAMs) delivering the plasmid into the host cells, which express the encoded products, such as the IL-15 and engineered STING.

The immunostimulatory bacteria provided herein are consumed by tumor-resident immune cells, and deliver their contents, including the plasmid encoding thetherapeutic products, which are expressed and produced in the immune cells and tumor microenvironment, to generate anti-tumor immunity. The bacteria that are *asd*$^-$ and that do not include a complementing gene encoded on the plasmid for expression under host cell control, do not replicate in the host. Bacteria are provided that are *thyA*$^-$, by virtue of genome modifications, such as deletion, insertion, transposition, or

modification resulting in inactive or missing gen product, requiring thymine supplementation for growth, similarly deliver payloads into phagocytic cells, but cannot replicate in such cells.

**1. Bacterial Cancer Immunotherapy**

Many solid tumor types have evolved a profoundly immunosuppressive microenvironment that renders them highly refractory to approved checkpoint therapies, such as anti-CTLA-4, anti-PD-1 and anti-PD-L1 therapies. One mechanism by which tumors have evolved resistance to checkpoint therapies is through their lack of intratumoral T-cells and tumor antigen cross-presenting dendritic cells (DCs), described as T-cell excluded, non-inflamed, or "cold tumors" (Sharma *et al.* (2017) *Cell* 168(4):707-723). For the small number of patients whose tumors are T-cell inflamed and respond to checkpoint immunotherapies, they often experience severe autoimmune toxicities, and many will eventually relapse and become checkpoint refractory (see, *e.g.*, Buchbinder *et al.* (2015) *J. Clin. Invest.* 125:3377-3383; Hodi *et al.* (2010) *N. Engl. J. Med.* 363(8):711-723; and Chen *et al.* (2015) *J. Clin. Invest.* 125:3384-3391). Tumors initiate multiple mechanisms to evade immune surveillance, reprogram anti-tumor immune cells to suppress immunity, exclude and inactivate anti-tumor T-cells, and develop emerged resistance to the targeted cancer therapies (see, *e.g.*, Mahoney *et al.* (2015) *Nat. Rev. Drug Discov.* 14(8):561-584). Solving this problem will require immunotherapies that can properly inflame these tumors, and generate anti-tumor immunity that can provide long-lasting tumor regressions. In addition, intratumoral therapies are intractable and will be quite limiting in a metastatic disease setting. Systemically-administered therapies that properly inflame each individual metastatic lesion and overcome multiple pathways of immunosuppression are required. By virtue of their ability to specifically target tumor-resident immune cells, and to express multiple complementary genetic payloads/therapeutic products, the immunostimulatory bacteria provided herein are designed to address these issues.

**2. Prior Therapies that Target the Tumor Microenvironment**

A number of therapies that target the tumor microenvironment (TME) and attempt to promote anti-tumor immunity have been developed. Each has its own

challenges and shortcomings, which are addressed by the immunostimulatory bacteria provided herein.

**a. Limitations of Autologous T-Cell Therapies**

Several systemically-administered therapeutic platforms have been investigated clinically, with the goal of accessing the highly immunosuppressive tumor microenvironment and inducing the proper immune responses to inflame tumors and promote anti-tumor immunity. These platforms include chimeric antigen receptors T-cells (CAR-T cells), which are produced by harvesting T-cells from patients and re-engineering them to fuse the T-cell receptor to an antibody Ig variable extracellular domain specific for a particular tumor antigen. This confers upon the cells the antigen-recognition properties of antibodies with the cytolytic properties of activated T-cells (see, *e.g.*, Sadelain *et al.* (2015) *J. Clin. Invest.* 125(9):3392-3400). Despite the promise and potency of this technology, such as the FDA approvals of the CD19 CAR-Ts tisagenlecleucel (such as those under the trademark Kymriah®) and axicabtagene ciloleucel (under the trademark Yescarta®), success has been limited to $CD19^+$ hematopoietic malignancies, and at the cost of deadly immune-related adverse events (see, *e.g.*, Jackson *et al.* (2016) *Nat. Rev. Clin. Oncol.* 13(6):370-383). Tumors can mutate rapidly to downregulate the targeted tumor antigens for solid tumors, including the antigen CD19, thereby fostering immune escape (see, *e.g.*, Mardiana *et al.* (2019) *Sci. Transl. Med.* 11(495):eaaw2293). There is not a plethora of tumor-specific target antigens. Solid tumor targets that are not expressed in healthy tissue are a major impediment to CAR-T therapy. Beyond that, CAR-T therapies suffer from other impediments to accessing solid tumor microenvironments, due to the lack of sufficient T-cell chemokine gradients, which are required for proper T-cell infiltration into tumors. In addition, once they have infiltrated tumors, they are rapidly inactivated (see, *e.g.*, Brown *et al.* (2019) *Nat. Rev. Immunol.* 19(2):73-74). Should the safety of CAR-T cells be significantly improved and the efficacy expanded to solid tumors, the feasibility and costs associated with these labor-intensive therapies still limit their broader adoption.

**b. Viral Vaccine Platforms**

Oncolytic viruses (OVs) have natural and engineered properties to induce tumor cell lysis, recruit T-cells to the tumor, and deliver genetic material that can be

read by tumor cells to produce immunomodulatory proteins. For example, the oncolytic virus designated Talimogene laherparepvec (T-VEC), is a modified herpes simplex virus encoding anti-melanoma antigens and the cytokine GM-CSF (granulocyte-macrophage colony-stimulating factor), that is intratumorally administered. It is FDA-approved for metastatic melanoma (see, *e.g.*, Bastin *et al.* (2016) *Biomedicines* 4(3):21). T-VEC has demonstrated clinical benefit for some melanoma patients, and with fewer immune toxicities than the immune checkpoint antibodies or the FDA-approved systemic cytokines, such as IL-2 and interferon-alpha (see, *e.g.*, Kim *et al.* (2006) *Cytokine Growth Factor Rev.* 17(5):349-366; and Paul *et al.* (2015) *Gene* 567(2):132-137).

Oncolytic viruses (OVs) possess a number of limitations as anti-cancer therapies. First, oncolytic viruses are rapidly inactivated by the human complement system in blood. It has proven difficult to deliver enough virus through systemic administration to have a desired therapeutic effect. Intratumoral delivery is limiting in a metastatic setting (where lesions are spread throughout the body), is intractable for most solid tumor types (*e.g.*, lung and visceral lesions), and requires interventional, guided radiology for injection, which limits repeat dosing. Viruses can be difficult to manufacture at commercial scale and to store. Most OV-based vaccines, such as those based on paramyxovirus, reovirus and picornavirus, among others, have similar limitations (see, *e.g.*, Chiocca *et al.* (2014) *Cancer Immunol. Res.* 2(4):295-300). Oncolytic viruses are inherently immunogenic and rapidly cleared from human blood, and T-cells that traffic into the tumor have a much higher affinity for viral antigens over weaker tumor neoantigens (see, *e.g.*, Aleksic *et al.* (2012) *Eur. J. Immunol.* 42(12):3174-3179). Thus, in addition to the recognized technical limitations of the platform, OVs can have limited capacity to stimulate durable anti-tumor immunity (see, *e.g.*, Kedl *et al.*(2003) *Curr Opinion Immunol. 15*:120-127; and Aleksic *et al.*, (2012) *Eur. J. Immunol. 42*:3174-3179, which show that TCRs that bind viral antigens have higher HLA-A2 affinity that those that bind cancer related antigens).

**c. Bacterial Cancer Therapies**

A number of bacterial species have demonstrated preferential replication within solid tumors when injected from a distal site in preclinical animal studies. These include, but are not limited to, species of *Salmonella*, *Bifodobacterium*,

*Clostridium*, and *Escherichia*. The tumor-homing properties of the bacteria, combined with the host's innate immune response to the bacterial infection, can mediate an anti-tumor response. This tumor tissue tropism reduces the size of tumors to varying degrees. One contributing factor to the tumor tropism of these bacterial species is the ability to replicate in anoxic and hypoxic environments. A number of these naturally tumor-tropic bacteria have been further engineered to increase the potency of the anti-tumor response (reviewed in Zu *et al.* (2014) *Crit. Rev. Microbiol.* 40(3):225–235; and Felgner *et al.* (2017) *Microbial Biotechnology* 10(5):1074–1078). Despite proof-of-concept in animal studies, complement factors in human serum, that are not present in animal models, can inactivate the bacteria, limiting their use as therapies to treat cancer.

To be administered orally or systemically, the bacterial strains are attenuated so that they do not cause systemic disease and/or septic shock, but still maintain some level of infectivity for effective tumor colonization, and resistance to inactivation by complement. A number of different bacterial species, including *Clostridium* (see, *e.g.*, Dang *et al.* (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98(26):15155-15160; U.S. Patent Publication Nos. 2017/0020931 and 2015/0147315; and U.S. Patent Nos. 7,344,710 and 3,936,354), *Mycobacterium* (see, *e.g.*, U.S. Patent Publication Nos. 2015/0224151 and 2015/0071873), *Bifidobacterium* (see, *e.g.*, Dang *et al.* (2001); and Kimura *et al.* (1980) *Cancer Res.* 40:2061-2068), *Lactobacillus* (see, *e.g.*, Dang *et al.* (2001)), *Listeria monocytogenes* (see, *e.g.*, Le *et al.* (2012) *Clin. Cancer Res.* 18(3):858-868; Starks *et al.* (2004) *J. Immunol.* 173:420-427; and U.S. Patent Publication No. 2006/0051380) and *Escherichia coli* (see, *e.g.*, U.S. Patent No. 9,320,787), have been studied as possible agents for anti-cancer therapy.

The immunostimulatory bacteria provided herein include genome modifications that address problems with prior bacteria developed for treating tumors. Modifications generally include changes in the genome that render a gene or gene product inactive. This can be effected by deleting a gene or a portion thereof, or disrupting a gene, or any other such change that results in an inactive product. The genome modifications improve the targeting to or accumulation of bacteria in the tumor microenvironment, and in particular, are designed so that the bacteria preferentially or only infect tumor-resident immune cells and do not infect healthy

tissues, thereby decreasing toxicity and improving delivery of encoded products. The immunostimulatory bacteria also are designed to deliver therapeutic products, including combinations thereof, designed to eliminate immune suppressive effects of tumors, enhance a host's anti-tumor response, and provide anti-tumor products.

**i. *Listeria***

*Listeria monocytogenes*, a live attenuated intracellular bacterium capable of inducing potent $CD8^+$ T-cell priming to expressed tumor antigens in mouse models of cancer, has also been explored as a bacterial cancer vector (see, *e.g.*, Le *et al.* (2012) *Clin. Cancer Res.* 18(3):858-868). In a clinical trial of the *L. monocytogenes*-based vaccine incorporating the tumor antigen mesothelin, together with an allogeneic pancreatic cancer-based GVAX vaccine in a prime-boost approach, a median survival of 6.1 months was noted in patients with advanced pancreatic cancer, versus a median survival of 3.9 months for patients treated with the GVAX vaccine alone (see, *e.g.*, Le *et al.* (2015) *J. Clin. Oncol.* 33(12):1325-1333). These results were not replicated in a larger phase 2b study, however, pointing to the difficulties in humans of subverting peripheral immune surveillance towards low affinity tumor neoantigens. *L. monocytogenes* also has shown limited immune responses to the encoded tumor antigens due to the requirement for bacteria to be lysed after phagocytosis, a pre-requisite to efficient plasmid transfer, which has not been demonstrated to occur by *L. monocytogenes* in human macrophages.

**ii. *Salmonella* Species**

*Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is exemplary of a bacterial species for use as an anti-cancer therapeutic. *S. typhimurium* is a Gram-negative facultative anaerobe, which preferentially accumulates in hypoxic and necrotic areas due to the availability of nutrients from tissue necrosis, the leaky tumor vasculature, and their increased likelihood to survive in the immunosuppressed tumor microenvironment (see, *e.g.*, Baban *et al.* (2010) *Bioengineered Bugs* 1(6):385-394). As a facultative anaerobe, *S. typhimurium* is able to grow under aerobic and anaerobic conditions, and is therefore able to colonize both small tumors that are less hypoxic, and large tumors that are more hypoxic.

*S. typhimurium* transmission through the fecal-oral route causes localized gastrointestinal infections. The bacterium can also enter the bloodstream and

lymphatic system, infecting systemic tissues such as the liver, spleen and lungs. Systemic administration of wild-type *S. typhimurium* overstimulates TNF-α and IL-6, leading to a cytokine cascade and septic shock, which, if left untreated, can be fatal. As a result, pathogenic bacterial strains, such as *S. typhimurium*, must be attenuated to prevent systemic infection, without completely suppressing their ability to effectively colonize tumor tissues. Attenuation often is achieved by mutating a cellular structure that can elicit an immune response through pathogen pattern recognition, such as the bacterial outer membrane, or by limiting the bacterium's ability to replicate in the absence of supplemental nutrients.

*S. typhimurium* is an intracellular pathogen that is rapidly taken up by phagocytic myeloid cells such as macrophages, or it can directly invade non-phagocytic cells, such as epithelial cells, through its *Salmonella* pathogenicity island 1 (SPI-1)-encoded type III secretion system (T3SS1). Once inside cells, it can replicate within a *Salmonella*-containing vacuole (SCV) through SPI-2 regulation, and can also escape into the cytosol of some epithelial cells (see, *e.g.*, Agbor *et al.* (2011) *Cell Microbiol.* 13(12):1858-1869; and Galan and Wolf-Watz (2006) *Nature* 444:567-573). Genetically modified bacterial strains of *S. typhimurium* have been described as anti-tumor agents to elicit direct tumoricidal effects and/or to deliver tumoricidal molecules (see, *e.g.*, Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002; Bermudes, D. *et al.* (2002) *Curr. Opin. Drug Discov. Devel.* 5:194-199; Zhao, M. *et al.* (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102:755-760; and Zhao, M. *et al.* (2006) *Cancer Res.* 66:7647-7652).

Various methods for attenuation of bacterial pathogens are known in the art. Auxotrophic mutations, for example, render bacteria incapable of synthesizing an essential nutrient, and deletions/mutations in genes such as *aro*, *pur*, *gua*, *thy*, *nad* and *asd* (see, *e.g.*, U.S. Patent Publication No. 2012/0009153) are used. Nutrients produced by the biosynthesis pathways involving these genes are often unavailable in host cells, and as such, bacterial survival is challenging. For example, attenuation of *Salmonella* and other species can be achieved by deletion or disruption of the *aroA* gene, which is part of the shikimate pathway, connecting glycolysis to aromatic amino acid biosynthesis (see, *e.g.*, Felgner *et al.* (2016) *mBio* 7(5):e01220-16). Deletion or disruption of *aroA* results in bacterial auxotrophy for aromatic amino acids and

subsequent attenuation (see, *e.g.*, U.S. Patent Publication Nos. 2003/0170276, 2003/0175297, 2012/0009153, and 2016/0369282; and International Application Publication Nos. WO 2015/032165 and WO 2016/025582). Similarly, other enzymes involved in the biosynthesis pathway for aromatic amino acids, including *aroC* and *aroD* have been deleted to achieve attenuation (see, *e.g.*, U.S. Patent Publication No. 2016/0369282; and International Application Publication No. WO 2016/025582). For example, *S. typhimurium* strain SL7207 is an aromatic amino acid auxotroph (*aroA*⁻ mutant), and strains A1 and A1-R are leucine-arginine auxotrophs.

Mutations that attenuate bacteria also include, but are not limited to, mutations in genes that alter the biosynthesis of lipopolysaccharide (LPS), such as *rfaL*, *rfaG*, *rfaH*, *rfaD*, *rfaP*, *rFb*, *rfa*, *msbB*, *htrB*, *firA*, *pagL*, *pagP*, *lpxR*, *arnT*, *eptA*, *and lpxT*; mutations that introduce a suicide gene, such as *sacB*, *nuk*, *hok*, *gef*, *kil*, or *phlA*; mutations that introduce a bacterial lysis gene, such as *hly* and *cly*; mutations in genes that encode virulence factors, such as *IsyA*, *pag*, *prg*, *iscA*, *virG*, *plc*, and *act*; mutations in genes that modify the stress response, such as *recA*, *htrA*, *htpR*, *hsp*, and *groEL*; mutations in genes that disrupt the cell cycle, such as *min*; and mutations in genes that disrupt or inactivate regulatory functions, such as *cya*, *crp*, *phoP/phoQ*, and *ompR* (see, *e.g.*, U.S. Patent Publication Nos. 2012/0009153, 2003/0170276, and 2007/0298012; U.S. Patent No. 6,190,657; International Application Publication No. WO 2015/032165; Felgner *et al.* (2016) *Gut Microbes* 7(2):171-177; Broadway *et al.* (2014) *J. Biotechnology* 192:177-178; Frahm *et al.* (2015) *mBio* 6(2):e00254-15; Kong *et al.* (2011) *Infection and Immunity* 79(12):5027-5038; and Kong *et al.* (2012) *Proc. Natl. Acad. Sci. U.S.A.* 109(47):19414-19419). In general, attenuating mutations are gene deletions to prevent spontaneous compensatory mutations that might result in reversion to a virulent phenotype.

Another way to attenuate *S. typhimurium* for safety is to use the PhoP/PhoQ operon system, which is a typical bacterial two-component regulatory system, composed of a membrane-associated sensor kinase (PhoQ), and a cytoplasmic transcriptional regulator (PhoP) (see, *e.g.*, Miller, S. I. *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5054-5058; and Groisman, E. A. *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:7077-7081). PhoP/PhoQ is required for virulence; its deletion results in poor survival of this bacterium in macrophages, and a marked attenuation in mice and

humans (see, *e.g.*, Miller, S. I. *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5054-5058; Groisman, E. A. *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:7077-7081; Galan, J. E. and Curtiss, R. III. (1989) *Microb. Pathog.* 6:433-443; and Fields, P. I. *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:5189-5193). PhoP/PhoQ deletion strains have been employed as vaccine delivery vehicles (see, *e.g.*, Galan, J. E. and Curtiss, R. III. (1989) *Microb. Pathog.* 6:433-443; Fields, P. I. *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:5189-5193; and Angelakopoulos, H. and Hohmann, E. L. (2000) *Infect. Immun.* 68:2135-2141). As described herein, however, it is disadvantageous for a strain to have limited survival in macrophages if the bacteria are not attempting to transfer plasmids.

These attenuated bacterial strains have been found to be safe in mice, pigs, and monkeys when administered intravenously (IV) (see, *e.g.*, Zhao, M. *et al.* (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102:755-760; Zhao, M. *et al.* (2006) *Cancer Res.* 66:7647-7652; Tjuvajev J. *et al.* (2001) *J. Control. Release* 74:313-315; and Zheng, L. *et al.* (2000) *Oncol. Res.* 12:127-135), and certain live attenuated *Salmonella* strains have been shown to be well tolerated after oral administration in human clinical trials (see, *e.g.*, Chatfield, S. N. *et al.* (1992) *Biotechnology* 10:888-892; DiPetrillo, M. D. *et al.* (1999) *Vaccine* 18:449-459; Hohmann, E. L. *et al.* (1996) *J. Infect. Dis.* 173:1408-1414; and Sirard, J. C. *et al.* (1999) *Immunol. Rev.* 171:5-26).

Other strains of *S. typhimurium* that have been attenuated for therapy are, for example, the leucine-arginine auxotroph A-1 (see, *e.g.*, Zhao *et al.* (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102(3):755-760; Yu *et al.* (2012) *Scientific Reports* 2:436; U.S. Patent No. 8,822,194; and U.S. Patent Publication No. 2014/0178341) and its derivative AR-1 (see, *e.g.*, Yu *et al.* (2012) *Scientific Reports* 2:436; Kawaguchi *et al.* (2017) *Oncotarget* 8(12):19065-19073; Zhao *et al.* (2006) *Cancer Res.* 66(15):7647-7652; Zhao *et al.* (2012) *Cell Cycle* 11(1):187-193; Tome *et al.* (2013) *Anticancer Research* 33:97-102; Murakami *et al.* (2017) *Oncotarget* 8(5):8035-8042; Liu *et al.* (2016) *Oncotarget* 7(16):22873-22882; and Binder *et al.* (2013) *Cancer Immunol. Res.* 1(2):123-133); the *aroA*$^-$ mutant *S. typhimurium* strain SL7207 (see, *e.g.*, *Guo et al.* (2011) *Gene Therapy* 18:95-105; and U.S. Patent Publication Nos. 2012/0009153, 2016/0369282 and 2016/0184456), and its obligate anaerobe derivative YB1 (see, *e.g.*, International Application Publication No. WO 2015/032165; Yu *et al.* (2012)

*Scientific Reports* 2:436; and Leschner *et al.* (2009) *PLoS ONE* 4(8):e6692); the *aroA*$^-$*/aroD*$^-$ mutant *S. typhimurium* strain BRD509, a derivative of the SL1344 (wild-type) strain (see, *e.g.*, Yoon *et al.* (2017) *Eur. J. Cancer* 70:48-61); the *asd*$^-$*/cya*$^-$*/crp*$^-$ mutant *S. typhimurium* strain χ4550 (see, *e.g.*, Sorenson *et al.* (2010) *Biologics: Targets & Therapy* 4:61-73) and the *phoP*$^-$*/phoQ*$^-$ *S. typhimurium* strain LH430 (see, *e.g.*, International Application Publication No. WO 2008/091375).

Attenuation, however, impacts the ability of the bacteria to accumulate in tumor-resident immune cells, the tumor microenvironment, and tumor cells. This problem is solved herein. The immunostimulatory bacteria, such as the *Salmonella* strains exemplified herein, are attenuated by virtue of modifications, that can include some of those described above, but also have other modifications and properties described herein that enhance the effectiveness as a cancer therapeutic.

Attenuated strains of *S. typhimurium* possess the innate ability to deliver DNA following phagocytosis and degradation (see, *e.g.*, Weiss, S. (2003) *Int. J. Med. Microbiol.* 293(1):95-106). They have been used as vectors for gene therapy. For example, *S. typhimurium* strains have been used to deliver and express a variety of genes, including those that encode cytokines, angiogenesis inhibitors, toxins and prodrug-converting enzymes (see, *e.g.*, U.S. Patent Publication No. 2007/0298012; Loeffler *et al.* (2008) *Cancer Gene Ther.* 15(12):787-794; Loeffler *et al.* (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104(31):12879-12883; Loeffler *et al.* (2008) *J. Natl. Cancer Inst.* 100:1113-1116; Clairmont, C. *et al.* (2000) *J. Infect. Dis.* 181:1996-2002; Bermudes, D. *et al.* (2002) *Curr. Opin. Drug Discov. Devel.* 5:194-199; Zhao, M. *et al.* (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102:755-760; Zhao, M. *et al.* (2006) *Cancer Res.* 66:7647-7652; and Tjuvajev J. *et al.* (2001) *J. Control. Release* 74:313-315).

*S. typhimurium* has been modified to deliver the tumor-associated antigen (TAA) survivin (SVN) to antigen presenting cells (APCs) to prime adaptive immunity (see, *e.g.*, U.S. Patent Publication No. 2014/0186401; and Xu *et al.* (2014) *Cancer Res.* 74(21):6260-6270). SVN is an inhibitor of apoptosis protein (IAP), which prolongs cell survival and provides cell cycle control, and is overexpressed in all solid tumors and poorly expressed in normal tissues. This technology uses SPI-2 and its type III secretion system to deliver the TAAs into the cytosol of APCs, which then are activated to induce TAA-specific $CD8^+$ T-cells and anti-tumor immunity (see, *e.g.*, Xu

*et al.* (2014) *Cancer Res.* 74(21):6260-6270). Similar to the *Listeria*-based TAA vaccines, this approach has shown promise in mouse models, but has not demonstrated effective tumor antigen-specific T-cell priming in humans.

In addition to the delivery of DNA that encodes proteins, *S. typhimurium* also has been used for the delivery of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) for cancer therapy. For example, attenuated *S. typhimurium* has been modified to express certain shRNAs, such as those that target the immunosuppressive gene indolamine dioxygenase (IDO). Silenced IDO expression in a murine melanoma model resulted in tumor cell death and significant tumor infiltration by neutrophils (see, *e.g.*, Blache *et al.* (2012) *Cancer Res.* 72(24):6447-6456; International Application Publication No. WO 2008/091375; and U.S. Patent No. 9,453,227). Co-administration of this vector with a hyaluronidase showed positive results in the treatment of murine pancreatic ductal adenocarcinoma (see, *e.g.*, Manuel *et al.* (2015) *Cancer Immunol. Res.* 3(9):1096-1107; and U.S. Patent Publication No. 2016/0184456). In another study, an *S. typhimurium* strain attenuated by a *phoP/phoQ* deletion, and expressing a signal transducer and activator of transcription 3 (STAT3)-specific shRNA, inhibited tumor growth and reduced the number of metastatic organs, extending the life of C57BL/6 mice (see, *e.g.*, Zhang *et al.* (2007) *Cancer Res.* 67(12):5859-5864). In another example, *S. typhimurium* strain SL7207 has been used for the delivery of shRNA targeting *CTNNB1*, the gene that encodes β-catenin (see, *e.g.*, Guo *et al.* (2011) *Gene Therapy* 18:95-105; and U.S. Patent Publication Nos. 2009/0123426 and 2016/0369282). The *S. typhimurium* strain VNP20009 has been used for the delivery of shRNA targeting STAT3 (see, *e.g.*, Manuel *et al.* (2011) *Cancer Res.* 71(12):4183-4191; U.S. Patent Publication Nos. 2009/0208534, 2014/0186401 and 2016/0184456; and International Application Publication Nos. WO 2008/091375 and WO 2012/149364). siRNAs targeting the autophagy genes *Atg5* and *Beclin1* have been delivered to tumor cells using *S. typhimurium* strains A1-R and VNP20009 (see, *e.g.*, Liu *et al.* (2016) *Oncotarget* 7(16):22873-22882).

It has been found, however, that these strains do not effectively stimulate an anti-tumor immune response, nor effectively colonize tumors for delivery of therapeutic doses of encoded products. Improvement of such strains is needed so that they more effectively stimulate an anti-tumor immune response, such as the

immunostimulatory bacteria provided herein. Further and alternative modifications of various bacteria have been described in published International PCT Application No. WO 2019/014398 and in U.S. Publication No. 2019/0017050 A1. The bacteria described in each of these publications, also described herein, can be modified as described herein to further improve their immunostimulatory and tumor-targeting properties.

**iii. VNP20009 (YS1646)**

Exemplary of a therapeutic bacterium that can be used as a starting strain for modification as described herein is the strain designated as VNP20009 (ATCC # 202165, YS1646). This virus was a clinical candidate. VNP20009 (ATCC # 202165, YS1646) was at least 50,000-fold attenuated for safety by deletion of the *msbB* and *purI* genes (see, *e.g.*, Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002; Low *et al.* (2003) *Methods in Molecular Medicine*, Vol. 90, *Suicide Gene Therapy: Methods and Reviews*, pp. 47-59; and Lee *et al.* (2000) *International Journal of Toxicology* 19:19-25). Deletion or disruption to prevent expression of the *msbB* gene alters the composition of the lipid A domain of lipopolysaccharide, the major component of Gram-negative bacterial outer membranes (see, *e.g.*, Low *et al.* (1999) *Nat. Biotechnol.* 17(1):37-41). This prevents lipopolysaccharide-induced septic shock, attenuating the bacterial strain and lowering systemic toxicity, while reducing the potentially harmful production of TNFα (see, *e.g.*, Dinarello, C.A. (1997) *Chest* 112(6 Suppl):321S-329S; and Low *et al.* (1999) *Nat. Biotechnol.* 17(1):37-41). Deletion or disruption to prevent expression of the *purI* gene renders the bacteria auxotrophic for purines, which further attenuates the bacteria and enriches them in the tumor microenvironment (see, *e.g.*, Pawelek *et al.* (1997) *Cancer Res.* 57:4537-4544; and Broadway *et al.* (2014) *J. Biotechnology* 192:177-178). As shown herein, VNP20009 also is auxotrophic for the immunosuppressive nucleoside adenosine. Adenosine can accumulate to pathologically high levels in the tumor and contribute to an immunosuppressive tumor microenvironment (see, *e.g.*, Peter Vaupel and Arnulf Mayer, *Oxygen Transport to Tissue XXXVII*, Advances in Experimental Medicine and Biology 876 chapter 22, pp. 177-183).

When VNP20009 was administered into mice bearing syngeneic or human xenograft tumors, the bacteria accumulated preferentially within the extracellular

components of tumors at ratios exceeding 300-1000 to 1, and demonstrated tumor growth inhibition, as well as prolonged survival compared to control mice (see, *e.g.*, Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002). VNP20009 demonstrated success in tumor targeting and tumor growth suppression in animal models, while eliciting very little toxicity (see, *e.g.*, Broadway *et al.* (2014) *J. Biotechnology* 192:177-178; Loeffler *et al.* (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104(31):12879-12883; Luo *et al.* (2002) *Oncology Research* 12:501-508; and Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002).

Results from the Phase 1 clinical trial in human metastatic melanoma revealed that, while VNP20009 was relatively safe and well tolerated, very limited anti-tumor activity was observed (see, *e.g.*, Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152). While the use of VNP20009 resulted in no significant changes in metastatic disease burden, it did demonstrate evidence of tumor colonization at the maximum tolerated dose (MTD). Higher doses, which would be required to effect any anti-tumor activity, were not possible due to toxicity that correlated with high levels of pro-inflammatory cytokines.

The immunostimulatory bacteria provided herein provide numerous improvements and advantages that strain VNP20009 lacks. For exemplified strains the VNP20009 strain (YS1646) is used as the parental strain that is further modified by introduction of additional genome modifications, including those that eliminate flagella. The strain also is improved by completely deleting purI and/or *msbB*. Other genome modifications include elimination or inactivation of the curli fimbriae, and rendering the strain *thyA*$^-$, so that the bacteria do not replicate *in vivo*, and/or *ansB*$^-$ to eliminate asparaginase activity, which inactivates or reduces activity of T-cells. These genome modifications individually and together improve the ability of the bacteria to accumulate in or infect phagocytic cells, and also to increase a host's anti-viral type immune response. The immunostimulatory bacteria are modified to include various payloads that stimulate the immune system and/or reduce immunosuppression and provide therapeutic products and immunizing antigens and products.

The immunostimulatory bacteria deliver encoded genetic payloads in a tumor-specific manner, to tumor-resident myeloid cells. The immunostimulatory bacteria, by virtue of genomic modifications, such as deletions or disruptions of genes, and other

modifications of the genome, exhibit reduced TLR2-, TLR4-, and TLR5-mediated inflammation, for example, by virtue of the elimination of the flagella, the modifications of the LPS, and the elimination of the curli fimbriae and reduced biofilm formation. As shown and described herein, elimination of TLR2, and also of TLR2/4/5- mediated activities and response promotes or enhances type I interferon production and/or reduces any reduction or inhibition of type I IFN that occurs when these receptors are activated or by virtue of TLR responses. The immunostimulatory bacteria enhance T-cell function and activities and effects, such as by virtue of the elimination of the expression of L-asparaginase II, and facilitate, provide, permit, and support plasmid maintenance. The bacteria accumulate in (or target) only, or substantially only, myeloid cells, particularly tumor-resident myeloid cells, providing highly efficient plasmid delivery after phagocytosis. The immunostimulatory bacteria provided herein colonize the tumor microenvironment, and can be administered systemically. The immunostimulatory bacteria provided herein exhibit at least 15-fold improved $LD_{50}$ compared to VNP20009. Thus, a much higher dose, if needed, of the immunostimulatory bacteria provided herein can be administered without toxic effects, compared to VNP20009 (see, *e.g.*, the table below in the section F.5. describing exemplary dosages and administration).

It is shown and described herein that immunostimulatory bacteria modified as described herein, including elimination of flagella, LPS modifications, and other modifications, preferentially accumulate in or target myeloid cells, particularly tumor-resident myeloid cells. The Examples demonstrate that the immunostimulatory bacteria accumulate in such cells following systemic, such as intravenous, administration. The Examples also describe and show plasmid transfer from the immunostimulatory bacteria into tumor-resident myeloid cells, and durable protein expression following bacterial cell death, thereby delivering therapeutic products, including products that result in an anti-cancer response and phenotype.

### iv. Wild-Type Strains

Accumulation of VNP20009 in tumors results from a combination of factors including: the inherent invasiveness of the parental strain, ATCC 14028, its ability to replicate in hypoxic environments, and its requirement for high concentrations of purines that are present in the interstitial fluid of tumors. As described herein, it is not

necessary to use an attenuated strain, such a VNP20009, as a starting bacterial strain. By virtue of the modifications described herein, the bacteria are rendered non-toxic or attenuated. The parental strain, ATCC 14028, or another wild-type strain, can be used as a starting strain, and modified as described herein. As described herein, the immunostimulatory bacteria provided herein, by virtue of genome modifications, infiltrate and colonize tumors and tumor-resident immune cells, and also tumor-resident macrophages. When used as vaccines in subjects who do not have tumors, the bacteria accumulate in phagocytic cells, such as macrophages.

### 3. Limitations of Prior Bacterial Cancer Immunotherapies

As shown herein, and also evidenced in light of the knowledge in the art, TLRs inhibit or prevent induction of type I IFN. The immunostimulatory bacteria provided herein, include modifications the reduce or inhibit or eliminate TLR2/4/5 responses/activities, thereby overcoming the inhibition of type I IFN. The immunostimulatory bacteria provided herein, also include properties and/or payloads that enhance type I IFN expression or render type I IFN induction constitutive.

In contrast, many classes of immunotherapies have significant limitations that limit their safety and efficacy, as well as complicated platforms that are not likely to be widely used. Bacteria, particularly those provided herein, have numerous advantageous properties for use as anti-cancer therapeutics, compared to, for example, oncolytic viruses. These include the ease with which they can be propagated, manufactured, stored, and eliminated from a host when treatment is completed. Viruses, however, also have advantageous properties, including the host response. The response to a bacterial infection is an innate inflammatory response, which is not advantageous for an anti-cancer therapeutic. The response to a viral infection is similar to an anti-cancer response. This is summarized in the following table.

| | **Bacteria** | **Viruses** |
|---|---|---|
| Innate Recognition by: | TLR2, TLR4 and TLR5 | TLR3, TLR7/8, RIG-I and STING |
| Inflammatory Cytokine Profile: | Promote innate immunity; Suppress adaptive immunity | Promote innate immunity; Promote adaptive immunity |
| Chemokine Gradients: | Attract neutrophils to clear infection | Attract T-cells, monocytes to clear infection |

| | Bacteria | Viruses |
|---|---|---|
| Generation of Immunity: | No | Yes |
| Immunogenicity: | Not immunogenic | Highly immunogenic |

A limitation of prior bacteria as a microbial anti-cancer platform, thus, derives from the specific immune program that is initiated upon sensing of bacteria, even intracellular bacteria, by the immune system, compared to viral-sensing pathways, which are more akin to anti-cancer pathways. The sensing programs that recognize viruses permit the generation of highly effective vaccines and durable adaptive immunity. Vaccinating against bacteria, however, has been met with limited success. For example, the FDA-approved vaccine for typhoid fever against *Salmonella typhi* is only 55% effective (see, *e.g.*, Hart *et al.* (2016) *PLoS ONE* 11(1):e0145945), despite *S. typhi* containing a highly immunogenic Vi capsule and O:9 antigen, which do not occur in less immunogenic bacterial strains, such as *L. monocytogenes* and *S. typhimurium*, against which there are no vaccines.

Bacteria and viruses contain conserved structures known as Pathogen-Associated Molecular Patterns (PAMPs), which are sensed by host cell Pattern Recognition Receptors (PRRs). Recognition of PAMPs by PRRs triggers downstream signaling cascades that result in the induction of cytokines and chemokines, and initiation of a specific immune response (see, *e.g.*, Iwasaki and Medzhitov (2010) *Science* 327(5963):291-295). The manner in which the innate immune system is engaged by PAMPs, and from what type of infectious agent, determines whether an appropriate innate or adaptive response is generated to combat the invading pathogen.

A class of PRRs, known as Toll Like Receptors (TLRs), recognize PAMPs derived from bacterial and viral origins, and are located in various compartments within the cell. TLRs recognize a variety of ligands, including lipopolysaccharide (TLR4), lipoproteins (TLR2), flagellin (TLR5), unmethylated CpG motifs in DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7 and TLR8) (see, *e.g.*, Akira *et al.* (2001) *Nat. Immunol.* 2(8):675-680; and Kawai and Akira (2005) *Curr. Opin. Immunol.* 17(4):338-344). DNA and RNA-based viruses can be sensed either in host cytosolic compartments after phagocytosis, or directly in the cytosol. Type I interferons (IFN-α, IFN-β) are the signature cytokines induced by host

recognition of single-stranded and double-stranded DNA and RNA, either of viral origin, or from the uptake of damaged host cell DNA. For example, the synthetic dsRNA analog polyinosinic:polycytidylic acid (poly(I:C)) is an agonist for endosomal TLR3; the more stable version, poly ICLC (such as that sold under the trademark Hiltonol®), of a dsRNA, has been in clinical development (see, *e.g.*, Caskey *et al.* (2011) *J. Exp. Med.* 208(12):2357-2366). Similarly, single-stranded RNA (ssRNA) in the endosome is sensed by TLR7 and TLR8 (only in humans), and its known synthetic ligands, resiquimod and imiquimod, are FDA-approved topical cancer immunotherapies.

In the cytosol, double-stranded RNA (dsRNA) is sensed by RNA helicases such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5), leading to induction of type I IFN (see, *e.g.*, Ireton and Gale (2011) *Viruses* 3(6):906-919). The cytosolic sensor for dsDNA is mediated through Stimulator of Interferon Genes (STING), an ER-resident adaptor protein that is the central mediator for sensing cytosolic dsDNA from infectious pathogens or aberrant host cell damage (see, *e.g.*, Barber (2011) *Immunol. Rev.* 243(1):99-108). STING signaling activates the TANK binding kinase 1 (TBK1)/ interferon regulatory factor 3 (IRF3) axis, and the NF-κB signaling axis, resulting in the induction of IFN-β and other pro-inflammatory cytokines and chemokines that strongly activate innate and adaptive immunity (see, *e.g.*, Burdette *et al.* (2011) *Nature* 478(7370):515-518). Sensing of cytosolic dsDNA through STING requires cyclic GMP-AMP synthase (cGAS), a host cell nucleotidyl transferase that directly binds dsDNA, and in response, synthesizes a cyclic dinucleotide (CDN) second messenger, cyclic GMP-AMP (cGAMP), which binds and activates STING (see, *e.g.*, Sun *et al.* (2013) *Science* 339(6121):786-791; and Wu *et al.* (2013) *Science* 339(6121):826-830).

STING also can bind to bacterially-derived CDNs, such as c-di-AMP produced from intracellular *L. monocytogenes*, or c-di-GMP from *S. typhimurium*. Cyclic GMP-AMP synthase (cGAS) produces a non-canonical CDN that can activate human STING alleles that are non-responsive to bacterially-derived canonical CDNs. Unlike the CDNs produced by bacteria, in which the two purine nucleosides are joined by a phosphate bridge with 3'-3' linkages, the internucleotide phosphate bridge in the cGAMP synthesized by cGAS is joined by a non-canonical 2'-3' linkage. These

2'-3' molecules bind STING with 300-fold better affinity than bacterial 3'-3' c-di-GMP, and thus, are more potent physiological ligands of STING (see, *e.g.*, Civril *et al.* (2013) *Nature* 498(7454):332-337; Diner *et al.* (2013) *Cell Rep.* 3(5):1355-1361; Gao *et al.* (2013) *Sci. Signal* 6(269):pl1; and Ablasser *et al.* (2013) *Nature* 503(7477):530-534). The cGAS/STING signaling pathway in humans appears to have evolved to preferentially respond to viral pathogens over bacterial pathogens.

Thus, viral-sensing PRRs and TLRs, such as STING and RIG-I, induce type I IFN, and the cytokines and chemokines that lead to effective T-cell mediated adaptive immunity. In the tumor setting, type I IFN signaling is required to induce T-cell trafficking chemokines, such as CXCL10, and also to activate DC cross-presentation of tumor antigens to prime $CD8^+$ T-cells (see, *e.g.*, Diamond *et al.* (2011) *J. Exp. Med.* 208(10):1989–2003; and Fuertes *et al.* (2011) *J. Exp. Med.* 208(10):2005–2016).

In contrast, host surveillance of bacteria, such as *S. typhimurium*, is largely mediated through TLR2, TLR4, and TLR5 (see, *e.g.*, Arpaia *et al.* (2011) *Cell* 144(5):675-688). These TLRs signal through MyD88 (myeloid differentiation primary response protein 88) and TRIF (Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β) adaptor molecules to mediate induction of the NF-κB-dependent pro-inflammatory cytokines TNF-α and IL-6 (see, *e.g.*, Pandey *et al.* (2015) *Cold Spring Harb. Perspect. Biol.* 7(1):a016246). *S. typhimurium* was shown to activate the NLRP3 inflammasome pathway, resulting in the cleavage of caspase-1 and the induction of the pro-inflammatory cytokines IL-1β and IL-18 that lead to pyroptotic cell death. Engagement of TLR2, TLR4 and TLR5, and inflammasome activation, induces chemokines and cytokines that lead to bacterial clearance by neutrophils and macrophages. Evidence that *S. typhimurium* is cleared by T-cells is limited, and antibodies that are generated against it are non-neutralizing (see, *e.g.*, McSorley (2014) *Immunol. Rev.* 260(1):168-182). Further, *S. typhimurium* has mechanisms to directly suppress T-cell function, impairing any potential anti-tumor T-cell response from being generated (see, *e.g.*, Kullas *et al.* (2012) *Cell Host Microbe* 12(6)791-798). As a result, bacterial cancer therapies, such as *S. typhimurium*, lead to recruitment and clearance by neutrophils and macrophages, which are not the T-cells that are required to generate adaptive anti-tumor immunity. It is described and shown herein that these differences can explain why prior bacterial

anti-cancer vaccines, even those harboring host tumor antigens, are poor T-cell priming vectors in humans. Many of the TLRs have been reported to induce type I interferon (IFN), but is found and described herein that this dogma is not necessarily correct with respect to TLR2/3/4/5/7 in primary human monocyte-derived macrophages. Experiments were performed using TLR agonists to assess the effects on type I IFN. The results showed that TLR3 and TLR4 agonists do not induce type I IFN in primary human monocytes unless pretreated with IFNα. Agonizing TLR2 does not induce type I IFN even with pretreatment with IFNα. In fact, TLR2 can inhibit induction of type I IFN. This is a heretofore unidentified problem with bacterial-based therapeutics.

These problems are among those addressed by the immunostimulatory bacteria provided herein. The immunostimulatory bacteria provided herein are engineered to have advantageous properties that were previously only provided by viral therapeutics, and also, to retain the advantageous properties of bacterial therapeutics. The immunostimulatory bacteria provided herein can be systemically administered, can localize to tumors, tumor-resident immune cells, and/or the tumor microenvironment, overcome immunosuppression, and properly activate anti-tumor immunity, while also limiting the autoimmune-related toxicities of existing systemic immunotherapies. The bacteria provided herein effectively localize to tumor-resident immune cells, and encode therapeutic anti-cancer products, and can encode a plurality of such products. For example, the bacteria provided herein can encode complementary therapeutic products. The immunostimulatory bacteria provided herein are modified to reduce or eliminate activation of TLR2, 4 and 5. As a result, they do not inhibit the induction of type I IFN. Such immunostimulatory bacteria provided herein encode payloads that induce type I IFN. This finding is generalized such that vaccines and delivery vehicles that are provided herein are designed so that TLR2 response is eliminated or reduced so that it does not prevent or inhibit type I IFN. In some embodiments, TLR4 and 5 responses are reduced or eliminated, thereby providing vaccines and other immune-stimulating therapeutics that do not inhibit type I IFN.

Provided herein is a superior microbial anti-cancer, vaccine, RNA deliver platform, engineered to retain the beneficial properties of bacteria, while eliciting a

viral-like immune response that induces effective adaptive immunity. As described herein, bacteria, such as strains of *Salmonella* and other species, can be modified as described herein to have reduced inflammatory effects, and thus, to be less toxic. As a result, for example, higher dosages can be administered. Any of these strains of *Salmonella*, as well as other species of bacteria, known to those of skill in the art and/or listed above and herein, can be modified as described herein.

The immunostimulatory bacteria provided herein are modified to have increased colonization of the tumor microenvironment, tumor-resident immune cells, and tumors. They are engineered so that they have reduced toxicity, and other properties that target them to the tumor microenvironment, including adenosine auxotrophy. The strains provided herein also are engineered so that they are not inactivated by complement. These characteristics, particularly those that result in colonization/infection of phagocytic cells also render the bacteria useful as vaccine platforms and as RNA delivery vehicles. They can be adapted for each use by virtue of the payloads selected, the regulatory sequences employed, depending upon whether bacterial or host cell transcriptional./translational machinery is to be used for expression of encoded products, and the locus of the host cells or tissues in which the products are produced.

The bacterial strains provided herein are engineered to deliver therapeutic products. The bacterial strains herein deliver immunostimulatory proteins, including cytokines, chemokines and co-stimulatory molecules, as well as modified gain-of-function cytosolic DNA/RNA sensors that can constitutively evoke or induce type I IFN expression, and other therapeutic products, such as, but not limited to, antibodies and fragments thereof, TGF-β and IL-6 binding decoy receptors, TGF-β polypeptide antagonists, bispecific T-cell engagers (BiTEs®), RNAi, and complementary combinations thereof, that promote an anti-tumor immune response in the tumor microenvironment. The bacterial strains also include genomic modifications that reduce pyroptosis of phagocytic cells, thereby providing for a more robust immune response, and/or reduce or eliminate the ability to infect/invade epithelial cells, but retain the ability to infect/invade phagocytic cells, so that they accumulate more effectively in tumors, the tumor microenvironment and in tumor-resident immune cells. The bacterial strains also can be modified to be resistant to inactivation by

complement factors in human serum. The bacterial strains also can be modified to encode therapeutic products, including, alone or in combinations, for example, cytokines, chemokines, co-stimulatory molecules, constitutively active inducers of type I IFN, and monoclonal antibodies (and fragments thereof) to immune checkpoints, and also to other such targets.

Provided is an anti-cancer therapeutic product that delivers a genetic payload encoding a truncated co-stimulatory molecule (receptor or ligand; *e.g.*, 4-1BBL, CD80, CD86, CD27L, B7RP1, OX40L), with a full or partial cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC), where the truncated gene product is capable of constitutive immuno-stimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC due to a deleted or truncated cytoplasmic domain.

The bacteria also can encode antigens, such as tumor antigens, and pathogen antigens or proteins, in additional to (or in lieu of) the immunostimulatory protein(s), such as STING and cytokines.

## C. MODIFICATIONS AND ENHANCEMENTS OF IMMUNOSTIMULATORY BACTERIA TO INCREASE THERAPEUTIC INDEX AND TO INCREASE ACCUMULATION IN TUMOR-RESIDENT MYELOID CELLS

Provided herein are enhancements, including modifications to the bacterial genome, or to the immunostimulatory bacteria, that, for example, reduce toxicity and improve the anti-tumor activity, such as by increasing accumulation in tumor-resident myeloid cells, improving resistance to complement inactivation, reducing immune cell death, promoting adaptive immunity, and enhancing T-cell function. The modifications are described and exemplified with respect to *Salmonella*, particularly *S. typhimurium*; it is understood that the skilled person can effect similar enhancements/modifications in other bacterial species, such as *Listeria*, and *E. coli*, and in other *Salmonella* strains to achieve similar properties and/or effects, and to express the same encoded payloads. Exemplary of such enhancements/modifications are the following.

### 1. Deletions in Genes in the LPS Biosynthetic Pathway

The lipopolysaccharide (LPS) of Gram-negative bacteria is the major component of the outer leaflet of the bacterial membrane. It is composed of three

major parts, lipid A, a non-repeating core oligosaccharide, and the O antigen (or O polysaccharide). O antigen is the outermost portion on LPS and serves as a protective layer against bacterial permeability, however, the sugar composition of O antigen varies widely between strains. The lipid A and core oligosaccharide vary less, and are more typically conserved within strains of the same species. Lipid A is the portion of LPS that contains endotoxin activity. It is typically a disaccharide decorated with multiple fatty acids. These hydrophobic fatty acid chains anchor the LPS into the bacterial membrane, and the rest of the LPS projects from the cell surface. The lipid A domain is responsible for much of the toxicity of Gram-negative bacteria. Typically, LPS in the blood is recognized as a significant pathogen associated molecular pattern (PAMP), and induces a profound pro-inflammatory response. LPS is the ligand for a membrane-bound receptor complex comprising CD14, MD2, and TLR4. TLR4 is a transmembrane protein that can signal through the MyD88 and TRIF pathways to stimulate the NF-κB pathway and result in the production of pro-inflammatory cytokines, such as TNF-α and IL-6, the result of which can be endotoxic shock, which can be fatal. LPS in the cytosol of mammalian cells can bind directly to the caspase recruitment domains (CARDs) of caspases 4, 5, and 11, leading to autoactivation and pyroptotic cell death (see, *e.g.*, Hagar *et al.* (2015) *Cell Research* 25:149-150). The composition of lipid A and the toxigenicity of lipid A variants is well documented. For example, a monophosphorylated lipid A is much less inflammatory than lipid A with multiple phosphate groups. The number and length of the acyl chains on lipid A also can have a profound impact on the degree of toxicity. Canonical lipid A from *E. coli* has six acyl chains, and this hexa-acylation is potently toxic. *S. typhimurium* lipid A is similar to that of *E. coli*; it is a glucosamine disaccharide that carries four primary and two secondary hydroxyacyl chains (see, *e.g.*, Raetz *et al.* (2002) *Annu. Rev. Biochem.* 71:635-700).

**a. *msbB* Deletion**

The enzyme lipid A biosynthesis myristoyltransferase, encoded by the *msbB* gene in *S. typhimurium*, catalyzes the addition of a terminal myristoyl group to the lipid A domain of lipopolysaccharide (LPS) (see, *e.g.*, Low *et al.* (1999) *Nat. Biotechnol.* 17(1):37-41). Deletion of *msbB*, thus, alters the acyl composition of the lipid A domain of LPS, the major component of the outer membranes of Gram-

negative bacteria. For example, deletion of *msbB* in the *S. typhimurium* strain VNP20009 results in the production of a predominantly penta-acylated lipid A, which is less toxic than native hexa-acylated lipid A, and allows for systemic delivery without the induction of toxic shock (see, *e.g.*, Lee *et al.* (2000) *International Journal of Toxicology* 19:19-25). This modification significantly reduces the ability of the LPS to induce septic shock, attenuating the bacterial strain, and thus, increasing the therapeutic index of *Salmonella*-based immunotherapeutics (see, *e.g.*, U.S. Patent Publication Nos. 2003/0170276, 2003/0109026, 2004/0229338, 2005/0255088, and 2007/0298012). Importantly, *msbB* mutants that do not express the *msbB* product are unable to replicate intracellularly, as exemplified herein (see, *e.g.*, Example 2), which is a requirement for *Salmonella* virulence (see, *e.g.*, Leung *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:11470-11474).

Other LPS mutations, including replacements, deletions, or insertions that alter LPS expression, can be introduced into the bacterial strains provided herein, including the *Salmonella* strains, that dramatically reduce virulence, and thereby provide for lower toxicity, and permit the administration of higher doses. As exemplified herein, the *msbB*$^-$ locus can be partially deleted, or interrupted, or translocated. It also can be completely deleted, which can improve growth of the strain.

Corresponding genes, encoding homologs or orthologs of lipid A biosynthesis myristoyltransferase in other bacterial species, also can be deleted or disrupted to achieve similar results. These genes include, but are not limited to, for example, *lpxM*, encoding myristoyl-acyl carrier protein-dependent acyltransferase in *E. coli*; and *msbB*, encoding lipid A acyltransferase in *S. typhi*.

**b. *pagP* Deletion or inactivation**

As described above, *msbB* mutants of *S. typhimurium* cannot undergo the terminal myristoylation of lipid A, and produce predominantly penta-acylated lipid A that is significantly less toxic than hexa-acylated lipid A. The modification of lipid A with palmitate is catalyzed by the enzyme lipid A palmitoyltransferase (PagP). Transcription of the *pagP* gene is under control of the PhoP/PhoQ system, which is activated by low concentrations of magnesium, *e.g.*, inside the SCV. Thus, the acyl content of *S. typhimurium* lipid A is variable, and with wild-type bacteria, it can be

hexa-acylated or penta-acylated. The ability of *S. typhimurium* to palmitate its lipid A increases resistance to antimicrobial peptides that are secreted into phagolysosomes.

In wild-type *S. typhimurium*, expression of *pagP* results in lipid A that is hepta-acylated. In an *msbB* mutant (in which the terminal acyl chain of the lipid A cannot be added), the induction of *pagP* results in a hexa-acylated lipid A (see, *e.g.*, Kong *et al.* (2011) *Infection and Immunity* 79(12):5027–5038). Hexa-acylated lipid A has been shown to be the most pro-inflammatory. While groups have sought to exploit this pro-inflammatory signal, for example, by deletion or disruption of *pagP* to allow only hexa-acylated lipid A to be produced (see, *e.g.*, Felgner *et al.* (2016) *Gut Microbes* 7(2):171-177; and Felgner *et al.* (2018) *Oncoimmunology* 7(2):e1382791), this can lead to poor tolerability, due to the TNF-α-mediated pro-inflammatory nature of the LPS, and paradoxically less adaptive immunity (see, *e.g.*, Kocijancic *et al.* (2017) *Oncotarget* 8(30):49988-50001).

LPS is a potent TLR4 agonist that induces TNF-α and IL-6. The dose-limiting toxicities in the I.V. VNP20009 clinical trial (see, *e.g.*, Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152) at 1E9 CFUs/$m^2$, were cytokine mediated (fever, hypotension), with TNF-α levels > 100,000 pg/ml, and IL-6 levels > 10,000 pg/ml in serum at 2 hours. Despite the *msbB* deletion in VNP20009 and its reduced pyrogenicity, the LPS still can be toxic at high doses, possibly due to the presence of hexa-acylated lipid A. Thus, a *pagP*$^-$/*msbB*$^-$ strain, which cannot produce hexa-acylated lipid A, and produces only penta-acylated lipid A, resulting in lower induction of pro-inflammatory cytokines, is better tolerated at higher doses, and will allow for dosing in humans at or above 1E9 CFUs/$m^2$. Higher dosing leads to increased colonization of tumors, tumor-resident immune cells, and the tumor microenvironment, enhancing the therapeutic efficacy of the immunostimulatory bacteria. Because of the resulting change in bacterial membranes and structure, the host immune response, such as complement activity, is altered so that the bacteria are not eliminated upon systemic administration. For example, it is shown herein that *pagP*$^-$/*msbB*$^-$ mutant strains have increased resistance to complement inactivation and enhanced stability in human serum.

Provided herein are immunostimulatory bacteria, exemplified by live attenuated *Salmonella* strains, such as the exemplary strain of *S. typhimurium*, that

only can produce LPS with penta-acylated lipid A, that contain a deletion or disruption of the *msbB* gene, and that further are modified by deletion or disruption of *pagP*. As discussed above, deletion of *msbB* expression prevents the terminal myristoylation of lipid A, while deletion of *pagP* expression prevents palmitoylation. A strain modified to produce LPS penta-acylated lipid A results in lower levels of pro-inflammatory cytokines, improved stability in the blood, resistance to complement fixation, increased sensitivity to antimicrobial peptides, enhanced tolerability, and increased anti-tumor immunity when further modified to express heterologous genetic payloads that stimulate the immune response in the tumor microenvironment.

Corresponding genes, encoding homologs and orthologs of lipid A palmitoyltransferase (PagP) in other bacterial species, also can be deleted or disrupted to achieve similar results. These genes include, but are not limited to, for example, *pagP*, encoding Lipid IVA palmitoyltransferase in *E. coli*; and *pagP*, encoding antimicrobial peptide resistance and lipid A acylation protein in *S. typhi*.

**2. Nutrient Auxotrophy**

The immunostimulatory bacteria provided herein can be attenuated by rendering them auxotrophic for one or more essential nutrients, such as purines (for example, adenine), nucleosides (for example, adenosine), amino acids (for example, aromatic amino acids, arginine, and leucine), adenosine triphosphate (ATP), or other nutrients as known and described in the art.

**a. *purI* Deletion/Disruption**

Phosphoribosylaminoimidazole synthetase, an enzyme encoded by the *purI* gene (synonymous with the *purM* gene), is involved in the biosynthesis pathway of purines. Disruption or deletion or inactivation of the *purI* gene, thus, renders the bacteria auxotrophic for purines. In addition to being attenuated, *purI*$^-$ mutants are enriched in the tumor environment and have significant anti-tumor activity (see, *e.g.*, Pawelek *et al.* (1997) *Cancer Research* 57:4537-4544). It was previously described that this colonization results from the high concentration of purines present in the interstitial fluid of tumors as a result of their rapid cellular turnover. Since the *purI*$^-$ bacteria are unable to synthesize purines, they require an external source of adenine, and it was thought that this would lead to their restricted growth in the purine-

enriched tumor microenvironment (see, *e.g.*, Rosenberg *et al.* (2002) *J. Immunotherapy* 25(3):218-225). While the VNP20009 strain was initially reported to contain a deletion of the *purI* gene (see, *e.g.*, Low *et al.* (2003) *Methods in Molecular Medicine* Vol. 90, *Suicide Gene Therapy: Methods and Reviews*, pp. 47-59), subsequent analysis of the entire genome of VNP20009 demonstrated that the *purI* gene is not deleted, but is disrupted by a chromosomal inversion (see, *e.g.*, Broadway *et al.* (2014) *Journal of Biotechnology* 192:177-178). The entire gene is contained within two parts of the VNP20009 chromosome that is flanked by insertion sequences, one of which has an active transposase. While disruption of the *purI* gene limits replication to the tumor tissue/microenvironment, it still permits intracellular replication and virulence. Deletion or disruption of each of the *msbB* and the *purI* genes, as exemplified herein (see, Example 2), is required to limit bacterial growth to the extracellular space in tumor tissue, and prevent intracellular replication. Provided herein are strains in which the coding portion of these genes are completely deleted to eliminate any possible reversion to wild-type by recombination. It is shown herein that such bacteria grow more effectively.

Besides *purI* gene deletions or disruptions, nutrient auxotrophy can be introduced into the immunostimulatory bacteria by deletions/mutations in genes such as *aro*, *gua*, *thy*, *nad*, and *asd*, for example. Nutrients produced by the biosynthesis pathways involving these genes are often unavailable in host cells, and as such, bacterial survival is challenging. For example, attenuation of *Salmonella* and other bacterial species can be achieved by deletion of the *aroA* gene, which is part of the shikimate pathway, connecting glycolysis to aromatic amino acid biosynthesis (see, *e.g.*, Felgner *et al.* (2016) *mBio* 7(5):e01220-16). Deletion of *aroA* results in bacterial auxotrophy for aromatic amino acids and subsequent attenuation (see, *e.g.*, U.S. Patent Publication Nos. 2003/0170276, 2003/0175297, 2012/0009153, and 2016/0369282; and International Application Publication Nos. WO 2015/032165 and WO 2016/025582). Similarly, other enzymes involved in the biosynthesis pathway for aromatic amino acids, including *aroC* and *aroD*, have been deleted to achieve attenuation (see, *e.g.*, U.S. Patent Publication No. 2016/0369282; and International Application Publication No. WO 2016/025582). For example, *S. typhimurium* strain SL7207 is an aromatic amino acid auxotroph (*aroA*$^-$ mutant); strains A1 and A1-R are

leucine-arginine auxotrophs; and VNP20009/YS1646 is a purine auxotroph (*purI*⁻ mutant) as well as being *msbB*⁻. As shown herein, VNP20009/YS1646 is also auxotrophic for the immunosuppressive nucleoside adenosine, and for ATP (see, *e.g.*, Example 1). Strains provided herein include strains derived from the strain designated YS1646, such as those that lack flagella, are *pagP*⁻ or modified to produce penta-acylated LPS, and include additional modifications, including complete deletion of purI and/*msbB*, as well as deletion of the curli fimbriae, such as by genome modifications that render the bacterium *csgD*⁻, and additional modifications that require various nutrients for growth, such as *thyA*⁻ strains. The strains also can be have genome modifications that render them *ansB*⁻ so that they do not produce asparagine synthase, which can inhibit T cells, thereby eliminating this immunosuppressive aspect of immunostimulatory bacteria. Exemplary strains include those designated YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI, and YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA. It is understood that these designations reference *Salmonella* genes; similar modifications can be effected in other bacterial species, such as *Listeria*, and *E. coli*, such as Nissle.

Corresponding genes, encoding homologs or orthologs of phosphoribosylaminoimidazole synthetase (PurI), and other genes required for purine synthesis in other bacterial species, also can be deleted or disrupted to achieve similar results. These genes include, but are not limited to, for example, *purM*, encoding phosphoribosylformylglycinamide cyclo-ligase in *E. coli*; *purM*, encoding phosphoribosylformylglycinamidine cyclo-ligase in *S. typhi*; *purA*, encoding adenylosuccinate synthetase, *purQ*, encoding phosphoribosylformylglycinamidine synthase II, and *purS*, encoding phosphoribosylformylglycinamidine synthase subunit PurS in *L. monocytogenes*; *purM* (BL1122), encoding phosphoribosylformylglycinamidine cyclo-ligase in *Bifidobacterium longum*; and NT01CX_RS09765, encoding AIR synthase, and NT01CX_RS07625 (*purM*), encoding phosphoribosylformylglycinamidine cyclo-ligase in *Clostridium novyi.*

**b. Adenosine Auxotrophy**

Metabolites derived from the tryptophan and adenosine triphosphate (ATP)/adenosine pathways are major drivers in forming an immunosuppressive environment within the tumor/tumor microenvironment (TME). Adenosine, which

exists in the free form inside and outside of cells, is an effector of immune function. Adenosine decreases T-cell receptor induced activation of NF-κB, and inhibits IL-2, IL-4, and IFN-γ. Adenosine decreases T-cell cytotoxicity, increases T-cell anergy, and increases T-cell differentiation to $Foxp3^+$ or $Lag3^+$ regulatory T-cells (T-reg cells, T-regs or Tregs). On natural killer (NK) cells, adenosine decreases IFN-γ production, and suppresses NK cell cytotoxicity. Adenosine blocks neutrophil adhesion and extravasation, decreases phagocytosis, and attenuates levels of superoxide and nitric oxide. Adenosine also decreases the expression of TNF-α, IL-12, and MIP-1α (CCL3) on macrophages, attenuates major histocompatibility complex (MHC) Class II expression, and increases levels of IL-10 and IL-6. Adenosine immunomodulation activity occurs after its release into the extracellular space of the tumor and activation of adenosine receptors (ADRs) on the surface of target immune cells, cancer cells or endothelial cells. The high adenosine levels in the tumor microenvironment result in local immunosuppression, which limits the capacity of the immune system to eliminate cancer cells.

Extracellular adenosine is produced by the sequential activities of membrane associated ectoenzymes, CD39 (ecto-nucleoside triphosphate diphosphohydrolase1, or NTPDase1) and CD73 (ecto-5'-nucleotidase), which are expressed on tumor stromal cells, together producing adenosine by phosphohydrolysis of ATP or ADP produced from dead or dying cells. CD39 converts extracellular ATP (or ADP) to 5'-AMP, which is converted to adenosine by CD73. Expression of CD39 and CD73 on endothelial cells is increased under the hypoxic conditions of the tumor microenvironment, thereby increasing levels of adenosine. Tumor hypoxia can result from inadequate blood supply and disorganized tumor vasculature, impairing delivery of oxygen (see, *e.g.*, Carroll and Ashcroft (2005) *Expert. Rev. Mol. Med.* 7(6), DOI: 10.1017/S1462399405009117). Hypoxia, which occurs in the tumor microenvironment, also inhibits adenylate kinase (AK), which converts adenosine to AMP, leading to very high extracellular adenosine concentrations. The extracellular concentration of adenosine in the hypoxic tumor microenvironment has been measured at 10-100 μM, which is up to about 100-1000 fold higher than the typical extracellular adenosine concentration of approximately 0.1 μM (see, *e.g.*, Vaupel *et al.* (2016) *Adv. Exp. Med. Biol.* 876:177-183; and Antonioli *et al.* (2013) *Nat. Rev.*

*Can.* 13:842-857). Since hypoxic regions in tumors are distal from microvessels, the local concentration of adenosine in some regions of the tumor can be higher than in others.

To direct effects to inhibit the immune system, adenosine also can control cancer cell growth and dissemination by effects on cancer cell proliferation, apoptosis and angiogenesis. For example, adenosine can promote angiogenesis, primarily through the stimulation of $A_{2A}$ and $A_{2B}$ receptors. Stimulation of the receptors on endothelial cells can regulate the expression of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on endothelial cells, maintain vascular integrity, and promote vessel growth (see, *e.g.*, Antonioli *et al.* (2013) *Nat. Rev. Can.* 13:842-857). Activation of one or more of $A_{2A}$, $A_{2B}$, or $A_3$ on various cells by adenosine can stimulate the production of the pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) or angiopoietin 2 (see, *e.g.*, Antonioli *et al.* (2013) *Nat. Rev. Can.* 13:842-857).

Adenosine also can directly regulate tumor cell proliferation, apoptosis, and metastasis through interaction with receptors on cancer cells. For example, studies have shown that the activation of $A_1$ and $A_{2A}$ receptors promote tumor cell proliferation in some breast cancer cell lines, and activation of $A_{2B}$ receptors have cancer growth-promoting properties in colon carcinoma cells (see, *e.g.*, Antonioli *et al.* (2013) *Nat. Rev. Can.* 13:842-857). Adenosine also can trigger apoptosis of cancer cells, and various studies have correlated this activity to activation of the extrinsic apoptotic pathway through $A_3$, or the intrinsic apoptotic pathway through $A_{2A}$ and $A_{2B}$ (see, *e.g.*, Antonioli *et al.* (2013)). Adenosine can promote tumor cell migration and metastasis, by increasing cell motility, adhesion to the extracellular matrix, and expression of cell attachment proteins and receptors to promote cell movement and motility.

The extracellular release of adenosine triphosphate (ATP) occurs from stimulated immune cells, and from damaged, dying, or stressed cells. The NLR family pyrin domain-containing 3 (NLRP3) inflammasome, when stimulated by this extracellular release of ATP, activates caspase-1 and results in the secretion of the cytokines IL-1β and IL-18, which in turn activate innate and adaptive immune responses (see, *e.*g., Stagg and Smyth (2010) *Oncogene* 29:5346–5358). ATP can

accumulate to concentrations exceeding 100 mM in tumor tissue, whereas levels of ATP found in healthy tissues are very low (~1-5 μM) (see, *e.*g., Song *et al.* (2016) *Am. J. Physiol. Cell Physiol.* 310(2):C99–C114). ATP is catabolized into adenosine by the enzymes CD39 and CD73. Activated adenosine acts as a highly immunosuppressive metabolite via a negative-feedback mechanism and has a pleiotropic effect against multiple immune cell types in the hypoxic tumor microenvironment (see, *e.*g., Stagg and Smyth (2010) *Oncogene* 29:5346–5358). Adenosine receptors $A_{2A}$ and $A_{2B}$ are expressed on a variety of immune cells and are stimulated by adenosine to promote cAMP-mediated signaling changes, resulting in immunosuppressive phenotypes of T-cells, B-cells, NK cells, dendritic cells (DCs), mast cells, macrophages, neutrophils, and natural killer T (NKT) cells. As a result, adenosine levels can accumulate to over one hundred times their normal concentration in pathological tissues, such as solid tumors, which have been shown to overexpress ecto-nucleotidases, such as CD73. Adenosine also has been shown to promote tumor angiogenesis and development. An engineered bacterium that is auxotrophic for adenosine would thus exhibit enhanced tumor-targeting and colonization.

Immunostimulatory bacteria, such as *Salmonella typhi*, can be made auxotrophic for adenosine, for example, by deletion of the *tsx* gene (see, *e.*g., Bucarey *et al.* (2005) *Infection and Immunity* 73(10):6210–6219) or by deletion of *purD* (see, *e.*g., Husseiny (2005) *Infection and Immunity* 73(3):1598–1605). In the Gram-negative bacteria *Xanthomonas oryzae*, a *purD* gene knockout was shown to be auxotrophic for adenosine (see, *e.*g., Park *et al.* (2007) *FEMS Microbiol. Lett.* 276:55–59). As exemplified herein, *S. typhimurium* strain VNP20009 is auxotrophic for adenosine due to its *purI* modification; hence, further modification to render it auxotrophic for adenosine is not required. Hence, embodiments of the immunostimulatory bacterial strains, as provided herein, are auxotrophic for adenosine. Such auxotrophic bacteria selectively replicate in the tumor microenvironment, further increasing accumulation and replication of the administered bacteria in tumors, and decreasing the levels of adenosine in and around tumors, thereby reducing or eliminating the immunosuppression caused by the accumulation of adenosine. Exemplary of such bacteria, provided herein, is a modified strain of *S. typhimurium* containing $purI^{-}/msbB^{-}$ mutations to provide

adenosine auxotrophy. For other strains and bacteria, the *purI* gene can be disrupted as it has been in VNP20009, or it can contain a deletion of all or a portion of the *purI* gene, which ensures that there cannot be a reversion to a wild-type gene. As described elsewhere herein, in strain VNP20009, the *purI* gene was inactivated by inversion. Similarly, the *msbB* gene in VNP20009 was not completely deleted. As exemplified herein, strains in which the *purI* and *msbB* genes have been completely deleted to eliminate any risk of reversion, demonstrate superior fitness as assessed by growth of cultures *in vitro*.

Immunostimulatory bacteria modified by rendering them auxotrophic for one or more essential nutrients, such as purines (for example, adenine), nucleosides (for example, adenosine), amino acids (for example, aromatic amino acids, arginine, and leucine), or adenosine triphosphate (ATP), are employed. In particular, in embodiments of the immunostimulatory bacteria provided herein, such as strains of *S. typhimurium*, the bacteria are rendered auxotrophic for adenosine, and optionally, for ATP, and preferentially accumulate in tumor microenvironments (TMEs). Hence, strains of immunostimulatory bacteria described herein are attenuated because they require purines, adenosine, and/or ATP for growth, and they preferentially colonize TMEs, which, as discussed below, have an abundance of these metabolites. Because adenosine accumulation that occurs in the tumor microenvironment of some tumors is immunosuppressive, adenosine auxotrophy eliminates the immunosuppression from adenosine that accumulates in the tumor microenvironment.

**c. Thymidine Auxotrophy**

Genome modifications can be introduced in place of or in addition to the inactivation/deletion (see section 3) discussed below. Other deletions or inactivation of genes or gene products required for growth, such as genes that produce nutrients, can be used in place of or in addition to, for example, the asd inactivation/deletion. These include, for example, modifications that render the bacteria *thyA-* (see, *e.g.*, Loessner *et al.* (2006) *FEBS Lett 265*:81-88). Immunostimulatory bacteria that are *ThyA*$^-$ have genome modifications, such as insertions, deletions, replacements, transpositions, and/or other changes, that result in inactive or eliminate production of thymidylate synthase. Thymidylate synthase catalyzes the reductive methylation of dUMP to dTMP, a DNA biosynthesis precursor (precursor to dTTP).

Elimination of expression or production or other attenuating mutations of the bacterial genome for production of such products results in release of encoded macromolecules upon bacterial cell death *in vivo* after administration. Asd, as discussed below, is an essential enzyme for bacterial cell wall synthesis; ThyA is an enzyme needed for DNA synthesis. Mutation of the respective genes renders the strain auxotrophic for diaminopimelic acid (DAP) or thymidine monophosphate precursors. Upon deprivation of the complementing substrates, such bacteria die by DAP-less or thymine-less death, resulting in release of bacterial proteins and plasmid. Inactivation or elimination of Asd, results in release of macromolecules. Elimination or inactivation of ThyA (to produce Δ*ThyA* bacteria, which includes those with insertions, deletions, and other modifications so that active enzyme is not produced) expression/activity does not result in release of macromolecules, including proteins and plasmid, upon thymidine starvation (Loessner *et al.*, (2006) *FEBS Lett 265*:81-88).

Thus, Δ*ThyA* bacteria, in which the genome is modified so that active enzyme is not produced, are advantageous, for example, for *in vivo* delivery of plasmids to host cells, since the bacteria do not prematurely release their contents. Since the bacteria provided herein infect or accumulate in myeloid cells, such as phagocytic cells, such as macrophages, dendritic cells, monocytes, and neutrophils, which consume bacteria, the intact Δ*ThyA* bacteria, release the plasmid encoding the payloads, such as therapeutic products, inside the targeted cells for, for example, expression, if RNA, or secretion or presentation, if protein. Thus, genome modifications that render the bacteria *thyA*$^-$ have advantages for particular applications, such as immunization, presentation on cells, delivery of RNA, and other such applications.

### 3. Plasmid Maintenance and Delivery

#### a. *asd* Deletion

As discussed above, the bacteria can be rendered *thyA*$^-$; they also can be rendered *asd*$^+$ instead or in addition. The selection of the particular genome modification depends upon the intended application of the bacteria. *thyA* inactivation is advantageous for some applications; for others rendering the bacteria *asd*$^+$ is advantageous; it is within the skill of the skilled person to select the particular

modification(s). The *asd* gene in bacteria encodes an aspartate-semialdehyde dehydrogenase. *asd*⁻ mutants of *S. typhimurium* have an obligate requirement for diaminopimelic acid (DAP), which is required for cell wall synthesis, and will undergo lysis in environments deprived of DAP. This DAP auxotrophy can be used for plasmid selection and maintenance of plasmid stability *in vivo*, without the use of antibiotics, when the *asd* gene is complemented *in trans* on a plasmid in the bacterium. Non-antibiotic-based plasmid selection systems are advantageous, and allow for 1) the use of administered antibiotics as a rapid clearance mechanism in the event of adverse symptoms, and 2) for antibiotic-free scale up of production, where such use is commonly avoided. The *asd* gene complementation system provides for such non-antibiotic-based plasmid selection (see, *e.g.*, Galán *et al.* (1990) *Gene* 94(1):29-35). The use of the *asd* gene complementation system to maintain plasmids in the tumor microenvironment is expected to increase the potency of *S. typhimurium* strains engineered to deliver plasmids encoding genetic payloads/therapeutic products, such as immunostimulatory proteins (*e.g.*, cytokines, chemokines, co-stimulatory molecules); cytosolic DNA/RNA sensors that induce type I IFN, such as STING and IRF3, and gain-of-function/constitutively active mutants thereof; antibodies and fragments thereof (*e.g.*, checkpoint inhibitors, or anti-IL-6 or anti-VEGF antibodies); bi-specific T-cell engagers (sold under the trademark BiTEs®); interfering RNAs; and other therapeutic products as discussed elsewhere herein and known in the art; and complementary combinations of all of the preceding therapeutic products.

An alternative use for an *asd* mutant of *S. typhimurium* is to exploit the DAP auxotrophy to produce an autolytic (or suicidal) strain, for delivery of therapeutic products/macromolecules to infected cells, without the ability to persistently colonize host tumors. Deletion of the *asd* gene makes the bacteria auxotrophic for DAP when grown *in vitro* or *in vivo*. An example described herein, provides an *asd* deletion strain that is auxotrophic for DAP, and that contains a plasmid suitable for delivery of immunomodulatory proteins, but that does not contain an *asd* complementing gene, resulting in a strain that is defective for replication *in vivo*. This strain is propagated *in vitro* in the presence of DAP, and grows normally, and then is administered as an immunotherapeutic agent to a mammalian host where DAP is not present. The

suicidal strain is able to invade host cells but is not be able to replicate due to the absence of DAP in mammalian tissues, lysing automatically, and delivering its cytosolic contents (*e.g.*, plasmids or proteins).

Corresponding genes, encoding homologs or orthologs of aspartate-semialdehyde dehydrogenase (*asd*) in other bacterial species, also can be deleted or disrupted to achieve similar results. These genes include, but are not limited to, for example, *asd*, encoding aspartate-semialdehyde dehydrogenase in *E. coli*; *asd* (STY4271), encoding aspartate-semialdehyde dehydrogenase in *S. typhi*; *asd* (lmo1437), encoding aspartate-semialdehyde dehydrogenase in *L. monocytogenes*; *asd* (BL0492), encoding aspartate-semialdehyde dehydrogenase in *Bifidobacterium longum*; and NT01CX_RS04325 (*asd*), encoding aspartate-semialdehyde dehydrogenase in *Clostridium novyi.* Similarly, as discussed, above, *thyA* can be inactivated or eliminated by genome modification in place of the asd modification. As discussed above, inactivation or elimination of thymidylate synthase expression/activity does not result in release of macromolecules, including proteins and plasmids, upon thymidine starvation.

**b. *endA* Deletion/Disruption**

The *endA* gene (see, for example, SEQ ID NO:250) encodes an endonuclease (DNA-specific endonuclease I; see, for example, SEQ ID NO:251) that mediates degradation of double-stranded DNA (dsDNA) in the periplasm of Gram-negative bacteria. Most common strains of laboratory *E. coli* are *endA*$^-$, as a mutation in the *endA* gene allows for higher yields of plasmid DNA. This gene is conserved among species. To facilitate intact plasmid DNA delivery, the *endA* gene of the engineered immunostimulatory bacteria is deleted or mutated to prevent its endonuclease activity. Exemplary of such mutations is an E208K amino acid substitution (see, *e.g.*, Durfee *et al.* (2008) *J. Bacteriol.* 190(7):2597-2606), or a corresponding mutation in the species of interest. *endA*, including residue E208, is conserved among bacterial species, including *Salmonella.* Thus, the E208K mutation can be used to eliminate endonuclease activity in other species, including *Salmonella* species. Those of skill in the art can introduce other mutations or deletions to eliminate *endA* activity. Effecting this mutation, or deleting or disrupting the gene to eliminate activity of *endA* in the immunostimulatory bacteria herein, such as in *Salmonella*, increases efficiency of

intact plasmid DNA delivery, thereby increasing expression of any one, or two, or more, immunomodulatory proteins/therapeutic products encoded on the plasmid, and enhancing the anti-tumor immune response and anti-tumor efficacy.

**4. Flagellin Knockout Strains**

Flagella are organelles on the surface of bacteria that are composed of a long filament that is attached, via a hook, to a rotary motor that can rotate in a clockwise or counterclockwise manner to provide a means for locomotion. Flagella, for example, in *S. typhimurium*, are important for chemotaxis and for establishing an infection via the oral route, due to the ability to mediate motility across the mucous layer in the gastrointestinal tract. While flagella have been demonstrated to be required for chemotaxis to and colonization of tumor cylindroids *in vitro* (see, *e.g.*, Kasinskas and Forbes (2007) *Cancer Res.* 67(7):3201-3209), and motility has been shown to be important for tumor penetration (see, *e.g.*, Toley and Forbes (2012) *Integr. Biol. (Camb)* 4(2):165-176), flagella are not required for tumor colonization in animals when the bacteria are administered intravenously (see, *e.g.*, Stritzker *et al.* (2010) *International Journal of Medical Microbiology* 300:449–456). Each flagellar filament is composed of tens of thousands of flagellin subunits. The *S. typhimurium* chromosome contains two genes, *fliC* and *fljB*, that encode antigenically distinct flagellin monomers. Mutants defective for both *fliC* and *fljB* are nonmotile and avirulent when administered via the oral route of infection, but maintain virulence when administered parenterally.

Flagellin is a major pro-inflammatory determinant of *Salmonella* (see, *e.g.*, Zeng *et al.* (2003) *J. Immunol.* 171:3668-3674), and is directly recognized by TLR5 on the surface of cells, and by NLCR4 in the cytosol (see, *e.g.*, Lightfield *et al.* (2008) *Nat. Immunol.* 9(10):1171–1178). Both pathways lead to pro-inflammatory responses resulting in the secretion of cytokines, including IL-1β, IL-18, TNF-α, and IL-6. Attempts have been made to make *Salmonella*-based cancer immunotherapy more potent by increasing the pro-inflammatory response to flagellin by engineering the bacteria to secrete *Vibrio vulnificus* flagellin B, which induces greater inflammation than flagellin encoded by *fliC* and *fljB* (see, *e.g.*, Zheng *et al.* (2017) *Sci. Transl. Med.* 9(376):eaak9537).

Herein, *Salmonella* bacteria, such as *S. typhimurium*, are engineered to lack both flagellin subunits *fliC* and *fljB*, to reduce TLR5-mediated pro-inflammatory signaling. Other bacteria that contain flagella similarly can be engineered to eliminate flagella, and to include other modifications that have the substantially the same effects as the exemplified modifications of *Salmonella*. For example, as shown herein, a *Salmonella* strain lacking *msbB* and/or *pagP*, which results in reduced TNF-alpha induction, is combined with elimination of flagella, which can be achieved in *Salmonella* by *fliC* and *fljB* knockouts. This results in a *Salmonella* strain that has a combined reduction in, among other effects, TNF-alpha induction and a reduction in TLR5 recognition. These bacterial modifications, $msbB^-$, $pagP^-$, $fliC^-$, and $fljB^-$, can be combined with an immunostimulatory plasmid, optionally containing CpGs, encoding therapeutic products, such as immunomodulatory proteins, and combinations of products. The resulting bacteria have reduced pro-inflammatory signaling, but robust anti-tumor activity. These genome modifications can be combined with others of the genome modifications described herein as well. For vaccines, the encoded products can include antigens and proteins against which an immmunoprotective or therapeutic response is intended.

For example, as exemplified and provided herein, a *fliC* and *fljB* double mutant was constructed in the *asd*-deleted strain of *S. typhimurium*, VNP20009. VNP20009, which is attenuated for virulence by disruption of *purI*/*purM*, and which contains a modification of the *msbB* gene (a partial deletion) that results the in production of a lipid A subunit that is less toxigenic than wild-type lipid A. This results in reduced TNF-α production in a mouse model after intravenous administration, compared to strains with wild-type lipid A. The resulting strain is exemplary of strains that are attenuated for bacterial inflammation by modification of lipid A to reduce TLR2/4 signaling, and by deletion of expression of the flagellin subunits to reduce TLR5 recognition and inflammasome induction.

Pathogenesis in certain bacterial species, including *Salmonella* species, such as *S. typhimurium*, involves a cluster of genes referred to as *Salmonella* pathogenicity islands (SPIs). *Salmonella* invades non-phagocytic intestinal epithelial cells using a type 3 secretion system (T3SS) encoded by the *Salmonella* pathogenicity island 1 (SPI-1), which forms a needle-like structure that injects effector proteins directly into

the cytosol of host cells. These effector proteins lead to rearrangement of the eukaryotic cell cytoskeleton to facilitate invasion of the intestinal epithelium, and also induces proinflammatory cytokines. The SPI designated SPI-1 mediates invasion of epithelial cells. SPI-1 genes include, but are not limited to: *avrA*, *hilA*, *hilD*, *invA*, *invB*, *invC*, *invE*, *invF*, *invG*, *invH*, *invI*, *invJ*, *iacP*, *iagB*, *spaO*, *spaP*, *spaQ*, *spaR*, *spaS*, *orgA*, *orgB*, *orgC*, *prgH*, *prgI*, *prgJ*, *prgK*, *sicA*, *sicP*, *sipA*, *sipB*, *sipC*, *sipD*, *sirC*, *sopB*, *sopD*, *sopE*, *sopE2*, *sprB*, and *sptP*. Deletion of one or more of these genes reduces or eliminates the ability of the bacterium to infect epithelial cells, but does not affect their ability to infect or invade phagocytic cells, including phagocytic immune cells. For example, it was demonstrated that deletion of both the *fliC* and *fljB* genes significantly reduced expression of SPI-1 genes, such as *hilA*, *hilD*, *invA*, *invF*, and *sopB*, thereby reducing the ability to invade non-phagocytic cells (see, *e.g.*, Elhadad *et al.* (2015) *Infect. Immun.* 83(9):3355-3368).

In bacteria such as *Salmonella*, flagellin, in addition to the SPI-1 type 3 secretion system (T3SS), is necessary for triggering pyroptosis in macrophages, and can be detected by the macrophage NLRC4 inflammasome. Elimination of flagellin subunits decreases pyroptosis in macrophages. For example, *S. typhimurium* with deletions in *fliC* and *fljB* results in significantly reduced IL-1β secretion compared to the wild-type strain, whereas cellular uptake and intracellular replication of the bacterium remains unaffected. This demonstrates that flagellin plays a significant role in inflammasome activation. Additionally, *S. typhimurium* strains engineered to constitutively express *fliC* were found to induce macrophage pyroptosis (see, *e.g.*, Li *et al.* (2016) *Scientific Reports* 6:37447; Fink and Cookson (2007) *Cellular Microbiology* 9(11):2562-2570; and Winter *et al.* (2015) *Infect. Immun.* 83(4):1546-1555).

The genome of the immunostimulatory bacteria herein can be modified to delete or mutate the flagellin genes *fliC* and *fljB* in *S. typhimurium*, leading to decreased cell death of tumor-resident immune cells, such as macrophages, and enhancing the anti-tumor immune response of the immunostimulatory bacteria.

Deletion of the flagellin subunits, combined with modification of the LPS, allows for greater tolerability in the host, limits uptake into only phagocytic cells and decreases their pyroptotic cell death, and directs the immunostimulatory response

towards delivery of therapeutic products, such as immunomodulatory proteins, to the TME, particularly tumor-resident myeloid cells. The resulting immunostimulatory bacteria elicit an anti-tumor response, and promote an adaptive immune response to the tumor.

Corresponding genes, encoding flagellin in other bacterial species, also can be deleted to achieve similar results. These genes include, but are not limited to, for example, *fliC*, encoding flagellar filament structural protein, and *fliE*, encoding flagellar basal-body protein FliE in *E. coli*; *fliC*, encoding flagellin and *flgB*, encoding flagellar basal-body rod protein FlgB, in *S. typhi*; *flaA* encoding flagellin, *fliE*, encoding flagellar hook-basal body protein FliE, and *flgB*, encoding flagellar basal-body rod protein FlgB, in *L. monocytogenes*; and NT01CX_RS04995, NT01CX_RS04990, NT01CX_RS05070 and NT01CX_RS05075, encoding flagellin, NT01CX_RS05080 (*flgB*), encoding flagellar basal body rod protein FlgB, NT01CX_RS05085 (*flgC*), encoding flagellar basal body rod protein FlgC, and NT01CX_RS05215 (*flgG*), encoding flagellar basal body rod protein FlgG, in *Clostridium novyi*.

### 5. Engineering Bacteria to Promote Adaptive Immunity and Enhance T-Cell Function

#### L-Asparaginase II (*ansB*) Deletion/Disruption

L-asparaginase II is an enzyme that catalyzes conversion of L-asparagine to ammonia and aspartic acid. Several bacterial strains, such as *E. coli* and *S. typhimurium*, utilize L-asparaginase to scavenge fructose-asparagine as a carbon and nitrogen source (see, *e.g.*, Sabag-Daigle *et al.* (2018) *Appl. Environ. Microbiol.* 84(5):e01957-17). Malignant T-cells, such as in acute lymphoblastic leukemia (ALL), require asparagine as they lack the enzymes to synthesize it. Administration of L-asparaginases has been a frontline therapy for ALL since the early 1970's (see, *e.g.*, Batool *et al.* (2016) *Appl. Biochem. Biotechnol.* 178(5):900-923). Production of L-asparaginase II by *S. typhimurium* is both necessary and sufficient for T-cell inhibition, as it directly induces T-cell receptor (TCR) downregulation, decreases T-cell cytokine production, and inhibits tumor cytolytic function (see, *e.g.*, Kullas *et al.* (2012) *Cell Host Microbe* 12(6)791-798; and van der Velden *et al.* (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102(49):17769-17774). Under rapid clonal expansion conditions,

such as those that occur during T-cell activation in the tumor microenvironment, asparagine is required, and its depletion by L-asparaginase II leads to T-cell suppression. L-asparaginase II, thus, has been used as an anti-cancer therapeutic for cancers in which T-cell suppression is a therapeutic modality.

In contrast to the prior uses of L-asparaginase as an anti-cancer therapeutic, it is shown herein that elimination of L-asparaginase activity in the immunostimulatory bacteria provided herein, and in other immunostimulatory bacteria and bacterial vaccines, such as the BCG vaccine used for vaccinating against tuberculosis (TB) and other diseases, enhances the function of T-cells in the tumor microenvironment. Elimination of L-asparaginase activity can be effected by modifying the bacterial genome to eliminate expression of active enzyme. Modifications include insertions, deletions, replacements, and other changes in the nucleic acids, so that the resulting encoded enzyme is not active, or not expressed, or is eliminated. It is shown herein that deletion of all or of a part of the gene that encodes L-asparaginase II, *ansB*, or disruption thereof, to eliminate expression of the encoded enzyme in the immunostimulatory bacteria, enhances the function of T-cells in a bacterially-colonized tumor microenvironment. Inhibition of L-asparaginase II activity is accomplished by deletion of all or of a part of, or interruption/disruption of, the gene *ansB* in the immunostimulatory bacteria, whereby L-asparaginase II is not produced. Thus, provided are immunostimulatory bacteria whose genomes are modified so that L-asparaginase II is not produced. Immunostimulatory bacteria provided herein are employed to colonize tumor-resident immune cells to enhance the anti-tumor immune response; included among the bacterial genome modifications are deletions, insertions, disruptions, and/or other modifications that eliminate the expression of L-asparaginase II.

As shown herein, the genome of the immunostimulatory bacteria herein can be modified to delete *ansB*, or to disrupt it or to otherwise modify it, to result in inactive encoded L-asparaginase II, or to eliminate the asparaginase, thereby preventing T-cell suppression, and enhancing the anti-tumor T-cell function *in vivo*. It is shown herein that strains in which *ansB* is intact induce profound T-cell immunosuppression in T-cells co-localized with the strain. Strains in which *ansB* is deleted do not induce immunosuppression, thus, solving another problem in the art in using bacteria to

deliver encoded therapeutic products to tumors. Immunostimulatory bacteria that combine modifications, such as deletions or disruptions, of the *ansB* gene, whereby functional encoded enzyme is not expressed, with other modifications described herein that result in increased accumulation in the tumor microenvironment and/or in tumor-resident immune cells, provide superior therapeutic immunostimulatory bacteria.

Corresponding genes, encoding homologs or orthologs of L-asparaginase II (ansB) in other bacterial species, also can be deleted or disrupted to achieve similar results. These genes include, but are not limited to, for example, *ansB*, encoding L-asparaginase 2 in *E. coli*; *ansB* (STY3259), encoding L-asparaginase in *S. typhi*; *ansB* (lmo1663), encoding asparagine synthetase in *L. monocytogenes*; and BL1142, encoding an L-asparaginase precursor in *Bifidobacterium longum.*

**6. Deletions/Disruptions in *Salmonella* Genes Required to Produce Curli Fimbriae**

Bacteria and fungi are capable of forming multicellular structures called biofilms. Bacterial biofilms are encased within a mixture of secreted and cell wall-associated polysaccharides, glycoproteins, and glycolipids, as well as extracellular DNA, known collectively as extracellular polymeric substances. These extracellular polymeric substances protect the bacteria from multiple insults, such as cleaning agents, antibiotics, and antimicrobial peptides. Bacterial biofilms allow for colonization of surfaces, and are a cause of significant infection of prosthetics, such as injection ports and catheters. Biofilms also can form in tissues during the course of an infection, which leads to increases in the duration of bacterial persistence and shedding, and limits the effectiveness of antibiotic therapies. Chronic persistence of bacteria in biofilms is associated with increased tumorigenesis, for example in *S. typhi* infection of the gall bladder (see, *e.g.*, Di Domenico *et al.* (2017) *Int. J. Mol. Sci.* 18:1887).

***csgD* Deletion**

In *Salmonella*, such as *S. typhimurium*, biofilm formation is regulated by the *csgD* gene, which activates the *csgBAC* operon, and results in increased production of the curli fimbriae subunits CsgA and CsgB (see, *e.g.*, Zakikhany *et al.* (2010) *Molecular Microbiology* 77(3):771–786). CsgA is recognized as a PAMP by TLR2,

and induces production of IL-8 from human macrophages (see, *e.g.*, Tukel *et al.* (2005) *Molecular Microbiology* 58(1):289–304). Also, *csgD* indirectly increases cellulose production by activating the *adrA* gene that encodes for di-guanylate cyclase. The small molecule cyclic di-guanosine monophosphate (c-di-GMP), generated by *adrA*, is a ubiquitous secondary messenger that occurs in almost all bacterial species. Increases in c-di-GMP enhance expression of the cellulose synthase gene *bcsA*, which in turn increases cellulose production via stimulation of the *bcsABZC* and *bcsEFG* operons, leading to cellulose biofilm formation. As a result, bacteria, such as *S. typhimurium*, can form biofilms in solid tumors as protection against phagocytosis by host immune cells. Bacterial mutants, such as *Salmonella* mutants, that cannot form biofilms, are taken up more rapidly by host phagocytic cells, and are more readily cleared from infected tumors (see, *e.g.*, Crull *et al.* (2011) *Cellular Microbiology* 13(8):1223-1233). This increase in intracellular localization within phagocytic cells can reduce the persistence of extracellular bacteria, and, as shown herein, can enhance the effectiveness of plasmid delivery of therapeutic products, such as immunomodulatory proteins and other anti-cancer therapeutics, as described herein. Reduction in the capability of immunostimulatory bacteria, such as *S. typhimurium*, to form biofilms, can be achieved through deletion or disruption of genes involved in biofilm formation, such as, for example, *csgD*, *csgA*, *csgB*, *adrA*, *bcsA*, *bcsB*, *bcsZ*, *bcsE*, *bcsF*, *bcsG*, *dsbA*, or *dsbB* (see, *e.g.*, Anwar *et al.* (2014) *PLoS ONE* 9(8):e106095).

It is shown herein that engineering the immunostimulatory bacteria to reduce biofilm formation increases clearance rates from tumors/tissues, increasing tolerability of the therapy, and prevents colonization of prosthetics in patients, thereby increasing the therapeutic benefit of these strains. It is known that adenosine mimetics inhibit *S. typhimurium* biofilm formation, indicating that the high adenosine concentration in the tumor microenvironment can contribute to tumor-associated biofilm formation (see, *e.g.*, Koopman *et al.* (2015) *Antimicrob. Agents Chemother.* 59:76-84). It is shown herein that *csgD*-deleted immunostimulatory bacterial strains demonstrate improved anti-tumor efficacy because of greater bacterial uptake into tumor-resident myeloid cells. Similar genome modifications can be effected in other bacterial strains, such as *E. coli* and *Listeria*, that alter biofilm formation and/or curli

fimbriae production, so that the bacteria can better infiltrate the vasculature, such as that present in tumors and the tumor microenvironment.

Corresponding genes, encoding homologs and orthologs of *csgD*, and other genes that are required for curli fimbriae and biofilm formation in other bacterial species, also can be deleted or disrupted or otherwise modified to achieve similar results. These genes include, but are not limited to, for example, *csgD*, encoding DNA-binding transcriptional dual regulator CsgD in *E. coli*; *csgD* (STY1179), encoding regulatory protein CsgD in *S. typhi*; and *lcp*, encoding the *Listeria* cellulose binding protein that is involved in biofilm formation in *L. monocytogenes*.

Modification of the bacterial genome, such as by deletion or disruption of genes to render the bacteria *csgD*$^-$, results changes, such as one or more of elimination of curli fimbriae and inflammatory cyclic dinucleotides (CDNs), and removes cellulose secretion. This eliminates inflammatory and immunosuppressive elements, prevents TLR4 recognition through altered lipid A acylation, and eliminates cellulose secretion, and, thus, possible biofilm formation, thereby increasing safety and efficacy.

As described herein, bacterial strains, such as *S. typhimurium* strains, that are engineered to be auxotrophic for adenosine; and are reduced in their ability to induce pro-inflammatory cytokines by modification of the LPS and/or deletion of flagellin; and/or that do not express L-asparaginase II to improve T-cell function; and/or that contain deletions or disruptions of genes required for biofilm formation; and/or that are further modified to maintain significant plasmid copy number per cell, at least low to medium copy number or higher, in the absence of antibiotic selection; and that deliver genetic expression cassettes encoding therapeutic products, promote robust anti-tumor immune responses. The plasmids include regulatory sequences to promote secretion of the encoded therapeutic products into the tumor microenvironment.

### 7. Improving Resistance to Complement

The complement system is the first line of immune defense against invading pathogens that directly activate the lectin pathway or the alternative pathway (AP) cascades in the human host. The complement system involves more than 30 soluble and cell-membrane bound proteins that function in the innate immune response to recognize and kill pathogens, such as bacteria, virus-infected cells, and parasites, and

also, that play a role in the antibody-mediated immune response. Activation of the complement cascade leads to opsonization of foreign microbes, release of chemotactic peptides, and finally, to disruption of bacterial cell membranes. Three homologous glycoproteins in the complement system, C3, C4, and C5, play a central role in complement function and interact with other complement components. C3b and C4b, generated from C3 and C4, respectively, are important components of convertases that promote activation of the complement cascade. The cleavage fragments of C5 are C5a, which induces migration of phagocytes into the infection site, and C5b, which initiates the formation of the membrane attack complex (MAC), and bacterial lysis (see, *e.g.*, Ramu *et al.* (2007) *FEBS Letters* 581:1716-1720).

To survive, pathogens have developed strategies to prevent deleterious consequences of complement activation. For example, members of the Ail/Lom family of outer membrane proteins provide protection from complement-dependent killing for a number of pathogenic bacteria. Members of the Ail/Lom family, which include Ail (attachment invasion locus) of *Yersinia* species, *e.g.*, *Y. enterocolitica* and *Y. pseudotuberculosis*, Rck (resistance to complement killing) and PagC of *Salmonella* species, and OmpX of *Escherichia coli*, are outer membrane proteins that share significant amino acid sequence similarity and identity, and have similar membrane topologies. While members of this family of proteins exhibit diverse functions, several of them, including Ail of *Y. enterocolitica* and *Y. pseudotuberculosis*, as well as Rck of *S. enterica*, function, at least in part, to protect bacteria from complement-mediated lysis (see, *e.g.*, Bartra *et al.* (2008) *Infection and Immunity* 76:612–622).

Another bacterial product that aids in avoiding or mitigating complement is the surface protease, designated PgtE (outer membrane serine protease) in *Salmonella*, and other members of the omptin family. The surface protease PgtE of *S. enterica* belongs to the omptin family of enterobacterial outer membrane aspartate proteases. PgtE and other omptins require rough LPS to be active, but are sterically inhibited by the O-antigen. Expression of *pgtE* is upregulated during the growth of *Salmonella* inside macrophages, and the bacteria released from macrophages exhibit strong PgtE-mediated proteolytic activity. PgtE proteolytically activates the mammalian plasma proenzyme plasminogen to plasmin, inactivates the main physiological inhibitor of

plasmin, alpha 2-antiplasmin, and mediates bacterial adhesion to extracellular matrices of human cells. This way, PgtE mediates the degradation of extracellular matrix components and generates potent, localized proteolytic activity, which can promote migration of *Salmonella* across extracellular matrices. PgtE also degrades alpha-helical antimicrobial peptides which can be important during intracellular growth of *Salmonella*. The omptin Pla of *Yersinia pestis* is a close ortholog of PgtE and shares functions with PgtE. Pla cleaves C3, and PgtE increases serum resistance of *Salmonella* by cleaving complement components C3b, C4b, and C5. The gene *pgtE*, and orthologs thereof from other bacterial species, can be included in the immunostimulatory bacteria herein to increase resistance to complement.

It is shown herein that the effects of complement in human serum explain the failure of therapeutic immunostimulatory bacteria, such as the *Salmonella* strain VNP20009, which had been shown to effectively colonize tumors in rodent models. Systemic administration of VNP20009 resulted in colonization of mouse tumors (see, *e.g.*, Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002; and Bermudes *et al.* (2001) *Biotechnol. Genet. Eng. Rev.* 18:219-33); whereas systemic administration of VNP20009 in human patients resulted in very little colonization. In the Phase 1 Study in advanced melanoma patients, very little VNP20009 was detected in human tumors after a 30 minute intravenous infusion (see, Toso *et al.* (2002) *J. Clin. Oncol.* 20:142-52). Patients that entered into a follow-up study evaluating a longer, four-hour infusion of VNP20009, also demonstrated a lack of detectable VNP20009 after tumor biopsy (see, Heimann *et al.* (2003) *J. Immunother.* 26:179–180). Following intratumoral administration, colonization of a derivative of VNP20009 was detected (see, Nemunaitis *et al.* (2003) *Cancer Gene Ther.* 10:737-744). Direct intratumoral administration of VNP20009 to human tumors resulted in much higher tumor colonization, indicating that human tumors can be colonized at a high level, and that the difference in tumor colonization between mice and humans occurs only after systemic administration.

It is shown and described herein, that, while not previously known to occur in wild-type *S. typhimurium*, VNP20009 is inactivated by human complement, which explains the low tumor colonization observed in humans upon systemic administration of VNP20009. Strains provided herein exhibit resistance to

complement. They can be modified to express Rck and other proteins involved in mediating complement resistance or avoidance, such as Ail of *Yersinia enterocolitica*, or PgtE of *Salmonella typhimurium*, or, if they natively express such a protein, they can be modified to overexpress Rck and/or other such proteins. Rck can be introduced into bacteria, such as *E. coli*, that lack a homolog.

***Rck* Expression**

Rck (resistance to complement killing) is a 17 kDa outer membrane protein encoded by the large virulence plasmid of *Salmonella* species, such as *S. enteritidis* and *S. typhimurium*, that induces adhesion to and invasion of epithelial cells. The Rck protein has been shown to protect *S. enterica* from complement by inhibiting C9 polymerization and subsequent assembly of a functional membrane attack complex. An *rck* mutant exhibited a 2-3 fold decrease in epithelial cell invasion compared to the wild-type strain, while *rck* overexpression in wild-type strains leads to increased invasion. The Rck protein induces cell entry by a receptor-mediated process, promoting local actin remodeling, and weak and closely adherent membrane extensions. Thus, *Salmonella* can enter cells by two distinct mechanisms: the Trigger mechanism mediated by the T3SS-1 complex, and a Zipper mechanism induced by *rck* (see, *e.g.*, Manon *et al.* (2012), *Salmonella*, Chapter 17, eds. Annous and Gurtler, Rijeka, pp. 339-364). Expression of *rck* on the *Salmonella* virulence plasmid confers a high level of resistance to neutralization by human complement, by preventing the formation of the membrane attack complex. When the *S. typhimurium* virulence plasmid containing *rck* was expressed in a highly serum-sensitive strain of *E. coli*, Rck was able to restore complement resistance.

The immunostimulatory bacteria provided herein retain, or are provided with, Rck to confer resistance to human complement. It is shown herein that immunostimulatory bacteria, such as *E. coli*, can be modified by encoding *rck* on a plasmid in the bacteria to thereby confer resistance to complement. Immunostimulatory bacteria provided herein encode *rck*, either endogenously, or can be modified to encode it in order to increase resistance to complement. Methods for conferring resistance to complement also are provided. For example, the therapeutic *E. coli* species described in U.S. Patent Application Publication Nos. 2018/0325963 and 2018/0273956, and U.S. Patent Nos. 9,889,164 and 9,688,967, can be improved

by modifying the bacteria therein, such as by introducing nucleic acid encoding the *Salmonella rck* gene on a plasmid therein, to thereby improve or provide resistance to complement. Bacteria that are resistant to complement can be systemically administered, and sufficient bacteria can survive to be therapeutically effective.

Nucleic acids encoding the *Salmonella rck* gene are introduced into bacteria, such as therapeutic *E. coli*, to thereby confer or increase complement resistance.

Other orthologs and homologs of *rck* from other bacterial species, similarly can be expressed in the immunostimulatory bacteria. For example, Ail is an Rck homolog from *Yersinia enterocolitica*, which enhances complement resistance under heterologous expression. PgtE is an *S. typhimurium* surface protease that has also been shown to enhance complement resistance under heterologous expression.

**8. Deletions of Genes Required for Lipoprotein Expression in *Salmonella* and Other Gram-Negative Bacteria**

The LPS and Braun (murein) lipoprotein (Lpp) are major components of the outer membrane of Gram-negative enteric bacteria that function as potent stimulators of inflammatory and immune responses. Braun (murein) lipoprotein (Lpp) is one of the most abundant components of the outer membrane in *S. typhimurium*, and leads to TLR2 induction of pro-inflammatory cytokines, such as TNFα, IL-6, and IL-8 (in humans). Two functional copies of the lipoprotein gene (*lppA* (SEQ ID NO:387) and *lppB* (SEQ ID NO:388)), that are located on the bacterial chromosome of *Salmonella*, contribute to bacterial virulence. Deletion of the *lppA* and *lppB* genes, and elimination of lipoprotein expression, reduces virulence and decreases pro-inflammatory cytokine production (see, *e.g.*, Sha *et al.* (2004) *Infect. Immun.* 72(7):3987-4003; and Fadl *et al.* (2005) *Infect. Immun.* 73(2):1081-1096). Deletion of the Lpp genes would be expected to reduce infection of cells, and, thus, decrease plasmid delivery and expression of the encoded therapeutic products or proteins. As shown in Example 18 below, however, while deletion of these genes did reduce tumor colonization, the amount of plasmid delivered to the targeted cells, *i.e.*, the tumor-resident immune cells, particularly macrophages, significantly was increased. As shown herein, deletion or disruption of these genes (*lppA* and *lppB*), thus, results in decreased virulence due to the inability to survive in infected macrophages, but results in enhanced plasmid delivery by the immunostimulatory bacteria, thereby increasing the

expression of encoded therapeutic genes in the targeted cells, *i.e.*, the tumor-resident immune cells, particularly macrophages.

Strains provided herein are ΔFLG (lack flagella), and/or Δ*pagP*, and/or Δ*ansB*, and/or Δ*csgD*. Additionally, the strains are one or more of Δ*purI* (Δ*purM*), Δ*msbB*, and Δ*asd* (in the bacterial genome). Exemplary strains are Δ*purI* (Δ*purM*), Δ*msbB*, Δ*pagP*, Δ*ansB*, Δ*asd*, or Δ*ThyA* in place of Δ*asd*. The strains also can be *lppA*$^-$ and/or *lppB*$^-$, *particularly lppA*$^-$/*lppB*$^-$. The plasmid is modified to encode therapeutic products under control of host-recognized promoters (*e.g.*, eukaryotic promoters, such as RNA polymerase II promoters, including those from eukaryotes, and from animal viruses). The plasmids can encode *asd* to permit bacterial replication *in vivo*, and can encode nucleic acids with other beneficial functions (such as CpGs), and can encode gene products, as described elsewhere herein.

The immunostimulatory bacteria provided herein can be modified to eliminate the ability to infect epithelial cells, such as by elimination of the flagella. Elimination of the ability to infect epithelial cells, as described elsewhere herein, also can be achieved by inactivating SPI-1-dependent invasion, through inactivation or knockout of one or more genes involved in the SPI-1 pathway. These genes include, but are not limited to, one more of: *avrA*, *hilA*, *hilD*, *invA*, *invB*, *invC*, *invE*, *invF*, *invG*, *invH*, *invI*, *invJ*, *iacP*, *iagB*, *spaO*, *spaP*, *spaQ*, *spaR*, *spaS*, *orgA*, *orgB*, *orgC*, *prgH*, *prgI*, *prgJ*, *prgK*, *sicA*, *sicP*, *sipA*, *sipB*, *sipC*, *sipD*, *sirC*, *sopB*, *sopD*, *sopE*, *sopE2*, *sprB*, and *sptP*. Additionally or alternatively, the immunostimulatory bacteria can contain knockouts or deletions in genes to inactivate products involved in SPI-1-independent infection/invasion, such as one or more of the genes *fljB*, *fliC*, *rck*, *pagN*, *hlyE*, *pefI*, *srgD*, *srgA*, *srgB*, and *srgC*, and/or the immunostimulatory bacteria can contain knockouts or deletions to inactivate products of genes that induce cell death of tumor-resident immune cells, such as genes that encode proteins that are directly recognized by the inflammasome, including *fljB*, *fliC*, *prgI* (needle protein), and *prgJ* (rod protein). The *rck* gene is desirable because it protects the bacteria against inactivation by complement. Bacteria that do not endogenously encode *rck*, can be modified to encode a heterologous *rck* gene.

The immunostimulatory bacteria are derived from suitable bacterial strains. Bacterial strains can be attenuated strains, or strains that are attenuated by standard

methods, or that, by virtue of the modifications provided herein, are attenuated in that their ability to colonize is limited primarily to immunoprivileged tissues and organs, particularly tumor-resident immune cells, the TME, and tumor cells, including solid tumors. Bacteria include, but are not limited to, for example, strains of *Salmonella*, *Shigella*, *Listeria*, *E. coli*, and *Bifidobacteriae*. For example, species include *Shigella sonnei*, *Shigella flexneri*, *Shigella dysenteriae*, *Listeria monocytogenes*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella gallinarum*, and *Salmonella enteritidis*. Other suitable bacterial species include *Rickettsia*, *Klebsiella*, *Bordetella*, *Neisseria*, *Aeromonas*, *Francisella*, *Corynebacterium*, *Citrobacter*, *Chlamydia*, *Haemophilus*, *Brucella*, *Mycobacterium*, *Mycoplasma*, *Legionella*, *Rhodococcus*, *Pseudomonas*, *Helicobacter*, *Vibrio*, *Bacillus*, and *Erysipelothrix*. For example, *Rickettsia rickettsii*, *Rickettsia prowazekii*, *Rickettsia tsutsugamuchi*, *Rickettsia mooseri*, *Rickettsia sibirica*, *Bordetella bronchiseptica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Aeromonas eucrenophila*, *Aeromonas salmonicida*, *Francisella tularensis*, *Corynebacterium pseudotuberculosis*, *Citrobacter freundii*, *Chlamydia pneumoniae*, *Haemophilus somnus*, *Brucella abortus*, *Mycobacterium intracellulare*, *Legionella pneumophila*, *Rhodococcus equi*, *Pseudomonas aeruginosa*, *Helicobacter mustelae*, *Vibrio cholerae*, *Bacillus subtilis*, *Erysipelothrix rhusiopathiae*, *Yersinia enterocolitica*, *Rochalimaea quintana*, and *Agrobacterium tumerfacium*.

Exemplary of the immunostimulatory bacteria provided herein are species of *Salmonella*. Exemplary of bacteria for modification as described herein are wild-type strains of *Salmonella*, such as the strain that has all of the identifying characteristics of the strain deposited in the American Type Culture Collection (ATCC) as accession #14028. Engineered strains of *Salmonella typhimurium*, such as strain YS1646 (ATCC catalog # 202165, also referred to as VNP20009; see, also International PCT Application Publication No. WO 99/13053), is engineered with plasmids to complement an *asd* gene knockout and to allow for antibiotic-free plasmid maintenance. The strains then are modified to delete the flagellin genes, and/or to delete *pagP*. The combination of flagella knockout and *pagP* deletion renders the strains highly resistant to human serum complement. The strains also are rendered auxotrophic for purines, particularly adenosine, and are $asd^-$ and $msbB^-$. As exemplified, strains in which the *purI* and *msbB* genes are completely deleted are

more fit (*e.g.*, grow faster) that strain VNP20009, in which these genes are not deleted, but are modified to eliminate expression. The *asd* gene can be provided on a plasmid for *in vivo* replication in the eukaryotic host. The strains also have a modification, such as a deletion, disruption, or other modification, in the *ansB* gene, preventing them from producing immunosuppressive L-asparaginase II, and improving tumor T-cell function. The strains also are modified to eliminate biofilm production, such as by a *csgD* deletion, which renders them unable to produce curli fimbriae, cellulose, and c-di-GMP, reducing unwanted inflammatory responses, and preventing the strains from forming biofilms.

These genomic deletions and plasmids are described and exemplified elsewhere herein. Any of the nucleic acid encoding therapeutic products, such as immunostimulatory proteins and other products, described elsewhere herein and/or known to those of skill in the art, can be included on the plasmid. The plasmid generally is present in low to medium copy number, as described elsewhere herein. Therapeutic products include gain-of-function mutants of cytosolic DNA/RNA sensors, that can constitutively evoke/induce type I IFN expression, and other immunostimulatory proteins, such as cytokines, chemokines, and co-stimulatory molecules, that promote an anti-tumor immune response in the tumor microenvironment, and other such products described herein. The plasmids also can encode antibodies, and fragments thereof, *e.g.*, single-chain antibodies that target immune checkpoints and other cancer targets, such as VEGF, IL-6, and TGF-β, and other molecules, such as bispecific T-cell engagers, or BiTEs®. The plasmids also can encode IL-6 binding decoy receptors, TGF-beta binding decoy receptors, and TGF-beta polypeptide antagonists.

### 9. Robust Immunostimulatory Bacteria Whose Genomes are Optimized for Anti-Tumor Therapy, and that Encode Therapeutic Products, Including a Plurality Thereof

As described herein, bacterial strains, such as *S. typhimurium* strains, that are engineered to be adenosine auxotrophic, and are reduced in their ability to induce pro-inflammatory cytokines by modification of the LPS and/or deletion of flagellin, and/or are modified by deletion or elimination of L-asparaginase II expression to improve T-cell function, and/or are modified by deletion or disruption of genes required for biofilm formation, and/or that demonstrate enhanced human serum

survival due to increased *rck* expression, are further modified to deliver therapeutic products, such as immunomodulatory proteins, and promote robust anti-tumor immune responses.

The table below summarizes the bacterial genotypes/modifications, with reference to *Salmonella* genes with the understanding that counterpart genes can be modified in other bacterial species, their functional effects, and some of the effects/benefits achieved herein.

| Genotype/Modification | Functional Effect | Effect/Benefit |
|---|---|---|
| Δ*asd* (in genome) | Plasmid maintenance | Improves plasmid delivery<br>Plasmid maintenance *in vivo* via *asd* cassette on plasmid |
| Δ*purI* | Purine/adenosine auxotrophy | Tumor-specific enrichment<br>Limited replication in healthy tissue |
| Δ*msbB* | LPS surface coat modification | Decreases TLR4 recognition<br>Reduces immunosuppressive cytokine profile (TNF-α)<br>Improves safety<br>Prevents intracellular replication |
| Δ*fliC*/Δ*fljB* (ΔFLG) | Flagella knockout | Removes major inflammatory and immune-suppressive element — lethal inflammasome activation<br>Eliminates TLR5 recognition<br>Reduces immunosuppressive cytokine profile, including suppression of type I IFN<br>Improves safety<br>Reduces ability to invade non-phagocytic cells (*e.g.*, stromal and tumor cells) |
| Δ*pagP* | LPS surface coat modification | Removes major inflammatory and immunosuppressive element, including suppression of type I IFN<br>Decreases TLR4 recognition<br>Reduces IL-6 production<br>Improves safety |
| Δ*ansB* | L-asparaginase II knockout | Enhances tumor T-cell function |
| Δ*csgD* | Removes curli fimbriae, cellulose production, c-di-GMP | Reduces inflammation, including IL-10 suppression of type I IFN,<br>Prevents possible biofilm formation<br>Enhances phagocytic cell uptake |
| Plasmid | Expresses gene products under control of host-recognized promoter | Eukaryotic promoter limits expression to cells containing the plasmid<br>Long term expression in the TME (*i.e.*, *asd* encoded on plasmid under control of host-recognized promoter)<br>Expression of any combination of therapeutic product(s) with large capacity |

## 10. Vaccines and Bacteria that Deliver RNA, including mRNA and other Forms of RNA, for Expression in a Eukaryotic Host

### Immunostimulatory Bacteria as Delivery Vehicles for RNA

Provided are immunostimulatory bacteria that contain or that express encoded heterologous RNA, where the bacteria infect eukaryotic host cells, and release the encoded RNA into the cytoplasm of the host cell (see, *e.g.*, U.S. Patent No. 7,390,646). The RNA can be a therapeutic product, or the RNA is translated by the host cell machinery into a therapeutic product. Such products include immunostimulatory proteins and antigens from pathogens and/or tumor antigens that can be expressed in cells, such as phagocytic cell for presentation to induce immune responses including T-cell responses, memory T-cells, and antibodies. For immunization, the bacteria can be administered to the site or by a route that is the same as naturally-occurring route of the pathogen, such as by inhalation or nasal administration for respiratory viruses, oral administration for gastric and enteric pathogens, intramuscular administration, such as for viruses, and also systemic routes. This can result in *in situ* immune responses that can provide lifelong immunity.

Any of the immunostimulatory bacteria provided herein can be modified so that the bacteria produce the exogenous or heterologous RNA within the bacterial cells *in vitro*, such as during *in vitro* cell culture, or after entry of the bacterial cells into the host eukaryotic cells, or both during *in vitro* cell culture, and after entry of the bacterial cells into the host eukaryotic cells. Following infection of eukaryotic host cells, the bacteria release the exogenous RNA into the cytoplasm of the host cells. The RNA can be encoded on a plasmid in the bacteria, and can encode a plurality of therapeutic products. In particular, the forms of RNA delivered are any that can be translated by eukaryotic cell machinery. This includes mRNA, long non-coding RNA, RNAi, dsRNA, circular RNA (eRNA; see, *e.g.*, U.S. Patent No. 10,953,033).

Provided are immunostimulatory bacteria and genomically-modified bacteria that contain or express encoded heterologous RNA, where, when the bacteria infect eukaryotic host cells, the encoded RNA, such as mRNA, is released into the cytoplasm of the host cell. The bacteria include any of the immunostimulatory bacteria provided herein, and particularly, those that include genomic modifications, so that they have reduced TLR2, TLR4, and TLR5 signaling. The bacteria also can

encode a therapeutic product or a plurality thereof, and particularly, encode immunostimulatory proteins, including cytokines and STING proteins, including the modified STING proteins provided herein, which can act as adjuvants if the RNA is encoding an antigen or protein for vaccination, or for a treatment for which immune stimulation is advantageous.

The nucleic acid encoding the RNA is modified so that it is transcribed in the bacteria, but is not translated. This can be effected by designing the nucleic acid so that the transcribed RNA product is not recognized by or cannot be translated by prokaryotic ribosomes. The encoding nucleic acid further is designed such that the transcript is recognized by and is translated by eukaryotic ribosomes, such as those present in a host, such as a human. This can be effected by, for example, including nucleic acid encoding an internal ribosome entry site (IRES) that is not recognized by prokaryotic ribosomes, but is recognized by eukaryotic ribosomes The encoded RNA typically is mRNA, but can be other forms of RNA that can be translated into therapeutic products or immunizing antigens or proteins. Other forms, of RNA include, but are not limited to, eRNA, which is circular RNA. eRNA is a circular polyribonucleotide that: (a) contains an expression sequence encoding a polypeptide, such as an antigen; (b) contains an internal ribosome entry site (IRES) that is not recognized by prokaryotic ribosomes, and a termination element; and (c) lacks a poly-A sequence, a free 3' end, and an RNA polymerase recognition motif (see, *e.g.*, U.S. Patent No. 10,953,033, which describes such circular RNA). The bacteria are modified so that the transcribed RNA is circularized *in vivo*.

The RNA can be a therapeutic product, or the RNA is translated by the host cell machinery into a therapeutic product. The RNA can encode an antigen, whereby it is encoded into a protein when delivered into the host cells, to immunize the host against the antigen, and the pathogen or the tumor from which the antigen is derived. The nucleic acid can be modified so that the encoded antigen is modified to improve its properties for immunization, such as by stabilizing a conformation of the antigen against which antibodies and other adaptive immune responses are directed. This has been done, for example, for the spike proteins, on corona viruses, so that the conformation that binds to host cell receptors is stabilized, resulting in a more robust antibody response.

Any of the immunostimulatory bacteria provided herein can be modified so that the bacteria produce the exogenous or heterologous RNA within the bacterial cells *in vitro*, such as during *in vitro* cell culture, or after entry of the bacterial cells into the host eukaryotic cells, or both during *in vitro* cell culture and after entry of the bacterial cells into the host eukaryotic cells. Following infection of eukaryotic host cells, the bacteria release the exogenous RNA into the cytoplasm of the host cells. The RNA can be encoded on a plasmid in the bacteria, and can encode a plurality of therapeutic products.

The nucleic acid encoding the RNA is operably linked to a promoter recognized by the bacteria, or the bacteria are modified to encode the RNA polymerase that recognizes the promoter, such as a bacterial promoter, or a bacteriophage promoter that is recognized by phage T7 RNA polymerase. The RNA can be mRNA, or other RNA molecules that contain regulatory sequences for expression in a eukaryotic host cell. The promoter can be an inducible promoter. The bacteria can contain a plurality of copies of the encoded RNA, which, when introduced into a eukaryotic host, such as a human, is released into the host cell cytoplasm, where, if it is mRNA, it can be translated. Bacterial transcription is decoupled from bacterial translation, so that gene products are transcribed, but not translated. In some embodiments, rather than including an IRES sequence, the nucleic acid encoding the RNA lacks a Shine-Dalgarno (SD) sequence upstream of the start codon, whereby the RNA cannot be translated by bacterial or archaeal ribosomes, but can be translated by eukaryotic ribosomes. The RNA can contain sequences, such as a Kozak sequence, with a start codon recognized by eukaryotic ribosomes. Shine-Dalgarno sequences include, for example SEQ ID NOs: 389-392:

(1) ...AUAAAGGAGGUAAAUA

(2) ...AUAAAGGAAAUAAAUA

(3) ...AUAACAGAGGUAAAUA

(4) ...AUAACAGGAGUAAAUA

Shine-Dalgarno

(see, *e.g.*, parts.igem.org/File:RBSAlignedSpacing.png). The RNA also can contain one or more Internal Ribosome Entry Site(s) (IRES), which block or inhibit

translation by prokaryotic ribosomes. Thus, the RNA is produced in the bacteria, which infect or have infected myeloid cells, thereby delivering RNA to the myeloid cells, where, if it is mRNA, it is translated in the myeloid cells, which recognize the RNA for translation because of the Shine-Dalgarno sequence, or IRES, or other such regulatory sequence. Inserting, for example, an IRES between a promoter and a gene sequence, de-couples transcription from translation. The immunostimulatory bacteria, thus, serve as an RNA delivery system.

In embodiments, bacterial systems, such as the immunostimulatory bacteria provided herein, are used to deliver genetic payloads to tissue-resident and/or tumor-resident myeloid cells. In this context, the bacteria are dosed *in vivo*; the bacteria are, for example, *asd*$^-$, and do not include a plasmid containing a complementing asd gene cassette, so that they cannot replicate *in vivo*. To grow the bacteria, they are cultured *in vitro*; diaminopimelic acid (DAP) is added to the culture medium to facilitate bacterial replication in the absence of a functional *asd* gene. In this context, when such immunostimulatory bacteria are administered, one bolus of nucleic acid is delivered to tissue-resident or tumor resident myeloid cells. After phagocytosis and intracellular destruction of the bacteria, the RNA encoding the therapeutic product(s) is released into the cytoplasm of the myeloid cells for translation, to produce the therapeutic product(s) that is/are secreted from the myeloid cells into the surrounding environment, such as the tumor microenvironment, when the infected cells are tumor-resident myeloid cells.

Bacteria cultured *in vitro* encode RNA, but lack the signals/regulatory sequences, such as the Shine-Dalgarno sequence, that are required to translate the RNA, or the encoding nucleic acid contains sequences, such as an IRES, that blocks or does not provide for translation by prokaryotic ribosomes, but that are recognized by eukaryotic ribosomes, so that the bacteria produce, but do not translate, the mRNA. The bacteria, which are modified as described herein, so that they infect or accumulate in tissue-resident or tumor-resident immune cells, particularly tissue-resident or tumor-resident myeloid cells, deliver the RNA to the myeloid cells, where it is translated. Any RNA can be delivered, including RNA encoding antigens, such as from pathogens or tumors, and/or encoding anti-viral therapeutics, which are expressed under control of a prokaryotic promoter. For such applications, the

plasmids can be present in higher copy number (*e.g.*, 150 copies or greater, such as 200, 300, 400, 500, or more copies, such as 500-700 copies), so that large amounts of the RNA is produced and delivered to the myeloid cells.

Also, as described and exemplified herein and discussed above, the encoding and regulatory sequences can be prepared so that the RNA is produced in the bacterium, but the encoding nucleic acid includes regulatory sequences that inhibit or prevent translation by bacterial ribosomes, but that are recognized by, and/or enhance, promote, or permit translation by, eukaryotic host ribosomes. For example, the mRNA is transcribed under control of a prokaryotic promoter. An IRES sequence is included in the transcript, which blocks translation in the prokaryotic bacterium, but, when delivered into a eukaryotic host cell, permits translation by eukaryotic ribosomes. The bacteria, thus, provide non-labile mRNA delivery systems. The bacteria can be grown in large quantities, and then can be lyophilized or frozen and/or stored at room temperature. They can be formulated into tablets, or powders, or can be micronized for inhalation.

The bacteria include those that contain genome modifications, whereby the response by toll-like receptors (TLRs) 2, 4, and 5 is reduced, compared to the bacterium without the genome modifications. These bacteria optionally can additionally contain genomic modifications so that they are auxotrophic for a required nutrient or factor, so that they are unable to replicate in a eukaryotic host, but can replicate *in vitro* when supplied with the nutrient or factor. These include deficiencies in adenosine biosynthesis, which has the ad added advantage of reducing immunosuppression when used for treatment of subjects with cancer who have tumors and a tumor microenvironment in which adenosine accumulates. Other deficiencies include thymine synthesis deficiencies, such as *thyA*$^{-}$, described and discussed in sections above, and exemplified below.

The bacteria contain a plasmid containing nucleic acid encoding a product, or comprise RNA encoding the product. The plasmid can be present in low, medium, or high copy number. When used to deliver RNA, the plasmid can be present in high copy number, such as 150 copies or greater. The product encoded by the nucleic acid or RNA is an antigenic sequence or sequences from a pathogenic virus, bacterium, parasite, or is a tumor antigen, whereby, upon expression of the encoded antigen in

the host, the host develops an immune-protective response or immunizing response against the pathogenic virus, bacterium, or parasite, or against the tumor antigen, or the product is a therapeutic product to be delivered to the host. Expression of the antigenic sequence(s) is/are under control of a prokaryotic promoter, so that RNA encoding the antigen(s) is produced in the bacterium, and, for RNA delivery, nucleic acid encoding the antigen comprises regulatory sequences that inhibit or prevent translation of encoded RNA by bacterial ribosomes, but that do not inhibit or prevent translation of the encoded RNA by eukaryotic host ribosomes, whereby translation is de-coupled from transcription in the bacterium. By virtue of the genomic modifications, the resulting bacterium is selective for infecting phagocytic cells when administered to a eukaryotic subject, and delivers the nucleic acid into the phagocytic cells, wherein the RNA is translated.

Of particular interest are the immunostimulatory bacteria provided herein that are modified so that they infect phagocytic cells, such as macrophages. In particular, the bacteria, which can be any suitable species/genus, such as *Salmonella*, *Listeria*, and *E. coli*, are so-modified so that they are one or more or all of modified to produce LPS with penta-acylated lipid A, modified to lack flagella, and modified to lack curli fimbriae; for example, the bacteria are *msbB*$^-$/*pagP*$^-$, flagellin$^-$, and *csgD*$^-$ (by modification by each of the encoding genes or equivalent genes in a particular species, so that the products are not produced, or, if produced, are inactive). The bacteria also are modified, such as by rendering them *asd*$^+$, so that the can be grown *in vitro* by providing the appropriate nutrient, such a DAP, but so that they do not replicate in a eukaryotic host, and ultimately die when the mRNA is delivered into the eukaryotic cells. The bacteria also can encode additional payloads, as described herein, such as modified STING proteins and/or cytokines (all as described and listed elsewhere herein), which serve to stimulate the host immune system, thereby acting as adjuvants. Such products can be encoded on plasmids in the bacterium, and can be encoded on a polycistronic construct with the mRNA encoding the antigen, and/or can be encoded under control of a eukaryotic promoter for transcription/translation into proteins, as described throughout the disclosure herein. These proteins can be designed for transcription, or, as appropriate, can be modified so that they are membrane anchored for presentations. The immunostimulatory bacteria, such as *E.*

*coli*, can be modified so that they encoded and express *rck* to increase resistance to complement.

**11. Bacterial Vaccines Against Particular Antigens, Including from Pathogens, and also Antigens from Tumors, for use as Anti-Pathogen Treatments and Vaccines, and For Anti-Cancer Treatment and/or Prevention**

Vaccines can be used for preventing (reducing the risk) of contracting a disese or developing a disorder or cancer or as treatments for diseases, disorders, or cancers, and combinations thereof. immunostimulatory bacteria provided herein and other vaccines can be used for these purposes.

Because of the similarity in the immune response between an anti-tumor response and an anti-viral response, the immunostimulatory bacteria provided herein also can be used to treat infectious diseases. The bacteria can encode an anti-viral or anti-bacterial therapeutic, such as an inhibitor of a viral or bacterial product, or an inhibitor of the expression of a viral or bacterial product, or a viral or bacterial antigen. The combination of the immune response from the immunostimulatory bacteria and the therapeutic anti-pathogen product, and also, to the immunostimulatory proteins and encoded immune-stimulating proteins, provides a therapeutic immunostimulatory bacterium for vaccinating against and/or treating infectious diseases, particularly diseases associated with viral infections, such as chronic viral infections and latent viral infections. Of interest are chronic viral infections, such as infections by hepatitis viruses, herpes viruses, varicella zoster virus (VZV), Epstein-Barr virus, human immunodeficiency virus (HIV), human T-cell leukemia virus (HTLV), Respiratory Syncytial Virus (RSV), measles virus, and other such viruses that chronically infect subjects. The immunostimulatory bacteria also can be used for the treatment of acute infections as well, such as initial infections with chronic influenza and coronaviruses, such as Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19). The immunostimulatory bacteria can encode an antigen from a pathogen, such as a viral antigen, and are used as a vaccine to prevent infection or to treat existing infections. The immunostimulatory bacterium, by virtue of its ability to accumulate in immune cells, such as antigen-presenting cells, ~~such as T-cells~~, can

promote a T-cell response to the virus. For example, immunostimulatory bacteria provided herein are deficient in asparaginase II, which enhances the function of T-cells to thereby promote the anti-viral response. The combination of expression of a viral antigen, such as an antigen from an essential viral protein, such as, for example, in the case of a coronavirus, an antigen from the Nucleocapsid, M and/or S proteins, which can result in neutralizing antibodies, and the enhancement of long-lived circulating and tissue-resident $CD8^+$ T-cells, and provides effective vaccines and treatments.

The efficacy of existing vaccines, such as inactivated or attenuated vaccines, such as the Bacillus Calmette–Guérin (BCG) vaccine for tuberculosis (TB), can be improved by modifying the genome so that asparaginase is not expressed, thereby eliminating the immune suppression. BCG vaccine effectiveness is often reduced. This is by virtue of the asparaginase secreted by the vaccine bacteria, which decreases T-cell activity, thereby having an immuno-suppressive effect. This can be mitigated by modifying the bacteria to reduce or eliminate asparaginase activity. As discussed elsewhere herein, othere modifications that reduce or eliminate TLR2 or TLR2/4/5 responses to the bacteria eliminate any blockade or inhibition of type I IFN by the activity or response of TLR2, or TLR4, or TLR5, or other toll-like receptors to bacteria. As described herein, the responses of the TLRs to bacteria can block or inhibit type I IFN; modifications of the bacteria herein by eliminating responses of these TLRs eliminate this undesirable effect.

As discussed in the section above, the immunostimulatory bacteria can encode an anti-viral therapeutic or an anti-bacterial therapeutic, or an antigen for immunization. Such therapeutics include inhibitors of viral genes and proteins, such as proteins required for replication and/or packaging, or the immunostimulatory bacteria can encode a therapeutic that prevents binding or interaction of a virus with a receptor or receptors that facilitate or provide for viral entry into a target cell. In some embodiments, the therapeutic protein can be under the control of a prokaryotic promoter. As discussed in the section above, the bacteria can be those that deliver RNA.

As discussed above, the bacteria can be used to deliver RNA, including mRNA for vaccination against pathogens, such as SARS-CoV, SARS-CoV-2,

influenza, and other pathgens, and also, for anti-tumor therapy. The bacteria can also encode immunostimulants or other immune system enhancers, or inhibitors of immunosuppressors, such as immune checkpoint proteins. Exemplary of these are STING proteins, including the modified STING proteins provided herein, cytokines, and other such immune stimulating proteins. Any of the payloads described herein, including the combined payloads, that are for treating tumors, also can be encoded/delivered by the bacteria. Anti-tumor responses and anti-viral responses are similar. For the vaccines, the immunostimulatory payloads serve as adjuvants to enhance the immune response. The pathogen antigen-encoding nucleic acid is under control of a prokaryotic promoter; the other payloads also can be provided as a polycistronic construct with the pathogen antigen-encoding nucleic acid, or they can be encoded under control of a eukaryotic promoter, as described herein for the anti-tumor therapeutics.

Bacteria, such as those provided herein, that deliver payloads to phagocytic cells, and that contain the various genome modifications that improve their properties for delivery, can be used to deliver RNA. The bacteria are superior vehicles to nanoparticles and other such delivery vehicles, since the RNA is delivered in the bacterial cytoplasm and contains the adjuvants to stimulate type I IFN. Among the advantages of the bacteria provided herein for delivery of RNA are: 1) they allow for RNA delivery "without the RNA" — the bacterial delivery vehicle obviates the need for complex RNA stability/manufacturing; 2) they result in direct targeting of phagocytic antigen presenting-cells (APCs); 3) they allow for the inclusion of payloads that provide type I IFN adjuvant activity; 4) they contain genome modification so that the bacteria do not replicate in the eukaryotic (such as human) host, such as by rendering the bacteria *asd*$^-$ by inactivating endogenous *asd* activity and not encoding it on the plasmid; and 5) removal of bacterial products that are poor adjuvants by genome modification is possible, such as by eliminating the flagella, modifying the LPS, reducing or eliminating biofilm formation, as well as the elimination of asparaginase II activity. Exemplary of these bacteria are those that are *msbB*$^-$/*pagP*$^-$, and flagellin$^-$, and optionally, *ansB*$^-$, and *csgD*$^-$, with a complete (clean) deletion of the *purI* gene. Bacteria as vehicles also permit rapid engineering and deployment; bacteria are easily scalable and are easy to manufacture; a plurality of

different RNAs can be encoded in a single bacterium; and bacteria are stable, and they can be stored at room temperature, they can be lyophilized, and they can be formulated for intranasal delivery or delivery by inhalation, as well as for intravenous delivery, and can be formulated for other suitable routes of delivery. Retaining some of the TLR2 activity renders endothelial vascular cells leaky, as does including the genome modification that renders the bacteria *csgD*$^{-}$.

For delivery of anti-cancer therapeutic products to the tumor/tumor microenvironment, the encoded products can be operatively linked to trafficking signals, such as signals for secretion. The products also can be designed for expression on a cell surface, such as the cell surfaces of tumor-resident myeloid cells and other phagocytic cells. The gene products can be modified so that they are membrane anchored. For example, IL-12 has been modified herein so that it is membrane-anchored by adding a transmembrane domain to the C-terminus. Other proteins, including, for example, IL-2, IL-12, IL-12p35, IL-21, IL-15, and FLT-3L, can be similarly modified by adding a transmembrane domain or other such anchoring domain, such as a GPI anchor. Anchoring such proteins to the membranes of cells that are infected with the bacteria reduces toxicity so that they are not systemically secreted, but act in the tumor microenvironment. In particular, the immunostimulatory bacteria that encode such products and combinations thereof are those with genome modifications, such as, for example, modifications that result in the bacteria being *msbB*$^{-}$/*pagP*$^{-}$ and not having flagella, whereby the response by toll-like receptors (TLRs) 2, 4, and 5 is reduced, compared to the bacteria without the genome modifications.

**12. Conversion of M2 Phenotype Macrophages into M1 and M1-Like Phenotype Macrophages**

As described herein, the immunostimulatory bacteria provided herein accumulate in and/or target macrophages. Macrophages are phagocytic immune cells; they play a role in clearing senescent and apoptotic cells, as well as in the phagocytosis of immune-related complexes and pathogens, and in the maintenance of homeostasis. The phenotype and function of macrophages can be polarized by the microenvironment. There are two types: M1-type (classically activated) macrophages, and M2-type (alternatively activated) macrophages.

The role of M1 macrophages is to secrete pro-inflammatory cytokines and chemokines, and to present antigens, and thus, to participate in the positive immune response, and function as immune monitors. M1 macrophages produce pro-inflammatory cytokines, including IL-6, IL-12, and TNF-alpha. M2 macrophages secrete arginase 1, IL-10, TGF-β, and other anti-inflammatory cytokines, which have the function of reducing inflammation, and contributing to tumor growth and immunosuppressive function. Thus, for the treatment of cancers and other such diseases and disorders, the M1 or M1-like phenotype is advantageous.

M2 macrophages can be converted into M1 macrophages or into macrophages with an M1-like phenotype. Immunostimulatory bacteria provided herein, which infect macrophages, can convert M2 macrophages into macrophages with an M1 or M1-like phenotype. M1 macrophage phenotypic markers include CD80 (also known as B7, B7.1, or BB1), CD86 (also known as B7.2), CD64 (also known as high affinity immunoglobulin gamma Fc receptor I ), CD16, and CD32 (also known as low affinity immunoglobulin gamma Fc region receptor IIb). Expression of nitric oxide synthase (iNOS) in M1 macrophages can also serve as a phenotypic marker. CD163 and CD206 are markers for the identification of M2 macrophages. Arginase 1 (Arg1) and DECTIN-1 also are ideal phenotypic indicators for the identification of M2 macrophages. The phenotypic conversion can be monitored or assessed by virtue of expression of these markers, and/or other markers that are characteristic of such macrophage phenotypes.

Tumor-associated macrophages (TAMs) are associated with an immunosuppressive M2 phenotype. Immunostimulatory bacteria provided herein can convert such macrophages into macrophages with an M1 or M1-like phenotype. The immunostimulatory bacteria provided herein, that encode a therapeutic product that leads to expression of type I interferon (IFN), can effect such conversion. This a property unique to the immunostimulatory bacteria provided herein, and exploits the ability of the bacteria that include genomic modifications that result in the infection of macrophages. The encoded therapeutic products include those that are part of a cytosolic DNA/RNA sensor pathway, such as the STING variants (described in detail herein). The encoding immunostimulatory bacteria can effect conversion of the infected macrophages to an M1 phenotype (or an M1-like phenotype) upon infection

of the tumor-resident macrophages, and expression of the therapeutic product(s). This ability to convert macrophage phenotypes is demonstrated and exemplified in Example 12 below. The expression of a modified STING protein by immunostimulatory bacteria provided herein that infect macrophages and express the STING protein, converts the phenotype of M2 macrophages to an M1-like phenotype.

Immunostimulatory bacteria provided herein, that include genome modifications as descrbied herein, such as the elimination of flagella and LPS modification, convert infected M2 macrophages into those that induce some or all of the cytokine profiles characteristic of M1 macrophages. Immunostimulatory bacteria that express a variant STING protein that results in constitutive type I IFN expression in human primary M2 macrophages, convert these cells to M1-like (having phenotypic markers and/or expression profiles typical of M1 macrophages) type I IFN producing cells.

## D. IMMUNOSTIMULATORY BACTERIA WITH ENHANCED THERAPEUTIC INDEX ENCODING GENETIC PAYLOADS THAT STIMULATE THE IMMUNE RESPONSE IN THE TUMOR MICROENVIRONMENT

The immunostimulatory bacteria provided herein are modified so that they accumulate in the tumor microenvironment, and in tumor-resident myeloid cells, where encoded therapeutic products, under the control of eukaryotic promoters, are expressed. The bacteria encode therapeutic products, particularly anti-cancer products, including products that stimulate the immune system, and/or that reverse or mitigate the immunosuppressive effects of tumors. As described herein, the bacteria can encode a plurality of products, where expression of each product is under the control of a separate promoter, or expression of the products is under the control of one promotor, and the expression cassette can include sequences that result in expression of the discrete products, and, where appropriate, includes regulatory sequences to ensure secretion of the encoded products into the tumor microenvironment. The immunostimulatory bacteria express encoded therapeutic products on the plasmid. As discussed herein, the plasmid can encode one product, or a plurality thereof. Each product can be expressed under the control of a different eukaryotic promoter, or multiple encoded products can be expressed under the control of a single promoter, such as by including 2A self-cleaving peptides between the

coding portions, such as T2A (SEQ ID NO:327), P2A (SEQ ID NO:328), E2A (SEQ ID NO:329), and F2A (SEQ ID NO:330). The encoded products include those described herein, and they can be anti-cancer immune stimulating products whose activities are complementary. The immunostimulatory bacteria provided herein permit the combinatorial administration of multiple immunomodulatory products or payloads (multiplexed payloads) that would otherwise be too toxic if systemically administered. Exemplary of multiplexed payloads include one or more cytokine(s), an immunostimulatory protein to stimulate or induce expression of type I IFN, such as STING or a variant thereof that has increased activity or that is constitutively active, and a co-stimulatory molecule, such as an engineered 4-1BBL co-stimulatory molecule.

The immunostimulatory bacteria provided herein have strong anti-tumor effects, including provision of cures, such as after IV dosing with the multiplexed payloads or single agent payloads. The immunostimulatory bacteria, when systemically administered, infiltrate and enrich in solid tumors, in the TME, and in tumor-resident myeloid cells, in which the encoded therapeutic products are expressed and then locally delivered to the tumor microenvironment. Upon consumption (phagocytosis) by tumor-resident myeloid cells, the bacteria deliver a genetic payload-encoding plasmid, which allows for ectopic, single or multiplexed payload expression in a tumor-specific manner.

**1. Immunostimulatory Proteins**

The immunostimulatory bacteria herein can be modified to encode one or more of an immunostimulatory protein that promotes, induces, or enhances an anti-tumor response. As exemplified and described herein, the order in which the encoding nucleic acids are arranged on the plasmid can improve overall expression, and modifications to the plasmids, such as complete deletions or inactivation of genes, can improve the fitness of the bacteria that contain the plasmids encoding the proteins.

The immunostimulatory protein(s) can be encoded on a plasmid in the bacterium, under the control of a eukaryotic promoter, such as a promoter recognized by RNA polymerase II, for expression in a eukaryotic subject, particularly the subject for whom the immunostimulatory bacterium is to be administered, such as a human. The nucleic acid encoding the immunostimulatory protein(s) can include, in addition

to the eukaryotic promoter, other regulatory signals for expression or trafficking in the cells, such as for secretion or expression on the surface of a cell.

Immunostimulatory proteins are those that, in the appropriate environment, such as a tumor microenvironment (TME), can promote, or participate in, or enhance, an anti-tumor response by the subject to whom the immunostimulatory bacterium is administered. Immunostimulatory proteins include, but are not limited to, cytokines, chemokines, and co-stimulatory molecules. These include cytokines, such as, but not limited to, IL-2, IL-7, IL-12, IL-15, IL-18, IL-21, IL-23, IL-12p70 (IL-12p40 + IL-12p35), IL-15/IL-15R alpha chain complex, IL-36γ, GM-CSF, IFNα, IFNβ, IL-2 that has attenuated binding to IL-2Ra, and IL-2 that is modified so that it does not bind to IL-2Ra; chemokines, such as, but not limited to, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11; and/or co-stimulatory molecules, such as, but not limited to, CD40, CD40L, OX40, OX40L, 4-1BB, 4-1BBL, 4-1BBL with the cytoplasmic domain truncated or deleted (4-1BBLΔCyt), members of the TNF/TNFR superfamily (*e.g.*, CD27 and CD27L), and members of the B7-CD28 family (*e.g.*, CD80, CD86, ICOS, and ICOS ligand (B7RP1)).

Other such immunostimulatory proteins, that are used for the treatment of tumors, or that can promote, enhance or otherwise increase or evoke an anti-tumor response, that are known to those of skill in the art, are contemplated for encoding in the immunostimulatory bacteria provided herein. For example, the immunostimulatory bacteria can deliver a genetic payload encoding a truncated co-stimulatory molecule (*e.g.*, 4-1BBL, CD80, CD86, CD27L, B7RP1, and OX40L), with a full or partial cytoplasmic domain deletion, for expression on an APC, where the truncated gene product is capable of constitutive immuno-stimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC due to a deleted or truncated cytoplasmic domain. As described elsewhere herein, the modified truncated cytoplasmic domain, for example, of 4-1BBL, contains particular residues to ensure proper orientation of the protein domains, which increases expression of the protein. Deletion (full or partial), and modification, of the cytoplasmic domain of co-stimulatory molecules, potentiates the activation of the co-stimulatory molecule, without the immunosuppressive reverse signaling.

### a. Cytokines and Chemokines

In some embodiments, the immunostimulatory bacteria provided herein are engineered to express cytokines to stimulate the immune system, including, but not limited to, for example, IL-2, IL-7, IL-12, IL-12p70 (IL-12p40 + IL-12p35), IL-15, the IL-15/IL-15R alpha chain complex (IL-15Rα-IL-15sc), IL-18, IL-21, IL-23, IL-36γ, IL-2 that has attenuated binding to IL-2Ra, IL-2 that is modified so that it does not bind to IL-2Ra, IFN-α, and IFN-β. Cytokines stimulate immune effector cells and stromal cells at the tumor site, and enhance tumor cell recognition by cytotoxic cells. In some embodiments, the immunostimulatory bacteria can be engineered to express chemokines, such as, for example, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11.

**IL-2**

Interleukin-2 (IL-2), which was the first cytokine approved for the treatment of cancer, is implicated in the activation of the immune system by several mechanisms, including the activation and promotion of cytotoxic T lymphocyte (CTL) growth, the generation of lymphokine-activated killer (LAK) cells, the promotion of regulatory T-cell (Treg cell) growth and proliferation, the stimulation of tumor-infiltrating lymphocytes (TILs), and the promotion of T-cell, B cell, and NK cell proliferation and differentiation. Recombinant IL-2 (rIL-2) is FDA-approved for the treatment of metastatic renal cell carcinoma (RCC) and metastatic melanoma (see, *e.g.*, Sheikhi *et al.* (2016) *Iran J. Immunol.* 13(3):148-166).

**IL-7**

IL-7, which is a member of the IL-2 superfamily, is implicated in the survival, proliferation, and homeostasis of T-cells. Mutations in the IL-7 receptor have been shown to result in the loss of T-cells, and the development of severe combined immunodeficiency disease (SCID), highlighting the critical role that IL-7 plays in T-cell development. IL-7 is a homeostatic cytokine that provides continuous signals to resting naïve and memory T-cells, and which accumulates during conditions of lymphopenia, leading to an increase in both T-cell proliferation and T-cell repertoire diversity. In comparison to IL-2, IL-7 is selective for expanding $CD8^+$ T-cells over $CD4^+FOXP3^+$ regulatory T-cells. Recombinant IL-7 has been shown to augment antigen-specific T-cell responses following vaccination and adoptive cell therapy in

mice. IL-7 also can play a role in promoting T-cell recovery following chemotherapy of hematopoietic stem cell transplantation. Early phase clinical trials on patients with advanced malignancy have shown that recombinant IL-7 is well-tolerated and has limited toxicity at biologically active doses (*i.e.*, in which the numbers of circulating $CD4^+$ and $CD8^+$ T-cells is increased by 3-4 fold) (see, *e.g.*, Lee, S. and Margolin, K. (2011) *Cancers* 3:3856-3893). IL-7 has been shown to possess antitumor effects in tumors, such as gliomas, melanomas, lymphomas, leukemia, prostate cancer, and glioblastoma, and the *in vivo* administration of IL-7 in murine models resulted in decreased cancer cell growth. IL-7 also has been shown to enhance the antitumor effects of IFN-γ in rat glioma tumors, and to induce the production of IL-1α, IL-1β, and TNF-α by monocytes, which results in the inhibition of melanoma growth. Additionally, administration of recombinant IL-7 following the treatment of pediatric sarcomas resulted in the promotion of immune recovery (see, *e.g.*, Lin *et al.* (2017) *Anticancer Research* 37:963-968).

**IL-12 (IL-12p70 (IL-12p40 + IL-12p35))**

Bioactive IL-12 (IL-12p70), which promotes cell-mediated immunity, is a heterodimer, composed of p35 and p40 subunits, whereas IL-12p40 monomers and homodimers act as IL-12 antagonists. IL-12, which is secreted by antigen-presenting cells, promotes the secretion of IFN-γ from NK and T-cells, inhibits tumor angiogenesis, results in the activation and proliferation of NK cells, $CD8^+$ T-cells, and $CD4^+$ T-cells, enhances the differentiation of naïve $CD4^+$ T-cells into Th1 cells, and promotes antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells. IL-12 has been shown to exhibit antitumor effects in murine models of melanoma, colon carcinoma, mammary carcinoma, and sarcoma (see, *e.g.*, Kalinski *et al.* (2001) *Blood* 97:3466-3469; Sheikhi *et al.* (2016) *Iran J. Immunol.* 13(3):148-166; and Lee, S. and Margolin, K. (2011) *Cancers* 3:3856-3893).

**IL-15 and IL-15:IL-15Rα (IL-15/IL-15Rα)**

IL-15 is structurally similar to IL-2, and while both IL-2 and IL-15 provide early stimulation for the proliferation and activation of T-cells, IL-15 blocks IL-2 induced apoptosis, which is a process that leads to the elimination of stimulated T-cells and the induction of T-cell tolerance, limiting memory T-cell responses, and potentially limiting the therapeutic efficacy of IL-2 alone. IL-15 also supports the

persistence of memory $CD8^+$ T-cells for maintaining long-term antitumor immunity, and has demonstrated significant antitumor activity in pre-clinical murine models via the direct activation of $CD8^+$ effector T-cells in an antigen-independent manner. In addition to $CD8^+$ T-cells, IL-15 is responsible for the development, proliferation, and activation of effector natural killer (NK) cells (see, *e.g.*, Lee, S. and Margolin, K. (2011) *Cancers* 3:3856-3893; and Han *et al.* (2011) *Cytokine* 56(3):804-810).

Il-IL-15/IL-15R alpha chain complex can be used instead of IL-15 because has longer *in vivo* stability than monomeric IL-15, is 10- to 100-fold more active on immune cells than IL-15. IL-15/IL-15Rα stimulates the maturation, proliferation and anti-apoptotic maintenance of NK cells, γδ (gamma delta) T-cells and T-cells; and it promotes the survival of activated T-cells (CTLs) and generation of long-lived CD8+ $CD44^{hi}$ T-cell memory cells. Clinically, IL-15 is superior to IL-2 as it does not bind IL-2Ralpha, located on immunosuppressive Tregs, and does not induce activation-induced cell death (AICD) or extensive capillary leak syndrome. It is shown herein that there is synergy between the type I interferon, induced by the engineered STING, to enhance CD8+ cell functions. There is synergy to enhance myheloid- mediated T-cell recruitment and CD8+ T-cell function. They act synergistincally to activate human dentritic cells (DCs) to induce anti-viral CD8+ T-cell responses to thereby induce anti-cancer immunity. It is shown herein that this combination induces a high cure rate in an animal model. Strong T-cell infliltration in murine orthotopic T-cell excluded tumors, and breakdown of the stromal tuamor associated macrophage (TAM) barrier. Significant T-cell and NK recruitment were observed.

IL-15 and IL-15 receptor alpha (IL-15Rα) are coordinately expressed by antigen-presenting cells, such as monocytes and dendritic cells, and IL-15 is presented *in trans* by IL-15Rα to the IL-15Rβγc receptor complex expressed on the surfaces of $CD8^+$ T-cells and NK cells. Soluble IL-15:IL15-Rα (IL-15/IL-15Rα) complexes have been shown to modulate immune responses via the IL-15Rβγc complex, and the biological activity of IL-15 has been shown to be increased 50-fold by administering it in a preformed complex of IL-15 and soluble IL-15Rα, which has an increased half-life compared to IL-15 alone. This significant increase in the therapeutic efficacy of IL-15 by pre-association with IL-15Rα has been demonstrated in murine tumor models (see, *e.g.*, Han *et al.* (2011) *Cytokine* 56(3):804-810).

**IL-18**

IL-18 induces the secretion of IFN-γ by NK and $CD8^+$ T-cells, enhancing their toxicity. IL-18 also activates macrophages and stimulates the development of Th1 helper $CD4^+$ T-cells. IL-18 has shown promising anti-tumor activity in several preclinical mouse models. For example, administration of recombinant IL-18 (rIL-18) resulted in the regression of melanoma or sarcoma in syngeneic mice through the activation of $CD4^+$ T-cells and/or NK cell-mediated responses. Other studies showed that IL-18 anti-tumor effects were mediated by IFN-γ, and involved antiangiogenic mechanisms. The combination of IL-18 with other cytokines, such as IL-12, or with co-stimulatory molecules, such as CD80, enhances the IL-18-mediated anti-tumor effects. Phase I clinical trials in patients with advanced solid tumors and lymphomas showed that IL-18 administration was safe, and that it resulted in immune modulatory activity and in the increase of serum IFN-γ and GM-CSF levels in patients, and in modest clinical responses. Clinical trials showed that IL-18 can be combined with other anti-cancer therapeutic agents, such as monoclonal antibodies, cytotoxic drugs, or vaccines (see, *e.g.*, Fabbi *et al.* (2015) *J. Leukoc. Biol.* 97:665-675; and Lee, S. and Margolin, K. (2011) *Cancers* 3:3856-3893).

It was found that an attenuated strain of *Salmonella typhimurium*, engineered to express IL-18, inhibited the growth of subcutaneous (S.C.) tumors or pulmonary metastases in syngeneic mice without any toxic effects following systemic administration. Treatment with this engineered bacterium induced the accumulation of T-cells, NK cells, and granulocytes in tumors, and resulted in the intratumoral production of cytokines (see, *e.g.*, Fabbi *et al.* (2015) *J. Leukoc. Biol.* 97:665-675).

**Chemokines**

Chemokines are a family of small cytokines that mediate leukocyte migration to areas of injury or inflammation, and that are involved in mediating immune and inflammatory responses. Chemokines are classified into four subfamilies, based on the position of cysteine residues in their sequences, namely XC-, CC-, CXC-, and CX3C-chemokine ligands, or XCL, CCL, CXCL, and CX3CL. The chemokine ligands bind to their cognate receptors and regulate the circulation, homing, and retention of immune cells, with each chemokine ligand-receptor pair selectively regulating a certain type of immune cell. Different chemokines attract different

leukocyte populations, and form a concentration gradient *in vivo*, with attracted immune cells moving through the gradient towards the higher concentration of chemokine (see, *e.g.*, Argyle D. and Kitamura, T. (2018) *Front. Immunol.* 9:2629; and Dubinett *et al.* (2010) *Cancer J.* 16(4):325-335). Chemokines can improve the anti-tumor immune response by increasing the infiltration of immune cells into the tumor, and facilitating the movement of antigen-presenting cells (APCs) to tumor-draining lymph nodes, which primes naïve T-cells and B cells (see, *e.g.*, Lechner *et al.* (2011) *Immunotherapy* 3(11):1317-1340). The immunostimulatory bacteria provided herein can be engineered to encode chemokines, including, but not limited to, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11.

**CCL3, CCL4, CCL5**

CCL3, CCL4, and CCL5 share a high degree of homology, and bind to CCR5 (CCL3, CCL4, and CCL5) and CCR1 (CCL3 and CCL5) on several cell types, including immature DCs and T-cells, in both humans and mice. Therapeutic T-cells have been shown to induce chemotaxis of innate immune cells to tumor sites, via the tumor-specific secretion of CCL3, CCL4, and CCL5 (see, *e.g.*, Dubinett *et al.* (2010) *Cancer J.* 16(4):325-335).

The induction of the T helper cell type 1 (Th1) response releases CCL3. *In vivo* and *in vitro* studies of mice have indicated that CCL3 is chemotactic for both neutrophils and monocytes; specifically, CCL3 can mediate myeloid precursor cell (MPC) mobilization from the bone marrow, and has MPC regulatory and stimulatory effects. Human ovarian carcinoma cells transfected with CCL3 showed enhanced T-cell infiltration and macrophages within the tumor, leading to an improved anti-tumor response, and indicated that CCL3-mediated chemotaxis of neutrophils suppressed tumor growth. DCs transfected with the tumor antigen human melanoma-associated gene (MAGE)-1 that were recruited by CCL3 exhibited superior anti-tumor effects, including increased lymphocyte proliferation, cytolytic capacity, and survival, and decreased tumor growth, in a mouse model of melanoma. A combinatorial use of CCL3 with an antigen-specific platform for MAGE-1 has also been used in the treatment of gastric cancer. CCL3 production by CT26, a highly immunogenic murine colon tumor, slowed *in vivo* tumor growth; this process was driven by the CCL3-dependent accumulation of natural killer (NK) cells, and thus, IFNγ, resulting in the

production of CXCL9 and CXLC10 (see, *e.g.*, Allen *et al.* (2017) *Oncoimmunology* 7(3):e1393598; and Schaller *et al.* (2017) *Expert Rev. Clin. Immunol.* 13(11):1049-1060).

CCL3 has been used as an adjuvant for the treatment of cancer. Administration of a CCL3 active variant, ECI301, after radiofrequency ablation in mouse hepatocellular carcinoma increased tumor-specific responses, and this mechanism was further shown to be dependent on the expression of CCR1. CCL3 has also shown success as an adjuvant in systemic cancers, whereby mice vaccinated with CCL3 and IL-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF), in a model of leukemia/lymphoma, exhibited increased survival (see, *e.g.*, Schaller *et al.* (2017) *Expert Rev. Clin. Immunol.* 13(11):1049-1060).

CCL3 and CCL4 play a role in directing $CD8^+$ T-cell infiltration into primary tumor sites in melanoma and colon cancers. Tumor production of CCL4 leads to the accumulation of $CD103^+$ DCs; suppression of CCL4 through a WNT/β-catenin-dependent pathway prevented $CD103^+$ DC infiltration of melanoma tumors (see, *e.g.*, Spranger *et al.* (2015) *Nature* 523(7559):231-235). CCL3 was also shown to enhance $CD4^+$ and $CD8^+$ T-cell infiltration to the primary tumor site in a mouse model of colon cancer (see, *e.g.*, Allen *et al.* (2017) *Oncoimmunology* 7(3):e1393598).

The binding of CCL3 or CCL5 to their receptors (CCR1 and CCR5), moves immature DCs, monocytes, and memory and T effector cells from the circulation into sites of inflammation or infection. For example, CCL5 expression in colorectal tumors contributes to T lymphocyte chemoattraction and survival. CCL3 and CCL5 have been used alone or in combination therapy to induce tumor regression and immunity in several preclinical models. For example, studies have shown that the subcutaneous injection of Chinese hamster ovary cells genetically modified to express CCL3, resulted in tumor inhibition and neutrophilic infiltration. In another study, a recombinant oncolytic adenovirus expressing *CCL5* (Ad-RANTES-E1A) resulted in primary tumor regression, and blocked metastasis in a mammary carcinoma murine model (see, *e.g.*, Lechner *et al.* (2011) *Immunotherapy* 3(11):1317-1340).

In a translational study of colorectal cancer, CCL5 induced an "antiviral response pattern" in macrophages. As a result of CXCR3-mediated migration of lymphocytes at the invasive margin of liver metastases in colorectal cancer, CCL5 is

produced. Blockade of CCR5, the CCL5 receptor, results in tumor death, driven by macrophages producing IFN and reactive oxygen species. While macrophages are present in the tumor microenvironment, CCR5 inhibition induces a phenotypic shift from an M2 to an M1 phenotype. CCR5 blockade also leads to clinical responses in colorectal cancer patients (see, *e.g.*, Halama *et al.* (2016) *Cancer Cell* 29(4):587-601).

CCL3, CCL4, and CCL5 can be used for treating conditions, including lymphatic tumors, bladder cancer, colorectal cancer, lung cancer, melanoma, pancreatic cancer, ovarian cancer, cervical cancer, or liver cancer (see, *e.g.*, U.S. Patent Publication No. US 2015/0232880; and International Application Publication Nos. WO 2015/059303, WO 2017/043815, WO 2017/156349, and WO 2018/191654).

**CXCL9, CXCL10, CXCL11**

CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (ITAC) are induced by the production of IFN-γ. These chemokines bind CXCR3, preferentially expressed on activated T-cells, and function both angiostatically, and in the recruitment and activation of leukocytes. Prognosis in colorectal cancer is strongly correlated to tumor-infiltrating T-cells, particularly Th1 and $CD8^+$ effector T-cells; high intratumoral expression of CXCL9, CXCL10 and CXCL11 is indicative of good prognosis. For example, in a sample of 163 patients with colon cancer, those with high levels of CXCL9 or CXCL11 showed increased post-operative survival, and patients with high CXC expression had significantly higher numbers of $CD3^+$ T-cells, $CD4^+$ T-helper cells, and $CD8^+$ cytotoxic T-cells. In liver metastases of colorectal cancer patients, CXCL9 and CXCL10 levels were increased at the invasive margin, and correlated with effector T-cell density. The stimulation of lymphocyte migration via the action of CXCL9 and CXCL10 on CXCR3 leads to the production of CCL5 at the invasive margin (see, *e.g.*, Halama *et al.* (2016) *Cancer Cell* 29(4):587-601; and Kistner *et al.* (2017) *Oncotarget* 8(52):89998-90012).

*In vivo*, CXCL9 functions as a chemoattractant for tumor-infiltrating lymphocytes (TILs), activated peripheral blood lymphocytes, natural killer (NK) cells, and Th1 lymphocytes. CXCL9 also is critical for T-cell-mediated suppression of cutaneous tumors. For example, when combined with systemic IL-2, CXCL9 has been shown to inhibit tumor growth via the increased intratumoral infiltration of $CXCR3^+$

mononuclear cells. In a murine model of colon carcinoma, a combination of the huKS1/4-IL-2 fusion protein with CXCL9 gene therapy achieved a superior anti-tumor effect and prolonged lifespan through the chemoattraction and activation of $CD8^+$ and $CD4^+$ T lymphocytes (see, *e.g.*, Dubinett *et al.* (2010) *Cancer J.* 16(4):325-335; and Ruehlmann *et al.* (2001) *Cancer Res.* 61(23):8498-8503).

CXCL10, produced by activated monocytes, fibroblasts, endothelial cells and keratinocytes, is chemotactic for activated T-cells, and can act as an inhibitor of angiogenesis *in vivo*. Expression of CXCL10 in colorectal tumors has been shown to contribute to cytotoxic T lymphocyte chemoattraction and longer survival. The administration of immunostimulatory cytokines, such as IL-12, has been shown to enhance the anti-tumor effects generated by CXCL10. A dendritic cell (DC) vaccine primed with a tumor cell lysate, and transfected with CXCL10 had increased immunological protection and effectiveness in mice; the animals showed a resistance to a tumor challenge, a slowing of tumor growth, and longer survival time. *In vivo* and *in vitro* studies in mice using the CXCL10-mucin-GPI fusion protein resulted in tumors with higher levels of recruited NK cells compared to tumors not treated with the fusion protein. Interferons (which can be produced by plasmacytoid dendritic cells; these cells are associated with primary melanoma lesions and can be recruited to a tumor site by CCL20) can act on tumor DC subsets, for example, $CD103^+$ DCs, which have been shown to produce CXCL9/10 in a mouse melanoma model, and have been associated with CXCL9/10 in human disease. CXCL10 also has shown higher expression in human metastatic melanoma samples relative to primary melanoma samples. Therapeutically, adjuvant IFN-α melanoma therapy upregulates CXCL10 production, whereas the chemotherapy agent cisplatin induces CXCL9 and CXCL10 (see, *e.g.*, Dubinett *et al.* (2010) *Cancer J.* 16(4):325-335; Kuo *et al.* (2018) *Front. Med. (Lausanne)* 5:271; Li *et al.* (2007) *Scand. J. Immunol.* 65(1):8-13; and Muenchmeier *et al.* (2013) *PLoS One* 8(8):e72749).

CXCL10/11 and CXCR3 expression has been established in human keratinocytes derived from basal cell carcinomas (BCCs). CXCL11 also is capable of promoting immunosuppressive indoleamine 2,3-dioxygenase (IDO) expression in human basal cell carcinoma, as well as enhancing keratinocyte proliferation, which

could reduce the anti-tumor activity of any infiltrating $CXCR3^+$ effector T-cells (see, *e.g.*, Kuo *et al.* (2018) *Front. Med. (Lausanne)* 5:271).

CXCL9, CXCL10 and CXCL11 can be encoded in oncolytic viruses for treating cancer (see, *e.g.*, U.S. Patent Publication No. 2015/0232880; and International Application Publication No. WO 2015/059303). Pseudotyped oncolytic viruses or a genetically engineered bacterium encoding the gene for CXCL10 also can be used to treat cancer (see, *e.g.*, International Application Publication Nos. WO 2018/006005 and WO 2018/129404).

**b. Co-Stimulatory Molecules**

Co-stimulatory molecules enhance the immune response against tumor cells, and co-stimulatory pathways are inhibited by tumor cells to promote tumorigenesis. The immunostimulatory bacteria herein can be engineered to express co-stimulatory molecules, such as, for example, CD40, CD40L, 4-1BB, 4-1BBL, 4-1BBL with a deletion of the cytoplasmic domain (4-1BBLΔcyt), 4-1BBL with a truncated cytoplasmic domain, OX40 (CD134), OX40L (CD252), other members of the TNFR superfamily (*e.g.*, CD27, CD27 ligand, GITR, CD30, Fas receptor, TRAIL-R, TNF-R, HVEM, and RANK), B7, CD80, CD86, ICOS, ICOS ligand (B7RP1), and CD28. Additionally, the immunostimulatory bacteria can encode and express truncated co-stimulatory molecules (*e.g.*, 4-1BBL, CD80, CD86, CD27L, B7RP1, OX40L), with a full or partial (complete, or truncated, or modified to ensure proper orientation when expressed in a cell) cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC). The gene product with a truncated cytoplasmic domain, including a full deletion, exhibits constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC due to a truncated or deleted (or otherwise modified as described herein) cytoplasmic domain. The truncation is sufficient to provide the signaling, and for the modified co-stimulatory molecule to be unable to counter-regulatory signal to the APC. The complete or partial deletion of the cytoplasmic domain of a co-stimulatory molecule, as described herein, potentiates the activation of the co-stimulatory molecule, without the immunosuppressive reverse signaling. The partial deletion (or truncation) of the cytoplasmic domain is a sufficient deletion to achieve

these effects, without affecting the expression of the co-stimulatory molecule, or the orientation of the expressed co-stimulatory molecule.

The co-stimulatory molecules also can be modified to eliminate or reduce the immunosuppressive intracellular/reverse signaling by modifications to the amino acids in the cytoplasmic domain, including insertions, deletions, and/or replacements. In particular, the co-stimulatory molecules are modified by modification, such as by replacement, of cytoplasmic domain phosphorylation sites. For example, replacing one or more Ser residues at an appropriate locus or loci, such as, for human 4-1BBL, Ser5 and Ser8, with a residue that reduces or eliminates reverse signaling.

The immunostimulatory bacteria herein also can be engineered to express agonistic antibodies against co-stimulatory molecules (*e.g.*, 4-1BBL) to enhance the anti-tumor immune response.

**TNF Receptor Superfamily**

The TNF superfamily of ligands (TNFSF) and their receptors (TNFRSF) are involved in the proliferation, differentiation, activation and survival of tumor and immune effector cells. Members of this family include CD30, Fas-L, TRAIL-R, and TNF-R, which induce apoptosis, and CD27, OX40L, CD40L, GITR-L, and 4-1BBL, which regulate B and T-cell immune responses. Other members include herpesvirus entry mediator (HVEM). The expression of TNFSF and TNFRSF by the immunostimulatory bacteria herein can enhance the anti-tumor immune response. It has been shown, for example, that the expression of 4-1BBL in murine tumors enhances immunogenicity, and that intratumoral injection of dendritic cells (DCs) with increased expression of OX40L can result in tumor rejection in murine models. Studies have also shown that injection of an adenovirus expressing recombinant GITR into B16 melanoma cells promotes T-cell infiltration and reduces tumor volume. Stimulatory antibodies against molecules such as 4-1BB, OX40 and GITR also can be encoded by the immunostimulatory bacteria to stimulate the immune system. For example, agonistic anti-4-1BB monoclonal antibodies have been shown to enhance anti-tumor CTL responses, and agonistic anti-OX40 antibodies have been shown to increase anti-tumor activity in transplantable tumor models. Additionally, agonistic anti-GITR antibodies have been shown to enhance anti-tumor responses and

immunity (see, *e.g.*, Lechner *et al.* (2011) *Immunotherapy* 3(11):1317-1340; and Peggs *et al.* (2009) *Clinical and Experimental Immunology* 157:9-19).

**CD40 and CD40L**

CD40, which is a member of the TNF receptor superfamily, is expressed by APCs and B cells, while its ligand, CD40L (CD154), is expressed by activated T-cells. Interaction between CD40 and CD40L stimulates B cells to produce cytokines, resulting in T-cell activation and tumor cell death. Studies have shown that anti-tumor immune responses are impaired with reduced expression of CD40L on T-cells or CD40 on dendritic cells. CD40 is expressed on the surface of several B-cell tumors, such as follicular lymphoma, Burkitt lymphoma, lymphoblastic leukemia, and chronic lymphocytic leukemia, and its interaction with CD40L has been shown to increase the expression of B7-1/CD80, B7-2/CD86, and human leukocyte antigen (HLA) class II molecules in the $CD40^+$ tumor cells, as well as enhance their antigen-presenting abilities. Transgenic expression of CD40L in a murine model of multiple myeloma resulted in the induction of $CD4^+$ and $CD8^+$ T-cells, local and systemic anti-tumor immune responses, and reduced tumor growth. Anti-CD40 agonistic antibodies also induced anti-tumor T-cell responses (see, *e.g.*, Marin-Acevedo *et al.* (2018) *Journal of Hematology & Oncology* 11:39; Dotti *et al.* (2002) *Blood* 100(1):200-207; and Murugaiyan *et al.* (2007) *J. Immunol.* 178:2047-2055).

**4-1BB and 4-1BBL**

4-1BB (CD137) is an inducible co-stimulatory receptor that is expressed primarily by T-cells and NK cells; it binds its ligand 4-1BBL, that is expressed on APCs, including DCs, B-cells, and monocytes, to trigger immune cell proliferation and activation. 4-1BB results in longer and more widespread responses of activated T-cells. Anti-4-1BB agonists and 4-1BBL fusion proteins have been shown to increase immune-mediated anti-tumor activity, for example, against sarcoma and mastocytoma tumors, mediated by $CD4^+$ Th1 and tumor-specific CTL activity (see, *e.g.*, Lechner *et al.* (2011) *Immunotherapy* 3(11):1317-1340; and Marin-Acevedo *et al.* (2018) *Journal of Hematology & Oncology* 11:39). 4-1BBL is negatively regulated by its cytoplasmic signaling domain. In the late-phase of 4-1BBL ligation on macrophages to T-cells, reverse signaling of the 4-1BBL cytoplasmic domain induces surface translocation of 4-1BBL to bind to form a signaling complex with TLR4. This induces

high levels of TNF-α, comparable to LPS activation of TLR4, that leads to immunosuppression of the adaptive immune response (see, *e.g.*, Ma *et al.* (2013) *Sci. Signaling* 295(6):1-11). Deletion of the cytoplasmic domain of 4-1BBL, as described herein, potentiates the activation of 4-1BBL without the immunosuppressive reverse signaling.

**OX40 and OX40L**

OX40 (CD134) is a member of the TNF receptor superfamily that is expressed on activated effector T-cells, while its ligand, OX40L is expressed on APCs, including DCs, B cells and macrophages, following activation by TLR agonists and CD40-CD40L signaling. OX40-OX40L signaling results in the activation, potentiation, proliferation and survival of T-cells, as well as the modulation of NK cell function and inhibition of the suppressive activity of Tregs. Signaling through OX40 also results in the secretion of cytokines (IL-2, IL-4, IL-5, and IFN-γ), boosting Th1 and Th2 cell responses. The recognition of tumor antigens by tumor-infiltrating lymphocytes (TILs) results in increased expression of OX40 by the TILs, which has been correlated with improved prognosis. Studies have demonstrated that treatment with anti-OX40 agonist antibodies or Fc-OX40L fusion proteins results in enhanced tumor-specific $CD4^+$ T-cell responses and increased survival in murine models of melanoma, sarcoma, colon carcinoma, and breast cancer, while Fc-OX40L incorporated into tumor cell vaccines protected mice from subsequent challenge with breast carcinoma cells (see, *e.g.*, Lechner *et al.* (2011) *Immunotherapy* 3(11):1317-1340; and Marin-Acevedo *et al.* (2018) *Journal of Hematology & Oncology* 11:39).

**B7-CD28 Family**

CD28 is a co-stimulatory molecule expressed on the surface of T-cells that acts as a receptor for B7-1 (CD80) and B7-2 (CD86), which are co-stimulatory molecules expressed on antigen-presenting cells. CD28-B7 signaling is required for T-cell activation and survival, and for the prevention of T-cell anergy, and results in the production of interleukins, such as IL-6.

Optimal T-cell priming requires two signals: (1) T-cell receptor (TCR) recognition of MHC-presented antigens, and (2) co-stimulatory signals resulting from the ligation of T-cell CD28 with B7-1 (CD80) or B7-2 (CD86) expressed on APCs. Following T-cell activation, CTLA-4 receptors are induced, which then outcompete

CD28 for binding to B7-1 and B7-2 ligands. Antigen presentation by tumor cells is poor due to their lack of expression of co-stimulatory molecules, such as B7-1/CD80 and B7-2/CD86, resulting in a failure to activate the T-cell receptor complex. As a result, upregulation of these molecules on the surfaces of tumor cells can enhance their immunogenicity. Immunotherapy of solid tumors and hematologic malignancies has been successfully induced by B7, for example, via tumor cell expression of B7, or soluble B7-immunoglobulin fusion proteins. The viral-mediated tumor expression of B7, in combination with other co-stimulatory ligands, such as ICAM-3 and LFA-3, has been successful in preclinical and clinical trials for the treatment of chronic lymphocytic leukemia and metastatic melanoma. Additionally, soluble B7 fusion proteins have demonstrated promising results in the immunotherapy of solid tumors as single agent immunotherapies (see, *e.g.*, Lechner *et al.* (2011) *Immunotherapy* 3(11):1317-1340; and Dotti *et al.* (2002) *Blood* 100(1):200-207).

### 2. Constitutively Active Proteins that Stimulate the Immune Response and/or Type I IFN, Non-Human STING Proteins, STING Chimeras, and Modified Forms

Type I interferons (IFNs; also referred to as interferon type 1), include IFN-α and IFN-β, and are pleiotropic cytokines with antiviral, anti-tumor, and immunoregulatory activities. IFN-β is produced by most cell types; IFN-α primarily is produced by hematopoietic cells, particularly plasmacytoid dendritic cells. Type I IFNs are produced following the sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). They are involved in the innate immune response against pathogens, mainly viral, and are potent immunomodulators that promote antigen presentation, mediate dendritic cell (DC) maturation, activate cytotoxic T lymphocytes (CTLs), natural killer (NK) cells and macrophages, and activate the adaptive immune system by promoting the development of high-affinity antigen-specific T-cell and B-cell responses and immunological memory.

Type I IFNs exhibit anti-proliferative and pro-apoptotic effects on tumors and have anti-angiogenic effects on tumor neovasculature. They induce the expression of MHC class I molecules on tumor cell surfaces, increase the immunogenicity of tumor cells, and activate cytotoxicity against them. Type I IFN has been used as a therapeutic for the treatment of cancers and viral infections. For example, IFN-α (sold

under the trademark Intron®/Roferon®-A) is approved for the treatment of hairy cell leukemia, malignant melanoma, AIDS-related Kaposi's sarcoma, and follicular non-Hodgkin's lymphoma; it also is used in the treatment of chronic myelogenous leukemia (CML), renal cell carcinoma, neuroendocrine tumors, multiple myeloma, non-follicular non-Hodgkin's lymphoma, desmoid tumors, and cutaneous T-cell lymphoma, although use is limited due to systemic immunotoxicity (see, *e.g.*, Ivashkiv and Donlin (2014) *Nat. Rev. Immunol.* 14(1):36-49; Kalliolias and Ivashkiv (2010) *Arthritis Research & Therapy* 12(Suppl 1):S1; and Lee, S. and Margolin, K. (2011) *Cancers* 3:3856-3893).

Expression of type I interferons in tumors and the tumor microenvironment is among the immune responses that the immunostimulatory bacteria herein are designed to evoke. Inducing or evoking type I interferon provides anti-tumor immunity for the treatment of cancer.

**a. Constitutive STING Expression and Gain-of-Function Mutations**

The induction of type I IFNs, proinflammatory cytokines and chemokines is necessary for mounting an immune response that prevents or inhibits infection by viral pathogens. This response also can be effective as an anti-tumor agent. The immunostimulatory bacteria provided herein encode proteins that constitutively induce type I IFNs. Among these proteins are those that occur in individuals with various diseases or disorders that involve the over-production of immune response modulators. For example, over-production or excessive production, or defective negative regulation of type I IFNs and pro-inflammatory cytokines, can lead to undesirable effects, such as inflammatory and autoimmune diseases. Disorders involving the overproduction, generally chronic, of type I IFNs, are referred to as interferonopathies (see, *e.g.*, Lu and MacDougall (2017) *Front. Genet.* 8:118; and Konno *et al.* (2018) *Cell Reports* 23:1112-1123). Disorders and clinical phenotypes associated with type I interferonopathies include Aicardi-Goutiéres syndrome (AGS), STING-associated vasculopathy with onset in infancy (SAVI), Singleton-Merten syndrome (SMS), atypical SMS, familial chilblain lupus (FCL), systemic lupus erythematosus (SLE), bilateral striatal necrosis (BSN), cerebrovascular disease (CVD), dyschromatosis symmetrica hereditaria (DSH), spastic paraparesis (SP), X-linked reticulate pigmentary disorder (XLPDR), proteasome-associated auto-

inflammatory syndrome (PRAAS), intracranial calcification (ICC), Mendelian susceptibility to mycobacterial disease (MSMD), and spondyloenchondrodysplasia (SPENCD) (see, *e.g.*, Rodero *et al.* (2016) *J. Exp. Med.* 213(12):2527-2538). These phenotypes are associated with particular genotypes, involving mutations in genes that lead to constitutive activities of products involved in the induction of type I IFNs.

The sustained activation of interferon signaling can be due to: 1) loss-of-function mutations leading to increased cytosolic DNA (*e.g.*, mutations in *TREX1* and *SAMHD1*), or increased cytosolic RNA/DNA hybrids (*e.g.*, mutations in *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, and *POLA1*); 2) loss-of-function mutations resulting in a defect in RNA editing and abnormal sensing of self-nucleic acid RNA species in the cytosol (*e.g.*, mutations in *ADAR1*); 3) gain-of-function mutations leading to constitutive activation of cytosolic IFN signaling pathways/increased sensitivity to cytosolic nucleic acid ligands (*e.g.*, mutations in RIG-I, MDA5 and STING); 4) loss-of-function mutations leading to aberrant RNA signaling via MAVS caused by a disturbance of the unfolded protein response (*e.g.*, mutations in *SKIV2L*); 5) loss-of-function mutations in molecules responsible for limiting IFN receptor (IFNAR1/2) signaling, leading to uncontrolled IFN-stimulated gene (ISG) production (*e.g.*, mutations in *USP18* and *ISG15*); 6) proteasomal dysfunction, leading to increased IFN signaling through an unknown mechanism (*e.g.*, mutations in *PSMA3*, *PSMB4* and *PSMB8*); and 7) loss-of-function mutations in TRAP/*ACP5* and C1q, where the mechanisms leading to type I IFN signaling remain unclear (see, *e.g.*, Rodero *et al.* (2016) *J. Exp. Med.* 213(12):2527-2538).

Of interest herein are mutations that lead to gain-of-function (GOF). There are known mutations in STING, MDA5 and RIG-I, that are associated with constitutive activation of the encoded proteins, and/or enhanced sensitivity or increased affinity or binding to endogenous ligands. GOF mutations in STING, for example, are linked to SAVI and FCL; GOF mutations in MDA5 are linked to AGS and SMS; and GOF mutations in RIG-I are linked to atypical SMS.

***TMEM173* STING Alleles**

Stimulator of interferon genes (STING) is encoded by the transmembrane protein 173 (*TMEM173*) gene, which is a ~7 kb-long gene. The human *TMEM173* gene is characterized by significant heterogeneity and population stratification of

alleles. The most common human *TMEM173* allele is referred to as *R232* (referencing the amino acid present at residue 232; see, *e.g.*, SEQ ID NOs:305-309, setting forth the sequences of various human *TMEM173* alleles). More than half the American population is not *R232/R232*. The second most common allele is *R71H-G230A-R293Q* (*HAQ*). Other common alleles include *AQ* (*G230A-R293Q*), *Q* (*Q293)* and *R232H* (named REF after the reference STING allele first identified and catalogued in the database by Glen Barber).

*R232/R232* is the most common genotype in Europeans, while *HAQ/R232* is the most common genotype in East Asians. Africans have no *HAQ/HAQ* genotypes, but have the *Q* allele, and ~4% of Africans are *AQ/AQ*, which is absent in other ethnic populations (see, *e.g.*, Patel and Jin (2018) *Genes & Immunity*, doi:10.1038/s41435-018-0029-9). The REF, *AQ* and *Q* alleles are highly refractory to bacterially-derived CDNs, such as 3'3' c-di-GMP (see, *e.g.*, Corrales *et al.* (2015) *Cell Reports 11*:1018-1030).

**STING Gain-of-Function Mutations**

Several activating or gain-of-function (GOF) mutations in *TMEM173*, the gene for STING, inherited and *de novo*, have been linked to the rare auto-inflammatory disease SAVI (STING-associated vasculopathy with onset in infancy). SAVI is an autosomal dominant disease and is characterized by systemic inflammation, interstitial lung disease, cutaneous vasculitis, and recurrent bacterial infection. SAVI with *de novo TMEM173* mutations typically is characterized by an early-onset (< 8 weeks) and severe phenotype, while familial mutations result in late-onset (teens to adults) and milder clinical symptoms. Inherited *TMEM173* activating mutations include G166E and V155M, whereas *de novo* mutations include N154S, V155M, V147M, V147L, C206Y, R284G, R281Q, and S102P/F279L (see, *e.g.*, Patel and Jin (2019) *Genes & Immunity* 20:82-89). Other activating *TMEM173* mutations that have been identified include R284M, R284K, R284T, E316Q, and R375A (see, *e.g.*, U.S. Application Publication No. 2018/0311343). Another gain-of-function mutation in *TMEM173* is R284S, which results in a highly constitutively active STING, and was found to trigger innate immune signaling in the absence of activating CDNs, leading to chronic production of pro-inflammatory cytokines (see, *e.g.*, Konno *et al.* (2018) *Cell Reports* 23:1112-1123).

*TMEM173* mutations, such as N154S, V155M and V147L, and/or any of the mutations listed in the table below, singly or in any combination, with these and any other such mutations, such as N154S/R284G, result in a gain-of-function STING that is constitutively active and does not require, or is hypersensitive to, ligand stimulation, leading to chronic activation of the STING-interferon pathway. This has been demonstrated (see, *e.g.*, Liu *et al.* (2014) *N. Engl. J. Med.* 371:507-518). Constructs of mutated *TMEM173* (with each of the replacements V147L, N154S, V155M, and the loss-of-function mutant V155R), and non-mutated *TMEM173*, were transfected into STING-negative HEK293T cells, and stimulated with the STING ligand, cGAMP. Cells transfected with the N154S, V155M and V147L mutants exhibited highly elevated *IFNB1* (the gene encoding IFN-β) reporter activity, which was not significantly boosted by stimulation with the STING ligand cGAMP. Cells that were transfected with the loss-of-function mutant (V155R), non-mutated *TMEM173*, or control plasmid, had no significant baseline activation. Stimulation with cGAMP resulted in a response in a dose-dependent manner in cells with non-mutated *TMEM173*, and resulted in a minimal response only at the highest cGAMP concentration, in cells expressing the loss-of-function mutant (see, *e.g.*, Liu *et al.* (2014) *N. Engl. J. Med.* 371:507-518). These results show that the activating *TMEM173* mutations result in constitutive activation of STING, even in the absence of stimulation by cGAMP.

G207E is another gain-of-function STING mutation that causes alopecia, photosensitivity, thyroid dysfunction, and SAVI-features. The G207E mutation causes constitutive activation of inflammation-related pathways in HEK cells, as well as aberrant interferon signature and inflammasome activation in patient peripheral blood mononuclear cells (PBMCs). Using STING variants with the R232 or H232 allele and the GOF mutation G207E, it was shown that after stimulation with CDN, the R232 + G207E variant resulted in slight increases of activity in the IFN-β and STAT1/2 pathways, while with the H232 + G207E variant, IFN-β levels remained constant, and STAT1/2 showed diminished activity. Both variants showed similar STAT3 and NF-κB pathway activation following stimulation. These results show that the residue R at position 232 is important for cGAMP binding and IFN induction, and show that G207E mutants result in constitutive activation of STING signaling pathways and

ligand-dependent hyperactivation of the NF-κB pathway. Patients with the R232 allele and the G207E mutation had more severe disease; this polymorphism strengthens the constitutive activation of the mutant STING, leading to the overexpression of downstream targets, such as IFN, IL1-β, and IL-18 (see, *e.g.*, Keskitalo *et al.* (2018), available from: doi.org/10.1101/394353).

67 amino acids in murine STING (SEQ ID NO:369) were mutated (see, Burdette *et al.* (2011) *Nature* 478(7370):515-518), either individually or in groups, to identify amino acids involved in cyclic di-GMP (c-di-GMP) binding and/or IFN induction. Among the mutants identified were hyperactive mutants R196A/D204A, S271A/Q272A, R309A/E315A, E315A, E315N, E315Q, and S271A (corresponding to R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q, and S272A, respectively, with reference to the sequence of human STING as set forth in SEQ ID NOs:305-309), that spontaneously induced IFN at low levels of transfection and did not respond to c-di-GMP, and the mutants R374A, R292A/T293A/E295A/E299A, D230A, R231A, K235A, Q272A, S357A/E359A/S365A, D230A/R231A/K235A/R237A, and R237A (corresponding to R375A, R293A/T294A/E296A (there is no equivalent to E299 in human STING), D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, and R238A, respectively, with reference to human STING, as set forth in SEQ ID NOs:305-309), that induced IFN when overexpressed but did not respond to c-di-GMP. These alleles can still respond to the endogenous CDN 2'3' c-di-GAMP, as it was later discovered that some human STING mutations have low affinity for the 3'3' CDNs produced by bacteria, such as c-di-GMP (see, *e.g.*, Corrales *et al.* (2015) *Cell Reports* 11:1018-1030).

The immunostimulatory bacteria provided herein that encode these proteins with gain-of-function mutations exploit the constitutive activation of these proteins to increase production of type I IFNs and pro-inflammatory cytokines. Tumor-targeting immunostimulatory bacteria are provided herein that encode STING, IRF3, IRF5, IRF7, MDA5, and/or RIG-I, with gain-of-function mutations. The immunostimulatory bacteria increase the production of type I IFN-mediated cytokines and chemokines in the tumor microenvironment, potentiating the anti-tumor immune response and improving the therapeutic efficacy of the immunostimulatory bacteria. The gene

encoding STING is referred to as *TMEM173*, the gene encoding MDA5 is *IFIH1*, and the gene encoding RIG-I is *DDX58*. There are numerous alleles for each gene, and known mutations that can occur in genes with any of the alleles, resulting in gain-of-function or constitutive activation. The mutations listed below can occur singly, or can be used in any combination. Other mutations that result in gain-of-function can be identified by routine screening/mutation protocols. The table below lists exemplary gain-of-function mutations in each of STING/TMEM173 (SEQ ID NOs:305-309), MDA5/IFIH1 (SEQ ID NO:310), RIG-I/DDX58 (SEQ ID NO:311), IRF3 (SEQ ID NO:312), and IRF7 (SEQ ID NO:313). Other mutations, such as deletion of, or replacement of, a phosphorylation site or sites, such as S324/L325/S326 → S324A/L325/S326A in STING, and other replacements to eliminate a phosphorylation site to reduce nuclear factor-κB (NF-κB) signaling in STING, or other proteins that employ such signaling, also can be introduced.

The resulting proteins can be encoded in the immunostimulatory bacteria provided herein. The proteins are encoded on plasmids in the immunostimulatory bacteria.

Administering nucleic acids encoding wild-type STING can induce an immune response; the administration of gain-of-function STING mutants, with constitutive activity as provided herein, in tumor-targeted immunostimulatory bacteria, leads to a more potent immune response and more effective anti-cancer therapeutic. The enhanced immune response by the tumor-targeted administration of constitutively active STING, or other such modified DNA/RNA sensors, such as gain-of-function mutants of MDA5, RIG-I, IRF3, or IRF7, as provided herein, provides a therapeutically more effective anti-cancer treatment. For example, as described herein, modifying the immunostimulatory bacteria so that they do not infect epithelial cells, but retain the ability to infect phagocytic cells, including tumor-resident immune cells, effectively targets the immunostimulatory bacteria to the tumor microenvironment, improving therapeutic efficiency and preventing undesirable systemic immune responses. These tumor-targeted bacteria are engineered to encode gain-of-function STING, MDA5, RIG-I, IRF3, or IRF7 mutants, which are constitutively active, for example, even in the absence of ligand stimulation,

providing a potent type I IFN response to improve the anti-cancer immune response in the tumor microenvironment.

Thus, for example, the administration of constitutively activated STING can provide an alternative means to boost STING signaling for the immunotherapeutic treatment of cancer. In certain embodiments, the tumor-targeting immunostimulatory bacteria provided herein can be modified to encode STING/TMEM173 (SEQ ID NOs: 305-309) with gain-of-function mutations, selected from S102P, V147L, V147M, N154S, V155M, G166E, R197A, D205A, R197A/D205A, C206Y, G207E, D231A, R232A, K236A, R238A, D231A/R232A/K236A/R238A, S272A, Q273A, S272A/Q273A, F279L, S102P/F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R293A, T294A, E296A, R293A/T294A/E296A, R310A, E316A, E316N, E316Q, R310A/E316A, S324A/S326A, S358A, E360A, S366A, S358A/E360A/S366A, and R375A, as well as conservative mutations thereof. In addition, combinations of STING gain-of-function mutations, such as N154S/R284G, significantly increase STING signaling, compared to the single mutation counterparts. These mutations result in constitutive activity of STING.

**Table of Exemplary Gain-Of-Function Mutants**

**Gain-of-Function Mutations Resulting in the Persistent Expression of Type I IFN**

| STING | RIG-I | MDA5 | IRF3 | IRF7 |
|---|---|---|---|---|
| V147L | E373A | T331I | S396D | S477D/S479D |
| N154S | C268F | T331R | S396D/S398D | S475D/S476D/S477D/S479D/S483D/S487D |
| V155M | | A489T | S396D/S398D/S402D/T404D/S405D | Δ247–467 |
| G166E | | R822Q | | S475D/S477D/S479D |
| C206Y | | G821S | | |
| G207E | | A452T | | |
| R281Q | | A946T | | |
| R284G | | R337G | | |
| R284S | | D393V | | |
| R284M | | G495R | | |
| R284K | | R720Q | | |
| R284T | | R779H | | |
| S102P | | R779C | | |
| F279L | | L372F | | |
| R197A | | | | |
| D205A | | | | |
| S102P/F279L | | | | |
| R197A/D205A | | | | |
| S272A/Q273A | | | | |
| R310A/E316A | | | | |
| R310A | | | | |

| STING | RIG-I | MDA5 | IRF3 | IRF7 |
|---|---|---|---|---|
| E316A | | | | |
| E316N | | | | |
| E316Q | | | | |
| S272A | | | | |
| R375A | | | | |
| R293A | | | | |
| T294A | | | | |
| E296A | | | | |
| R293A/T294A/E296A | | | | |
| D231A | | | | |
| R232A | | | | |
| K236A | | | | |
| Q273A | | | | |
| S358A | | | | |
| E360A | | | | |
| S366A | | | | |
| S358A/E360A/S366A | | | | |
| D231A/R232A/K236A/R238A | | | | |
| R238A | | | | |
| V147M | | | | |
| S324A/S326A | | | | |
| N154S/R284G | | | | |

Amino acid residues R197, D205, R310, R293, T294, E296, S272, Q273, E316, D231, R232, K236, S358, E360, S366, and R238, with reference to the sequence of human STING, as set forth in any of SEQ ID NOs:305-309, correspond to amino acid residues R196, D204, R309, R292, T293, E295, S271, Q272, E315, D230, R231, K235, S357, E359, S365, and R237, respectively, of the murine STING, as set forth in SEQ ID NO:369. Also included are conservative substitutions of each of the replacements (see, *e.g.*, the Table in the Definitions section, listing exemplary conservative mutations for each amino acid).

**b. Constitutive IRF3 Expression and Gain-of-Function Mutations**

IRF3 (interferon regulatory factor 3, or IRF-3) and IRF7 (or IRF-7) are key activators of type I IFN genes. Following virus-induced C-terminal phosphorylation (by TBK1), activated IRF3 and IRF7 form homodimers, translocate from the cytoplasm to the nucleus, and bind to IFN-stimulated response elements (ISREs) to induce type I IFN responses. IRF3 is expressed constitutively in unstimulated cells, and exists as an inactive cytoplasmic form, while IRF7 is not constitutively expressed in cells, and is induced by IFN, lipopolysaccharide and virus infection. Overexpression of IRF3 significantly increases the virus-mediated expression of type I IFN genes, resulting in the induction of an antiviral state. IRF3 activation also has

been shown to up-regulate the transcription of the CC-chemokine RANTES (CCL5) following viral infection (see, *e.g.*, Lin *et al.* (1999) *Mol. Cell Biol.* 19(4):2465-2474).

Residues S385, S386, S396, S398, S402, T404, and S405 in the C-terminal domain of IRF3 are phosphorylated after virus infection, inducing a conformational change that results in the activation of IRF3. IRF3 activation is induced, not only by viral infection, but also by lipopolysaccharide (LPS) and poly(I:C). Of the seven residues that can be phosphorylated in the C-terminal cluster of IRF3, a single point mutation, S396D, is sufficient for the generation of a constitutively active form of IRF3. IRF3(S396D) enhances the transactivation of IFNα1, IFN-β, and RANTES promoters by 13-, 14-, and 11-fold, respectively, compared to wild-type IRF3. Another mutant, IRF3(S396D/S398D), enhances the transactivation of IFNα1, IFN-β, and RANTES promoters by 13-, 12-, and 12-fold, respectively, compared to wild-type IRF3. Another constitutively active mutant of IRF3 is IRF3(5D), in which the serine or threonine residues at positions 396, 398, 402, 404, and 405 are replaced by phosphomimetic aspartic acid residues (IRF3(S396D/S398D/S402D/T404D/S405D)). Similar gain-of-function mutations, leading to constitutive activity of immune response mediators, such as induction of type I interferon, can be achieved by mutating serine residues to phosphomimetic aspartic acid in other proteins, such as RIG-I, MDA5 and STING, that are in immune response signaling pathways.

IRF3(5D) displays constitutive DNA binding and transactivation activities, dimer formation, association with the transcription coactivators p300 (also called EP300, or E1A binding protein p300)/ CBP (also known as CREB-binding protein, or CREBBP), and nuclear localization. Its transactivation activity is not induced further by virus infection. IRF3(5D) is a very strong activator of IFN-β and ISG-15 gene expression; IRF3(5D) alone stimulates IFN-β expression as strongly as virus infection, and enhances transactivation of IFNα1, IFN-β, and RANTES promoters by 9-fold, 5.5-fold, and 8-fold, respectively, compared to wild-type IRF3 (see, *e.g.*, Lin *et al.* (2000) *J. Biol. Chem.* 275(44):34320-34327; Lin *et al.* (1998) *Mol. Cell Biol.* 18(5):2986-2996; and Servant *et al.* (2003) *J. Biol. Chem.* 278(11):9441-9447). Any of positions S385, S386, S396, S398, S402, T404, and S405 can be mutated, alone or in combination, to produce constitutively active IRF3 mutants in the immunostimulatory bacteria provided herein.

### c. Non-Human STING Proteins, and Variants Thereof with Increased or Constitutive Activity, and STING Chimeras, and Variants Thereof with Increased or Constitutive Activity

As discussed above, cytosolic double-stranded DNA (dsDNA) stimulates the production of type I interferon (IFN) through the endoplasmic reticulum (ER)–resident adaptor protein STING (stimulator of IFN genes), which activates the transcription factor interferon regulatory factor 3 (IRF3). The TANK binding kinase 1 (TBK1)/IRF3 axis results in the induction of type I IFNs, and the activation of dendritic cells (DCs) and cross-presentation of tumor antigens to activate $CD8^+$ T cell-mediated anti-tumor immunity. STING signaling also activates the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling axis, resulting in a pro-inflammatory response, but not in the activation of the DCs and $CD8^+$ T-cells that are required for anti-tumor immunity.

Upon recognition of 2'3' cGAMP, STING translocates from the endoplasmic reticulum through the Golgi apparatus, allowing the recruitment of TANK-binding kinase 1 (TBK1), and the activation of the transcription factors IRF3 and NF-κB. The carboxyl-terminal tail (C-terminal tail or CTT) region of STING is necessary and sufficient to activate TBK1 and stimulate the phosphorylation of IRF3; it also is involved in NF-κB signaling. The CTT is an unstructured stretch of approximately 40 amino acids that contains sequence motifs required for STING phosphorylation and recruitment of IRF3. IRF3 and NF-κB downstream signaling is attributed to specific sequence motifs within the C-terminal tail (CTT) of STING that are conserved among vertebrate species. Modular motifs in the CTT, which include IRF3-, TBK1-, and TRAF6-binding modules, control the strength and specificity of cell signaling and immune responses.

Depending on the species and the respective characteristics of their STING CTT discrete elements, the IRF3 and NF-κB downstream responses can be affected, and sometimes opposite. The STING CTT elements dictate and finely tune the balance between the two signaling pathways, resulting in different biological responses. In human and mouse immune cells, for example, STING-dependent IRF3 activation results predominantly in a type I interferon response. STING signaling in human cells also drives a pro-inflammatory response through canonical and possibly

non-canonical NF-κB pathways via TRAF6 recruitment. Human STING residue S366 (see, *e.g.*, SEQ ID NOs:305-309) is a primary TBK1 phosphorylation site that is part of an LxIS motif in the CTT, which is required for IRF3 binding, while a second PxPLR motif, including residue L374, is required for TBK1 binding. The LxIS and PxPLR motifs are highly conserved in all vertebrate STING alleles. In other species, STING signaling results predominantly in the activation of the NF-κB signaling axis. For example, the zebrafish CTT, which is responsible for hyperactivation of NF-κB signaling, contains an extension with a highly conserved PxExxD motif at the extreme C-terminus that is not present in human and other mammalian STING alleles; this motif shares similarity with tumor necrosis factor receptor-associated factor 6 (TRAF6) binding sites. While the role of TRAF6 in human STING signaling is non-essential, TRAF6 recruitment is essential for zebrafish STING-induced NF-κB activation. A human-zebrafish STING chimera, in which human STING was engineered to contain the zebrafish STING CTT module DPVETTDY, induced more than 100-fold activation of NF-κB activation, indicating that this region is necessary and sufficient to direct enhanced NF-κB signal activation. The addition of the zebrafish CTT also resulted in an increased STING interferon response (see, de Oliveira Mann *et al.* (2019) *Cell Reports* 27:1165-1175).

The differences among species in the balance between IRF3 and NF-κB signaling is exploited herein to produce modified STING proteins that have reduced NF-κB signaling, and/or optionally, increased IRF3 signaling, so that when the STING protein is delivered to and expressed in the TME, the resulting response is an increased anti-tumor/anti-viral response, compared to the unmodified STING protein.

In some embodiments, STING proteins from species that have low or no NF-κB signaling activity are provided in delivery vehicles, including any of the immunostimulatory bacteria described herein or known to those of skill in the art, as well as in other delivery vehicles, such as viral vectors, including oncolytic vectors, minicells, exosomes, liposomes, and in cells, such as T-cells that are used in cell therapy and used to deliver vehicles, such as bacteria and oncolytic vectors.

The non-human STING proteins can be, but are not limited to, STING proteins from the following species: Tasmanian devil (*Sarcophilus harrisii*; SEQ ID NO:349), marmoset (*Callithrix jacchus*; SEQ ID NO:359), cattle (*Bos taurus*; SEQ ID

NO:360), cat (*Felis catus*; SEQ ID NO:356), ostrich (*Struthio camelus australis*; SEQ ID NO:361), crested ibis (*Nipponia nippon*; SEQ ID NO:362), coelacanth (*Latimeria chalumnae*; SEQ ID NOs:363-364), boar (*Sus scrofa*; SEQ ID NO:365), bat (*Rousettus aegyptiacus*; SEQ ID NO:366), manatee (*Trichechus manatus latirostris*; SEQ ID NO:367), ghost shark (*Callorhinchus milii*; SEQ ID NO:368), and mouse (*Mus musculus*; SEQ ID NO:369). These vertebrate STING proteins readily activate immune signaling in human cells, indicating that the molecular mechanism of STING signaling is shared in vertebrates (see, de Oliveira Mann *et al.* (2019) *Cell Reports* 27:1165-1175).

In other embodiments, the non-human STING proteins contain any of the constitutive STING activation and gain-of-function mutations, at corresponding loci in the non-human STING corresponding to those in human STING, described above (see, Example 17 below, which provides exemplary alignments and corresponding mutations in various species; see, also, Figures 1-13).

In other embodiments, chimeras of STING proteins are provided. In the chimeras, the CTT region, or portion(s) thereof that confers or participates in NF-κB signaling/activity, of a first species STING protein is replaced with the corresponding CTT or portion(s) thereof from a second species, whose STING protein has lower or very little, less than human, NF-κB signaling activity. Generally, the first species is human, and the replacing CTT or portion(s) thereof is from the STING of a species such as Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, and ghost shark, which have much lower NF-κB activity. This thereby results in a STING protein that induces type I interferon, which is important for anti-tumor activity, and that has limited or no NF-κB activity, which is not desirable in an anti-tumor therapy. The chimeras can further include the human constitutive STING activation and gain-of-function mutations in corresponding loci to increase or render type I interferon activity constitutive. In all embodiments, the TRAF6 binding motif can be deleted to further decrease or eliminate activity that is not desirable in an anti-tumor therapeutic. These non-human STING proteins, chimeras, and mutants are provided in delivery vehicles, such as any described herein or known to those of skill in the art, including oncolytic viral vectors, cells, such as stem cells and T-cells that are used in cell therapies, exosomes, minicells, liposomes,

and the immunostimulatory bacteria provided herein, which accumulate in tumor-resident immune cells, and deliver encoded proteins to the tumor microenvironment and to tumors. The non-human STING proteins, modified STING proteins, and STING chimeras, are for use as therapeutics for the treatment of tumors as described herein, or for use in other methods known to those of skill in the art. Pharmaceutical compositions containing the STING proteins, delivery vehicles, and encoding nucleic acids also are provided.

### d. Other Gene Products that Act as Cytosolic DNA/RNA Sensors and Constitutive Variants Thereof

Other gene products that sense or interact with cytosolic nucleic acids are the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which include RIG-I and MDA5 (melanoma differentiation-associated protein 5). RLRs are cytoplasmic sensors of viral dsRNA and nucleic acids secreted by bacteria, and include RIG-I, MDA5, and LGP2 (laboratory of genetics and physiology 2). Upon the binding of a ligand, such as a viral dsRNA, RIG-I and MDA5 activate the mitochondrial antiviral-signaling adaptor protein, or MAVS, which recruits tumor necrosis factor (TNF) receptor-associated factors (TRAFs), to assemble a signaling complex at the outer membranes of the mitochondria. Downstream signaling components further are recruited by TRAFs, resulting in the phosphorylation and activation of IRF3 (interferon regulatory factor 3), IRF7, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and AP-1 (activator protein 1). As a result, the expression of IFNs, proinflammatory cytokines, and other genes involved in pathogen clearance, is induced (see, *e.g.*, Lu and MacDougall (2017) *Front. Genet.* 8:118). Like STING, the constitutive activation of MDA5 and RIG-I due to gain-of-function mutations leads to the induction of type I IFNs, which can be leveraged to enhance the anti-tumor immune response in the immunostimulatory bacteria.

#### i. RIG-I

Retinoic acid-inducible gene I (RIG-I), also known as DDX58 (DEXD/H-box helicase 58), is another protein whose constitutive activation has been linked to the development of interferonopathies, such as atypical Smith-Magenis syndrome. RIG-I, like MDA5/IFIH1, is a member of the RIG-I-like receptor (RLR) family, and is a 925-residue cytosolic pattern recognition receptor that functions in the detection of viral

dsRNA. RIG-I initiates an innate immune response to viral RNA through independent pathways that promote the expression of type I and type III IFNs and proinflammatory cytokines (see, *e.g.*, Jang *et al.* (2015) *Am. J. Hum. Genet.* 96:266-274; and Lu and MacDougall (2017) *Front. Genet.* 8:118).

Atypical Smith-Magenis syndrome, without hallmark dental anomalies, but with variable phenotypes, including glaucoma, aortic calcification and skeletal abnormalities, has been found to be caused by mutations in the DEXD/H-box helicase 58 gene (*DDX58*), which encodes retinoic acid-inducible gene I (RIG-I). In particular, the mutations E373A and C268F in *DDX58* were identified as causing gain-of-function in RIG-I. Elevated amounts of mutated DDX58 were associated with a significant increase in the basal levels of NF-κB reporter gene activity, and this activity was further increased by stimulation with the dsRNA analog poly(I:C). The RIG-I mutations also induced IRF3 phosphorylation and dimerization at the basal level, and led to increased expression of *IFNB1*, interferon-stimulated gene 15 (*ISG15*), and chemokine (C-C motif) ligand 5 (*CCL5*) in both basal, and poly(I:C) transfected HEK293FT cells. These results indicate that the mutated DDX58/RIG-I results in constitutive activation, leading to increased IFN activity and IFN-stimulated gene expression (see, *e.g.*, Jang *et al.* (2015) *Am. J. Hum. Genet.* 96:266-274; and Lu and MacDougall (2017) *Front. Genet.* 8:118). Tumor-targeting immunostimulatory bacteria provided herein can be modified to encode RIG-I/DDX58 (see, *e.g.*, SEQ ID NO:311) with gain-of-function mutations such as, but not limited to, E373A and C268F, singly, and in combination.

**ii. MDA5/IFIH1**

Another interferonopathy gene is the IFN-induced with helicase C domain-containing protein 1 (IFIH1), also known as melanoma differentiation-associated protein 5 (MDA5), which is a member of the RIG-I-like family of cytoplasmic DExD/H box RNA receptors. MDA5, encoded by *IFIH1*, is a 1,025 amino acid cytoplasmic pattern-recognition receptor that senses viral double-stranded RNA (dsRNA) and secreted bacterial nucleic acids in the cytoplasm, and activates type I IFN signaling through an adaptor molecule, MAVS (mitochondrial antiviral-signaling protein). MAVS recruits tumor necrosis factor (TNF) receptor-associated factors (TRAFs), which in turn recruit downstream signaling components, resulting in the

phosphorylation and activation of IRF3 (interferon regulatory factor 3), IRF7, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and AP-1 (activator protein 1). This results in the expression of IFNs, proinflammatory cytokines, and other genes involved in pathogen clearance (see, *e.g.*, Rutsch *et al.* (2015) *Am. J. Hum. Genet.* 96:275-282; Rice *et al.* (2014) *Nat. Genet.* 46(5):503-509; and Lu and MacDougall (2017) *Front. Genet.* 8:118).

Gain-of-function (GOF) *IFIH1* variants occur in subjects with autoimmune disorders, including Aicardi-Goutiéres syndrome (AGS) and Singleton-Merten syndrome (SMS), which are characterized by prominent vascular inflammation. AGS is an inflammatory disease particularly affecting the brain and skin, and is characterized by an upregulation of interferon-induced transcripts. AGS typically occurs due to mutations in any of the genes encoding DNA exonuclease TREX1, the three non-allelic components of the RNase H2 endonuclease complex, the deoxynucleoside triphosphate triphosphohydrolase SAMHD1, and the double-stranded RNA editing enzyme ADAR1. Some patients with AGS do not have mutations in any of these genes, but have GOF mutations in *IFIH1*, indicating that this gene also is implicated in AGS. Singleton-Merten syndrome is an autosomal-dominant disorder characterized by abnormalities in the blood vessels (*e.g.*, calcification), teeth (*e.g.*, early-onset periodontitis, root resorption), and bones (*e.g.*, osteopenia, acro-osteolysis, osteoporosis). Interferon signature genes are upregulated in Singleton-Merten syndrome patients, which was linked to GOF mutations in *IFIH1* (see, *e.g.*, Rice *et al.* (2014) *Nat. Genet.* 46(5):503-509; and Rutsch *et al.* (2015) *Am. J. Hum. Genet.* 96:275-282).

The IFN-β reporter stimulatory activity of wild-type IFIH1, and six IFIH1 GOF mutants identified in AGS patients (R720Q, R779H, R337G, R779C, G495R, D393V), was compared in HEK293T cells, which express low levels of endogenous viral RNA receptors. Wild-type IFIH1 was induced upon binding of the long (> 1 kb) dsRNA analog polyinosinic-polycytidylic acid (poly(I:C)), but not by a short 162 bp dsRNA, and had minimal activity in the absence of exogenous RNA. The IFIH1 mutants displayed a significant induction of IFN signaling in response to the short 162 bp dsRNA, in addition to robust signaling in response to poly(I:C). The mutants also

displayed a 4-10 fold higher level of baseline signaling activity in the absence of exogenous ligand (Rice *et al.* (2014) *Nat. Genet.* 46(5):503-509).

Another gain-of-function *IFIH1* mutation, R822Q, was identified as causing Singleton-Merten syndrome by triggering type I IFN production, and leading to early arterial calcification, as well as dental inflammation and resorption. HEK293T cells (which have the lowest endogenous *IFIH1* expression levels) were used to overexpress wild-type and R822Q MDA5. Wild-type *IFIH1* expression led to an increase in the expression of *IFNB1* (interferon, beta 1, fibroblast) in a dose-dependent manner, whereas the mutated *IFIH1* led to approximately 20-fold more *IFNB1* expression. Following stimulation with the dsRNA analog poly(I:C), R822Q *IFIH1* resulted in higher levels of *IFNB1* expression than wild-type *IFIH1*, indicating that R822Q *IFIH1* is hyperactive to non-self dsRNA. There was also higher expression of interferon signature genes, such as *IFI27*, *IFI44L*, *IFIT1*, *ISG15*, *RSG15*, *RSAD2*, and *SIGLEC1* in whole-blood samples from Singleton-Merten syndrome patients, which was in agreement with the higher expression level of *IFNB1* by R822Q *IFIH1* (see, *e.g.*, Rutsch *et al.* (2015) *Am. J. Hum. Genet.* 96:275-282).

The interferon signature observed in patients with another *IFIH1* GOF mutation, A489T, is indicative of a type I interferonopathy; *IFIH1* A489T is associated with increased interferon production and phenotypes resembling chilblain lupus, AGS, and SMS (see, *e.g.*, Bursztejn *et al.* (2015) *Br. J. Dermatol.* 173(6):1505-1513). The A489T variant not only resulted in IFN induction following stimulation with the long dsRNA analog poly(I:C), but also with short dsRNA. Two additional gain-of-function mutations in *IFIH1*, T331I and T331R, were identified in patients with SMS phenotypes, who presented with a significant upregulation of IFN-induced transcripts. The T331I and T331R variants resulted in increased expression of IFN-β, even in the absence of exogenous dsRNA ligand, consistent with the observed constitutive activation of MDA5 (see, *e.g.*, Lu and MacDougall (2017) *Front. Genet.* 8:118).

A946T is another *IFIH1* GOF mutation that leads to the increased production of type I IFN, promoting inflammation and increasing the risk of autoimmunity. The A946T mutation in *IFIH1* results in additive effects when combined with the *TMEM173* R232 allele and the G207E GOF mutation in STING, leading to a severe

early-onset phenotype with features similar to SAVI (see, *e.g.*, Keskitalo *et al.* (2018) preprint, available from doi.org/10.1101/394353). G821S is a GOF mutation in *IFIH1* which has been shown to lead to spontaneously developed lupus-like autoimmune symptoms in a mouse model (see, *e.g.*, Rutsch *et al.* (2015) *Am. J. Hum. Genet.* 96:275-282), while the *IFIH1* missense mutations A452T, R779H, and L372F, identified in individuals with AGS, were shown to cause type I interferon overproduction (see, *e.g.*, Oda *et al.* (2014) *Am. J. Hum. Genet.* 95:121-125).

The tumor-targeting immunostimulatory bacteria provided herein can be modified to encode MDA5/IFIH1 (see, *e.g.*, SEQ ID NO:310) with gain-of-function mutations selected from T331I, T331R, R337G, L372F, D393V, A452T, A489T, G495R, R720Q, R779H, R779C, G821S, R822Q, and A946T, singly, or in any combination.

**iii. IRF7**

Constitutively active forms of IRF7 (or IRF-7) include mutants in which different C-terminal serines are substituted by phosphomimetic Asp, including IRF7(S477D/S479D), IRF7(S475D/S477D/S479D), and IRF7(S475D/S476D/S477D/S479D/S483D/S487D). IRF7(S477D/S479D) is a strong transactivator for IFNA and RANTES gene expression, and stimulates gene expression, even in the absence of virus infection. IRF7(S475D/S477D/S479D), and IRF7(S475D/S476D/S477D/S479D/S483D/S487D) do not further augment the transactivation activity of IRF7(S477D/S479D), but the transactivation activity of all three mutants is stimulated further by virus infection. The mutant IRF7(Δ247-467), which localizes to the nucleus in uninfected cells, is a very strong constitutive form of IRF7; it activates transcription more than 1500-fold higher than wild-type IRF7 in unstimulated and virus infected cells (see, *e.g.*, Lin *et al.* (2000) *J. Biol. Chem.* 275(44):34320-34327). The immunostimulatory bacteria provided herein, can encode and express constitutively active IRF7 mutants, including those with replacements at residues 475-477, 479, 483, and 487, and those with amino acid deletions. The immunostimulatory bacteria encode these proteins on plasmids under the control of promoters and, any other desired regulatory signals recognized by mammalian hosts, including humans.

**e. Other Type I IFN Regulatory Proteins**

Other proteins involved in the recognition of DNA/RNA that activate type I IFN responses can be mutated to generate constitutive type I IFN expression. The unmodified and/or modified proteins can be encoded in the immunostimulatory bacteria provided herein, to be used to deliver the protein to the tumor microenvironment, such as to tumor-resident immune cells, to increase expression of type I IFN.

These proteins include, but are not limited to, proteins designated TRIM56, RIP1, Sec5, TRAF2, TRAF3, TRAF6, STAT1, LGP2, DDX3, DHX9 (DDX9), DDX1, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200.

| Gene | Encoded Protein | Activity/Function |
|---|---|---|
| TRIM56 | Tripartite motif-containing protein 56/ E3 ubiquitin-protein ligase TRIM56 | Promotes dimerization of STING in response to dsDNA stimulation, resulting in production of IFN-β; potentiates extracellular dsRNA-induced expression of IFNB1 and IFN-stimulated genes ISG15, IFIT1/ISG56, CXCL10, OASL and CCL5; positive regulator of TLR3 signaling |
| RIP1/RIPK1 | Receptor-interacting serine/threonine protein (kinase) 1 | Transduces inflammatory and cell-death signals (programmed necrosis) following death receptor ligation, activation of pathogen recognition receptors and DNA damage; indirectly activates NF-κB; directs LPS-induced IFN-β synthesis in mice |
| Sec5 (EXOC2) | Exocyst complex component 2 | Component of exocyst complex, involved in docking of exocytic vesicles with fusion sites on plasma membrane; co-localizes with STING and TBK1 after intracellular DNA stimulation, inducing type I IFN production |
| TRAF2 | TNF receptor-associated factor 2 | Regulates activation of NF-κB and JNK/MAPK8; mediates type I IFN induction |
| TRAF3 | TNF receptor-associated factor 3 | Regulates activation of NF-κB and MAP kinases; mediates activation of IRF3; mediates type I IFN induction; mediates cytokine production |
| TRAF6 | TNF receptor-associated factor 6 | Activates NF-κB, JUN, and AP-1; induces type I IFN production in response to viral infection and intracellular dsRNA; induces production of proinflammatory cytokines |
| STAT1 | Signal transducer and activator of transcription 1 | Forms part of ISGF3 transcription factor, which binds IFN stimulated response elements (ISREs) to activate transcription of IFN-stimulated genes (ISGs) |
| LGP2 (DHX58) | Laboratory of genetics and physiology 2 / Probable ATP-dependent RNA helicase DHX58 | Regulates RIG-I/DDX58- and IFIH1/MDA5-mediated antiviral signaling |
| DDX3 (DDX3X) | ATP-dependent RNA helicase DDX3X | Promotes production of type I IFN; acts as viral RNA sensor; involved in TBK1 and IKBKE-dependent IRF3 activation, leading to induction of IFNB; associates with IFNB promoters; associates with MAVS and RIG-I to induce signaling in early stages of infection; binds MDA5 to enhance its recognition of dsRNA |

| Gene | Encoded Protein | Activity/Function |
|---|---|---|
| DHX9/DDX9 | DExD/H-box helicase 9 / ATP-dependent RNA helicase A | Senses viral nucleic acids; triggers host responses to non-self DNA in MyD88-dependent manner; interacts with MAVS to stimulate NF-κB-mediated innate immunity against virus infection and activate IRF3 and MAPK pathways; potentiates virus-triggered induction of IL-6 and IFN-β |
| DDX1 | ATP-dependent RNA helicase DDX1 | Component of a multi-helicase-TRIF complex that senses viral double-stranded RNA (dsRNA), activates the NF-κB signaling pathway, and induces production of type I IFN and proinflammatory cytokines |
| DDX21 | Nucleolar RNA helicase 2 | Component of a multi-helicase-TRIF complex that senses viral double-stranded RNA (dsRNA), activates the NF-κB signaling pathway, and induces production of type I IFN and proinflammatory cytokines |
| DHX15 (DDX15) | Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 | Viral RNA sensor that interacts with MAVS to induce type I IFN and proinflammatory cytokine production; activates IRF3, NF-κB, and MAPK signaling |
| DHX33 (DDX33) | ATP-dependent RNA helicase DHX33 | Viral dsRNA sensor that interacts with MAVS and triggers type I IFN response; activates NF-κB, IRF3, and MAPK signaling pathways; activates NLRP3 inflammasome, resulting in secretion of proinflammatory cytokines |
| DHX36 (DDX36) | ATP-dependent DNA/RNA helicase DHX36 | Component of a multi-helicase-TRIF complex that senses viral double-stranded RNA (dsRNA), activates the NF-κB signaling pathway, and induces production of type I IFN and proinflammatory cytokines |
| DDX60 | Probable ATP-dependent RNA helicase DDX60 | Senses viral RNA and DNA; forms complex with RIG-I like receptors to promote antivirus activity; positively regulates RIG-I and MDA5-dependent type I IFN and IFN-inducible gene expression in response to viral infection; binds ssRNA, dsRNA, and dsDNA; promotes binding of RIG-I to dsRNA |
| SNRNP200 | U5 small nuclear ribonucleoprotein 200 kDa helicase | Senses/binds viral RNA and interacts with TBK1 to promote IRF3 activation and type I IFN production |

Gain-of-function variants can be produced, such as by screening and/or by mutagenesis. Site-directed mutagenesis can be performed *in vitro* to identify mutations with enhanced activity, that lead to higher level and/or constitutive type I IFN expression. Intact genomic DNA can be obtained from non-related patients experiencing auto-immune and auto-inflammatory symptoms, and from healthy individuals, to screen for and identify other products whose expression leads to increased or constitutive type I IFN expression. Whole exome sequencing can be performed, and introns and exons can be analyzed, such that proteins with mutations in the pathways associated with the increased or constitutive expression of type I interferon are identified. After identification of mutations, cDNA molecules encoding the full-length gene, with and without the identified mutation(s), are transfected into a

reporter cell line that measures expression of type I interferon. For example, a reporter cell line can be generated where the expression of luciferase is placed under the control of the promoter for IFN-β. A gain-of-function mutant that is constitutively active will promote the expression of IFN-β, whereas the unstimulated wild-type protein will not. Stimulation can be by virus infection, bacterial infection, bacterial nucleic acids, LPS, dsRNA, poly(I:C), or by increasing exogenous levels of the protein's ligand (*e.g.*, CDNs). Identified proteins also include those that enhance an immune response to an antigen(s) of interest in a subject. The immune response comprises a cellular or humoral immune response characterized by one or more of: (i) stimulating type I interferon pathway signaling; (ii) stimulating NF-κB pathway signaling; (iii) stimulating an inflammatory response; (iv) stimulating cytokine production; (v) stimulating dendritic cell development, activity, or mobilization; (vi) any other responses indicative of a product whose expression enhances an immune response; and (vii) a combination of any of (i)-(vi).

### 3. Antibodies and Antibody Fragments

Advances in antibody engineering have led to the creation of recombinant antibody fragments that have many improvements over conventional monoclonal antibodies, especially in terms of manufacturing, tissue penetration, and ease of use. An example of these is the single-chain fragment variable (scFv), consisting of the variable regions of the heavy ($V_H$) and light ($V_L$) chains of the antibody binding site, joined together by a flexible peptide linker that is generally the $(G_4S)_3$ sequence (see, *e.g.*, Weisser *et al.* (2009) *Biotechnol. Adv.* 27(4):502-520). Other examples include scFv-Fc antibody fragments, in which the $V_H$ domain of the scFv is linked to an Fc region, as well as dimers and multimers and other combinations and forms of antibodies that are known to those of skill in the art. Antibody fragments and other constructs allow for targeting of antigens in a manner that can be encoded on a plasmid and delivered, as exemplified herein, by an immunostimulatory bacterium. Examples of antigens to target, include, tumor antigens, including those discussed in the Examples below, and other exemplary targets discussed below and elsewhere herein. Any target known to the skilled person is included, as are neo-antigens formed upon changes in conformation or structure.

#### a. TGF-β

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine with numerous roles in embryogenesis, wound healing, angiogenesis, and immune regulation. It exists in three isoforms in mammalian cells, TGF-β1, TGF-β2, and TGF-β3; TGF-β1 is the most predominant in immune cells (see, *e.g.*, Esebanmen *et al.* (2017) *Immunol. Res.* 65:987-994). TGF-β's role as an immunosuppressant is arguably its most dominant function. Its activation from a latent form in the tumor microenvironment, in particular, has profound immunosuppressive effects on DCs and their ability to tolerize antigen-specific T-cells. TGF-β also can directly convert Th1 $CD4^+$ T-cells to immunosuppressive Tregs, further promoting tumor tolerance (see, *e.g.*, Travis *et al.* (2014) *Annu. Rev. Immunol.* 32:51-82). Based on its tumor-specific immunosuppressive functions, and irrespective of its known cancer cell growth and metastasis-promoting properties, inhibition of TGF-β is a cancer therapy target. High levels of TGF-β signaling have been demonstrated in several human tumor types, including colorectal cancer (CRC), hepatocellular carcinoma (HCC), pancreatic ductal adenocarcinoma (PDAC), and non-small-cell lung cancer (NSCLC) (see, *e.g.*, Colak *et al.* (2017) *Trends Cancer* 3(1):56-71). Systemic inhibition of TGF-β can lead to unacceptable autoimmune toxicities, and its inhibition should be localized to the tumor microenvironment. One way to accomplish this is to create a soluble TGF-β receptor that acts as a decoy for binding TGF-β (see, *e.g.*, Zhang *et al.* (2008) *J. Immunol.* 181:3690-3697). As such, a tumor-targeting immunostimulatory bacteria, containing a TGF-β receptor decoy, provided herein, can bind and remove TGF-β from the tumor microenvironment, thereby breaking tumor immune tolerance and stimulating anti-tumor immunity.

In addition to TGF-beta binding decoy receptors, other TGF-beta polypeptide antagonists, that can bind to and eliminate or reduce TGF-β in the tumor microenvironment, thereby breaking tumor immune tolerance and stimulating anti-tumor immunity, are included. Exemplary of such are anti-TGF-beta antibodies or antibody fragments, anti-TGF-beta receptor antibodies or antibody fragments, and soluble TGF-beta antagonist polypeptides.

Immunostimulatory bacteria are provided herein that accumulate in the tumor microenvironment, in tumors, and in particular, in tumor-resident immune cells, and that contain plasmids encoding TGF-beta polypeptide antagonists, including, for

example, TGF-beta binding decoy receptors (TGF-β receptor decoys), anti-TGF-beta antibodies or antibody fragments, anti-TGF-beta receptor antibodies or antibody fragments, and soluble TGF-beta antagonist polypeptides. The antibody fragments can include any known in the art, or described herein, such as, but not limited to, scFvs and scFv-Fcs.

**b. Bispecific scFvs and T-Cell Engagers**

The use of scFvs has been improved by increasing the valency of binding to the target, often through the use of one or more scFv fragments (bis-specific, tri-specific, etc.), joined together by a long linker. Bi-specific T-cell engager (available under the trademark BiTE®) constructs are a class of artificial bispecific monoclonal antibodies that are utilized in cancer immunotherapy, and are formed by linking two single-chain variable fragments (scFvs), such that one scFv binds CD3 on the surface of cytotoxic T-cells, and the other binds a specific tumor-associated antigen (TAA). BiTEs® thus target T-cells to tumor cells, stimulating T-cell activation, cytokine production and tumor cell cytotoxicity independently of MHC class I or co-stimulatory molecules. Two examples of bi-specific T-cell engagers (BiTEs® have been approved by the FDA, including catumaxomab, which is directed against the tumor antigen EpCAM, and against CD3, and is used in the treatment of malignant ascites, and blinatumomab, a BiTE® antibody directed against CD19 and CD3, which is used for the treatment of relapsed, refractory acute lymphoblastic leukemia (ALL; see, *e.g.*, Ahamadi-Fesharaki *et al.* (2019) *Mol. Ther. Oncolytics* 14:38-56). Other bi-specific T-cell engagers (BiTEs®) target a variety of antigens, including, for example, carcinoembryonic antigen (CEA), prostate-specific membrane antigen (PSMA), EGFR, EphA2, HER2/neu, ADAM17/TACE, prostate stem cell antigen (PSCA), Delta-like ligand 3 (DDL3), and melanoma-associated chondroitin sulfate proteoglycan (MCSP). As exemplified herein, a BiTE® antibody also can be expressed from a plasmid following delivery by an immunostimulatory bacterium.

**c. Anti-PD-1, Anti-PD-L1 and Anti-CTLA-4 Antibodies**

**i. Anti-PD-1/Anti-PD-L1 Antibodies**

Programmed cell death protein 1 (PD-1) is an immune-inhibitory receptor that is involved in the negative regulation of immune responses. Its cognate ligand, programmed death-ligand 1 (PD-L1), is expressed on antigen-presenting cells

(APCs), and upon binding to PD-1 on T-cells, leads to loss of $CD8^+$ T-cell effector function, inducing T-cell tolerance. The expression of PD-L1 is often associated with tumor aggressiveness and reduced survival in certain human cancers (see, *e.g.*, Gao *et al.* (2009) *Clin. Cancer Res.* 15(3):971-979).

Antibodies designed to block immune checkpoints, such as anti-PD-1 (for example, pembrolizumab, and nivolumab) and anti-PD-L1 (for example, atezolizumab, avelumab, and durvalumab) antibodies can prevent T-cell anergy and break immune tolerance. Only a fraction of treated patients, however, exhibit clinical benefit, and those that do, often present with autoimmune-related toxicities (see, *e.g.*, Ribas (2015) *N. Engl. J. Med.* 373(16):1490-1492; and Topalian *et al.* (2012) *N. Engl. J. Med.* 366(26):2443-2454). Besides acquiring toxicity, anti-PD-1/anti-PD-L1 therapy often leads to resistance, and the concomitant use of anti-CTLA-4 antibodies (for example, ipilimumab) has shown limited success in clinical trials, with significantly additive toxicity. To limit the toxicity and enhance the potency of PD-1/PD-L1 blockade, an immunostimulatory bacterium, containing a plasmid encoding an antibody or antibody fragment, such as an scFv and scFv-Fc, and others known in the art and/or described herein, against PD-1 or against PD-L1, will synergize with activation of immune cells to potentiate anti-tumor immunity.

**ii. Anti-CTLA-4 Antibodies**

CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), also known as CD152 (cluster of differentiation 152), is another immune-inhibitory receptor that functions as an immune checkpoint, and downregulates immune responses. CTLA-4 is constitutively expressed in regulatory T-cells (Tregs, or $T_{regs}$), and contributes to their inhibitory function, but is upregulated in conventional T-cells only after activation. CTLA-4 functions as an immune checkpoint by transmitting inhibitory signals to T-cells. CTLA-4 is homologous to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 (also known as B7-1 or B7.1) and CD86 (also known as B7-2 or B7.2) ligands on antigen-presenting cells (APCs). The binding of CTLA-4 to the ligands transmits an inhibitory signal to T-cells, whereas the binding of CD28 transmits a stimulatory signal.

Following T-cell activation, CTLA-4 receptors are induced, which then outcompete CD28 receptors on T-cells, for binding to CD80 and CD86 ligands on the

surfaces of APCs. CTLA-4 binds to CD80 and CD86 with greater affinity and avidity than CD28, thus enabling it to outcompete CD28 for its ligands, resulting in the transmittal of inhibitory signals to T-cells, and an immune inhibitory response. T-cell activation through the T-cell receptor and CD28 leads to increased expression of CTLA-4.

Optimal T-cell priming requires co-stimulatory signals resulting from the ligation of T-cell CD28 with CD80 and/or CD86. The blockade of CTLA-4 from binding to these ligands thus enhances T-cell priming, and allows for the induction of an anti-tumor immune response.

In some embodiments, the immunostimulatory bacterial strains provided herein contain plasmids encoding anti-CTLA-4 antibodies, including fragments thereof, such as, but not limited to, anti-CTLA-4 scFvs (see, *e.g.*, copending PCT application International PCT Application No. PCT/US2020/060307 for sequences of exemplary human anti-CTLA-4 scFv and scFv-Fc fragments; see, also, Example 25).

**d. Additional Exemplary Checkpoint Targets**

Exemplary immune checkpoint targets for which an scFv, or any other recombinant antibody fragment against them can be prepared, or are exemplified herein include, but are not limited to, those listed in the table below:

| Checkpoint target |
|---|
| CTLA-4 |
| PD-L1 (B7-H1) |
| PD-L2 |
| PD-1, PD-2 |
| IDO1 |
| IDO2 |
| SIRP alpha (CD47) |
| VISTA (B7-H5) |
| LIGHT |
| HVEM |
| CD28 |
| LAG3, TIM3, TIGIT |
| Galectin-9 |
| CEACAM1, CD155, CD112, CD226, CD244 (2B4) |
| B7-H2, B7-H3, CD137, ICOS, GITR, B7-H4, B7-H6 |
| CD137, CD27, CD40, CD40L, CD48, CD70, CD80, CD86, CD137 (4-1BB), 4-1BBL, CD200, CD272 (BTLA), CD160 |

| Checkpoint target |
|---|
| A2a receptor, A2b receptor, HHLA2, ILT-2, ILT-4, gp49B, PIR-B |
| OX40, OX-40L, HLA-G, ILT-2/4 |
| KIR, TIM1, TIM4 |
| CLEVER-1/Stabilin-1 |

### 4. Combinations of Immunomodulatory Proteins can have Synergistic Effects and/or Complementary Effects

Cytokines are powerful modulators of the anti-tumor immune response. Cytokine combinations are known to have profound synergistic effects on different immune compartments involving T-cells, NK cells, and myeloid cells (including dendritic cells and macrophages). Cytokines are known to play major roles in antigen priming by dendritic cells, survival and proliferation of innate immune cells and antigen-specific T-cells, and the cytotoxic activity of NK and T-cells. Cytokine combinations must be properly chosen to maximize biological responses and enhance anti-tumor immunity. For example, in a murine model of hepatitis, IFN-α alone was found to enhance the $CD8^+$ T-cell cytolytic function of virally infected cells, while IL-15 alone enhanced the proliferation of activated lymphocytes. Together, they maximally suppressed hepatitis B (HBV) infection (see, *e.g.*, Di Scala *et al.* (2016) *J. Virol.* 90(19):8563-8574). In another example, combinations of the cytokines IL-15 + IL-18, and IL-15 + IL-21, were able to enhance the production of IFN-γ from human NK and T-cells (see, *e.g.*, Strengell *et al.* (2003) *J. Immunol.* 170(11):5464-5469). In another example, IL-2 + IL-18 synergized to enhance IFN-γ production and increase the cytolytic function of $CD4^+$ T-cells, $CD8^+$ T cells, and NK lymphocytes (see, *e.g.*, Son *et al.* (2001) *Cancer Res.* 61(3):884-888). Additionally, IL-12 and IL-18 were found to synergize to promote antigen-CD3 T-cell ligation-independent production of IFN-γ from human T-cells (see, *e.g.*, Tominaga *et al.* (2000) *Int. Immunol.* 12(2):151-160). Combinations of cytokines are powerful enhancers of T-cell function, but the FDA-approved anti-cancer cytokines are too toxic to be dosed systemically and are thus rarely used, and combinations of systemically-administered cytokines only compound the toxicity (see, *e.g.*, Conlon *et al.* (2019) *J. Interferon Cytokine Res.* 39(1):6-21).

The immunostimulatory bacteria provided herein solve these problems. Provided are immunostimulatory bacteria containing plasmids encoding multiple therapeutic products, such as immunomodulatory proteins, that allow for tumor-specific delivery of cytokine combinations and/or combinations with other therapeutic products, such as the inducers of type I interferon discussed herein, and others, including co-stimulatory molecules, chemokines, and antibodies and fragments thereof. These immunostimulatory bacteria achieve powerful and synergistic immuno-activation without the systemic toxicities and pharmacokinetic (PK) liabilities associated with direct IV administration of the cytokines and other therapeutic products.

Combinations of therapeutic products that can be encoded on the plasmids in the immunostimulatory bacteria provided herein include, but are not limited to, for example, two or more cytokines; one or more cytokines and an inducer of type I IFN (*e.g.*, STING, IRF3, IRF7, MDA5, RIG-I, and constitutively active, GOF variants thereof), and/or a co-stimulatory molecule (*e.g.*, 4-1BBL and 4-1BBLΔcyt); a TGF-β decoy receptor and one or more cytokines; a TGF-β decoy receptor and an inducer of type I IFN; a TGF-β decoy receptor, one or more cytokines, and/or an inducer of type I IFN, and/or a co-stimulatory molecule; an antibody (*e.g.*, against an immune checkpoint, such as CTLA-4) and one or more cytokines; an antibody and an inducer of type I IFN; an antibody, one or more cytokines, and/or an inducer of type I IFN, and/or a co-stimulatory molecule; a co-stimulatory molecule agonist (*e.g.*, a CD40 agonist) and one or more cytokines; a co-stimulatory molecule agonist and an inducer of type I IFN; and a co-stimulatory molecule agonist, one or more cytokines, and/or an inducer of type I IFN, and/or a co-stimulatory molecule.

As discussed below, the multiple therapeutic product expression cassettes can include single promoter constructs and/or dual/multiple promoter constructs, as well as post-transcriptional regulatory elements, and other regulatory elements, such as enhancers, polyadenylation signals, terminators, signal peptides, etc. The nucleic acid sequences can be codon optimized to increase protein expression, and generally, are under control of a eukaryotic promoter. Particular constructs and details thereof are described elsewhere herein.

Among the immunostimulatory bacteria provided herein are those that contain plasmids encoding immunostimulatory proteins (*e.g.*, cytokines, chemokines, co-stimulatory molecules), and/or gene products with gain-of-function mutations that increase immune responses in the tumor microenvironment (*e.g.*, cytosolic DNA/RNA sensors that induce type I IFN), and/or antibodies and fragments thereof, and/or other therapeutic products that enhance the anti-tumor response, such as TGF-β and/or IL-6 decoy receptors, and/or TGF-β antagonizing polypeptides. These immunostimulatory bacteria that encode the cytokines, gain-of-function products/type I IFN pathway proteins, and/or chemokines, and/or co-stimulatory molecules, and/or antibodies and fragments thereof, such as single-chain antibodies, and other therapeutic products discussed herein, include the immunostimulatory bacteria that preferentially infiltrate the tumor microenvironment, tumors, and tumor-resident immune cells. The immunostimulatory bacteria also include those in which the genome is modified so that they induce less cell death in tumor-resident immune cells, whereby the immunostimulatory bacteria accumulate in tumor-resident myeloid cells, to achieve high level ectopic expression of multiplexed genetic payloads in the target cells, and deliver the therapeutic products/immunomodulatory proteins to the tumor microenvironment (TME), to stimulate the immune response against the tumor. In particular, the immunostimulatory bacteria provided herein include up to about 8 or 8 modifications as described herein, including, but not limited to, adenosine auxotrophy, *csgD*$^-$, *pagP*$^-$, *msbB*$^-$, flagellin$^-$ (*fliC*$^-$/*fljB*$^-$), *purI*$^-$, *ansB*$^-$, *asd*$^-$, and any other modifications described herein or known to improve targeting to, or accumulation in, the tumor microenvironment and/or tumor-resident myeloid cells, or to improve safety and tolerability (allowing for a higher dose), reduce the immunosuppressive cytokine profile, improve T-cell quality and function, limit replication in healthy tissues, eliminate biofilms, and improve the anti-tumor immune response, or to impart any of the desirable and advantageous properties discussed elsewhere herein.

The immunostimulatory bacteria further can encode other therapeutic products, such as a tumor antigen from the subject's tumor, to enhance the response against the particular tumor. Any of the immunostimulatory bacteria provided herein and described above and below can be modified to encode the therapeutic products,

such as cytokines, chemokines, co-stimulatory molecules and gain-of-function type I IFN pathway product(s). The therapeutic products are encoded on a plasmid under control of a promoter recognized by the host, and any other desired regulatory sequences recognized in a eukaryotic, such as a human, or other animal, or mammalian, subject. Generally, the nucleic acid encoding the product is under the control of an RNA polymerase II promoter. Additionally, any of the bacteria described herein for modification, such as any of the strains of *Salmonella*, *Shigella*, *E. coli*, *Bifidobacteriae*, *Rickettsia*, *Vibrio*, *Listeria*, *Klebsiella*, *Bordetella*, *Neisseria*, *Aeromonas*, *Francisella*, *Cholera*, *Corynebacterium*, *Citrobacter*, *Chlamydia*, *Haemophilus*, *Brucella*, *Mycobacterium*, *Mycoplasma*, *Legionella*, *Rhodococcus*, *Pseudomonas*, *Helicobacter*, *Bacillus*, and *Erysipelothrix*, or an attenuated strain thereof, or a modified strain thereof, can be modified by introducing a plasmid containing, or by encoding on a plasmid in the bacteria, nucleic acid encoding the therapeutic product(s) under control of an RNA polymerase promoter recognized by the host. The therapeutic products are expressed in the infected subject's cells. The immunostimulatory bacteria include those that are modified, as described herein, to accumulate in, or to preferentially infect, tumors, the TME and/or tumor-resident myeloid cells. For example, immunostimulatory bacteria that encode gain-of-function products leading to the expression of, or the constitutive expression of, type I interferon (IFN), such as IFN-beta, and/or other therapeutic products as discussed herein, further are modified to have reduced ability or no ability to infect epithelial cells, but are able to infect phagocytic cells, including tumor-resident immune cells, and/or the immunostimulatory bacteria are modified so that they do not kill the infected phagocytic cells.

As described herein, genes involved in the SPI-1 pathway, and flagella, activate the inflammasome in phagocytic cells (immune cells), triggering pyroptosis. Knocking out SPI-1 genes and genes that encode flagella, decreases or eliminates pyroptosis of phagocytic cells, and also, eliminates infection of epithelial cells, resulting in increased infection of phagocytic cells. Provided are immunostimulatory bacteria that accumulate in phagocytic cells, particularly tumor-resident immune cells, such as, for example, myeloid-derived suppresser cells (MDSCs), tumor-associated macrophages (TAMs), and dendritic cells (DCs), in which they express the genetic

payloads/therapeutic products encoded on plasmids that are controlled by eukaryotic promoters, such as those recognized by RNA polymerase II, and include other eukaryotic regulatory signals, as discussed herein. Expressed therapeutic products include those that evoke immune responses, such as through pathways that increase or induce type I interferons, which increase the host response in the tumor microenvironment. The immunostimulatory bacteria also can encode immunostimulatory proteins, such as IL-2 and/or other cytokines, and/or other immunostimulatory proteins and therapeutic products, as discussed herein, further enhancing the immune response in the tumor microenvironment.

The immunostimulatory bacteria can encode products, referred to as cytosolic DNA/RNA sensors, that evoke immune responses when exposed to nucleic acids, such as RNA, DNA, nucleotides, dinucleotides, cyclic nucleotides, cyclic dinucleotides, and other such molecules, in the cytosol of cells. The immunostimulatory bacteria herein, encode modified therapeutic products that constitutively evoke immune responses, and do not require the presence of the DNA/RNA in the cytosol. Exemplary of such are components of pathways that induce type I interferon expression. The therapeutic products contemplated herein include modified forms of these cytosolic DNA/RNA sensors, that have constitutive activity or increased activity (*i.e.*, gain-of-function products), such that type I interferon(s) is/are expressed or produced in the absence of nucleotides, dinucleotides, cyclic nucleotides, cyclic dinucleotides, and other such ligands, in the cytosol of cells. Expression of these modified products in cells, particularly in tumor cells, including tumor-resident immune cells, leads to constitutive expression of type I interferons, including interferon-β, in the tumor microenvironment. Because the immunostimulatory bacteria that express these gain-of-function products accumulate in or preferentially infect tumor cells/the TME/tumor-resident immune cells, the therapeutic products are expressed in the tumor microenvironment, resulting in increased immune responses in the tumor microenvironment.

Exemplary gene products that can be encoded in the immunostimulatory bacteria and other vehicles, include, but are not limited to, proteins that sense or are involved in innate pathways that recognize cytosolic DNA/RNA and activate type I interferon production. Proteins involved in innate DNA/RNA recognition that activate

type I interferon include, but are not limited to: STING, RIG-I, MDA5, IRF3, IRF7, TRIM56, RIP1/RIPK1, Sec5/EXOC2, TRAF2, TRAF3, TRAF6, STAT1, LGP2/DHX58, DDX3/DDX3X, DHX9/DDX9, DDX1, DDX21, DHX15/DDX15, DHX33/DDX33, DHX36/DDX36, DDX60, and SNRNP200. Gain-of-function mutations in any of these proteins that result in constitutive type I interferon expression are known, or can be identified, and the mutants can be delivered by the immunostimulatory bacteria to the tumor microenvironment, such as by infection of phagocytic cells, or by targeting and binding to tumor cells.

The gain-of-function mutations include those identified from individuals with disorders resulting from constitutive type I interferon expression. Exemplary of gain-of-function products are those that occur in subjects with interferonopathies. As noted above, mutations can be identified by screening, to generate gain-of-function products as well.

The nucleic acids encoding the therapeutic products further can be modified to improve properties for expression. Modifications include, for example, codon optimization to increase transcriptional efficiency in a mammalian, particularly human, subject, such as reduction of GC content or CpG dinucleotide content, removal of cryptic splicing sites, adding or removing (generally removing) CpG islands, replacement of the Shine-Dalgarno (SD) sequence, and replacement of TATA box and/or terminal signals to increase transcriptional efficiency. Codons can be optimized for increasing translation efficiency by altering codon usage bias, decreasing GC content, decreasing mRNA secondary structure, removing premature PolyA sites, removing RNA instability motifs (ARE), reducing stable free energy of mRNA, modifying internal chi sites and ribosomal binding sites, and reducing RNA secondary structures. Additional modifications to improve expression, and to maintain or enhance bacterial fitness have been incorporated into the immunostimulatory bacteria. These are described in sections below, and detailed and exemplified in the working Examples below.

As described above, type I interferon induction pathways, mediated by host recognition of cytosolic nucleic acids, such as single-stranded and double-stranded RNA, cyclic di-nucleotides (CDNs), and other such forms of nucleic acids, induce type I IFN. There also are Toll-Like Receptor (TLR)-independent type I IFN

pathways, mediated by host recognition of single-stranded (ss) and double-stranded (ds) RNA in the cytosol. These are sensed by RNA helicases, including retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and through IFN-β promoter stimulator 1 (IPS-1) adaptor protein-mediated phosphorylation of the IRF3 transcription factor, leading to induction of IFN-β (see, *e.g.*, Ireton and Gale (2011) *Viruses* 3(6):906-919). As discussed herein, proteins in these pathways can be modified, or can exist as variants, that result in constitutive expression of type I interferons (also referred to as interferon type 1), which include IFN-α and IFN-β. Exemplary of such proteins are the modified STING proteins described, including those provided, herein. These include modified STING proteins with mutations that result in constitutive expression of the type I interferons so that the interferons are expressed in the absence of induction, including modified non-human STING proteins and human STING proteins, and also, chimeric STING proteins, such as those in which the a C-terminal tail (CTT) portion is replaced with a CTT portion from a STING protein from a second species, wherein the STING protein of the second species has lower NF-κB signaling activity than the NF-κB signaling activity of human STING, and the TRAF6 binding site in the CTT optionally is deleted, and chimeric STING proteins that further include one or more gain-of-function mutations.

Therapy with the immunostimulatory bacteria provided herein can be combined with any other anti-cancer therapy, including checkpoint inhibitor therapies and, as discussed above and elsewhere herein, other cancer treatments and chemotherapy.

**5. Molecules that Activate Prodrugs**

The plasmids in the immunostimulatory bacteria provided herein can include nucleic acids that encode molecules, such as enzymes, that activate, such as by cleavage of a portion of, therapeutic products, such as prodrugs, including chemotherapeutic prodrugs, particularly toxins, that are activated by enzymatic cleavage. As a result, the inactive prodrug can be administered systemically, and is inactive. The plasmid-encoded activating molecule, such as an enzyme, is expressed in the tumor microenvironment after delivery of the immunostimulatory bacteria provided herein, so that the inactive prodrug is activated in the tumor

microenvironment, where it exerts its anti-tumor effect. There are many examples of such prodrugs, including certain nucleosides, and toxin conjugates. Many such prodrugs and enzymes are known (see, *e.g.*, Malekshah *et al.*, (2016) *Curr. Pharmacol. Rep.* 2(6):299-308). These include prodrugs of 5-fluorouracil (5-FU), oxazaphosphorines, platinum drugs, and enzymes, such as deaminases, nitroreductases, phosphorylases, and cytochrome P450 enzymes, and many others.

**6. Immunostimulatory Bacteria that Deliver Combination Therapies**

The immunostimulatory bacteria herein can be used to provide more than one therapeutic product, particularly those that are for anti-cancer therapy. In general, the products are complementary products to enhance and re-program the anti-tumor immune response. The immunostimulatory bacteria, by virtue of the genomic modifications described herein, particularly the combination of several or of all of *asd*$^-$, flagellin$^-$ (such as *fliC*$^-$/*fljB*$^-$), *pagP*$^-$, *csgD*$^-$, *purI*$^-$, adenosine auxotrophy, *msbB*$^-$, *ansB*$^-$, and any other modifications described elsewhere herein or known to those of skill in the art, accumulate in the tumor microenvironment (TME) and infect tumor-resident immune cells (myeloid cells). The immunostimulatory bacteria contain plasmids, encoding complementary therapeutic products, under control of a promoter or promoters recognized by the host, and any other desired regulatory sequences recognized in a eukaryotic, such as a human, or other animal, or mammalian, subject, to effect expression of the encoded products, and, also, secretion of the products. The immunostimulatory bacteria accumulate in the TME, particularly in tumor-resident immune cells, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and dendritic cells (DCs), where the encoded therapeutic products are expressed and then secreted into the tumor microenvironment to achieve an anti-tumor effect. By appropriate combination of products, the anti-tumor effect can be enhanced by virtue of interactions of the various products with the host immune system.

As discussed elsewhere herein, the immunostimulatory bacteria, containing plasmids encoding therapeutic products, with a single promoter and open reading frame (ORF), can express two (or more) proteins through the use of viral internal ribosomal entry sites (IRES), which are cap-independent, or through translational read-through of 2A peptides (*e.g.*, T2A, P2A, E2A, or F2A), and subsequent self-

cleavage into equally expressed co-proteins. Alternatively, the genetic payloads/ therapeutic products can be expressed using dual or multiple promoter constructs, where each protein is expressed under the control of a separate promoter. A combination of single and dual/multiple promoter constructs, to express three or more proteins, also can be included on the plasmids. Generally, the nucleic acids encoding the therapeutic products are under the control of RNA polymerase II promoters. For example, promoters include, but are not limited to EF-1α, CMV, SV40, UBC, CBA, PGK, GUSB, GAPDH, EIF41A, CAG, CD68, and synthetic MND promoters. The plasmids can contain other regulatory elements, such as post-transcriptional regulatory elements (PREs; *e.g.*, WPRE, HPRE), polyadenylation signal sequences, terminators, enhancers, secretion signals (also known as signal peptides/sequences, leader peptides/sequences), DNA nuclear targeting sequences (DTS), and other regulatory elements, described elsewhere herein or known to those of skill in the art that can enhance or increase the expression and/or secretion of the encoded therapeutic products.

The genetic payloads, or therapeutic products, encoded on the plasmids include immunostimulatory proteins, such as cytokines, chemokines, and co-stimulatory molecules; cytosolic DNA/RNA sensors that induce type I IFN and gain-of-function/constitutively active mutants/variants thereof; antibodies and fragments thereof; bi-specific T-cell engagers (BiTEs®); soluble TGF-β receptors that act as decoys for binding TGF-β, or TGF-β antagonizing polypeptides; IL-6 binding decoy receptors; interfering RNAs (*e.g.*, siRNA, shRNA, miRNA); and other therapeutic products as discussed below and elsewhere herein, and as known in the art; and complementary combinations of all of the preceding therapeutic products. In some embodiments, the cytokines can be encoded on the plasmid within the immunostimulatory bacteria, with a membrane anchoring motif, such as a transmembrane domain, and a collagen-binding domain.

The immunostimulatory proteins, including cytokines, chemokines, and co-stimulatory molecules, that can be encoded on the plasmids include, but are not limited to, IL-2, IL-7, IL-12p70 (IL-12p40 + IL-12p35), IL-15, IL-15/IL-15Rα chain complex, IL-18, IL-21, IL-23, IL-36 gamma, interferon-α, interferon-β, IL-2 that has attenuated binding to IL-2Ra, IL-2 that is modified so that it does not bind to IL-2Ra,

CXCL9, CXCL10, CXCL11, CCL3, CCL4, CCL5, proteins that are involved in or that effect or potentiate the recruitment and/or persistence of T-cells, CD40, CD40 Ligand (CD40L), OX40, OX40 Ligand (OX40L), 4-1BB, 4-1BB Ligand (4-1BBL), 4-1BBL with a deletion in the cytoplasmic domain (4-1BBLΔcyt), ICOS, CD27, members of the B7-CD28 family, and members of the tumor necrosis factor receptor (TNFR) superfamily. The immunostimulatory proteins also include truncated co-stimulatory molecules, such as, for example, 4-1BBL, CD80, CD86, CD27L, B7RP1 and OX40L, with a cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC), where the truncated gene product is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC due to a deleted cytoplasmic domain.

The cytosolic DNA/RNA sensors, that induce or activate type I IFN production include, but are not limited to, STING, RIG-I, MDA5, IRF3, IRF5, and IRF7, and gain-of-function (GOF) or constitutively active variants thereof. Other proteins involved in the recognition of DNA/RNA that activate type I IFN responses, that can be mutated to generate constitutive type I IFN expression and can be encoded on the plasmids, include, but are not limited to, TRIM56, RIP1, Sec5, TRAF2, TRAF3, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200.

Other therapeutic products that can be encoded on the plasmids delivered by the immunostimulatory bacteria herein, or that can be co-administered with the bacteria, that enhance or increase the anti-tumor response, include, but are not limited to, antibodies and fragments thereof, for example, TGF-β inhibitory antibodies; anti-IL-6 antibodies; antibodies against checkpoint inhibitors, such as PD-1, PD-L1, and CTLA-4; and antibodies against, or inhibitors of, VEGF, CD73, CD38, Siglec-15, EGFR, Her2, Mesothelin, and BCMA. Also contemplated for expression on the plasmid, or for co-administration with the immunostimulatory bacteria herein, are bispecific T-cell engagers (BiTEs®), IL-6 binding decoy receptors, TGF-beta binding decoy receptors, and TGF-beta polypeptide antagonists. Any of these antibodies, inhibitors or decoy receptors can be co-administered with the immunostimulatory bacteria herein. In some embodiments, PARP (poly (ADP)-ribose polymerase)

inhibitors, histone deacetylase (HDAC) inhibitors, and/or chemotherapy, also can be co-administered with any of the therapeutic products listed above, alone, or in any combination.

Exemplary of complementary combinations of therapeutic products that can be encoded on the plasmids in the immunostimulatory bacteria herein include, but are not limited to:

IL-2 and IL-12p70; IL-2 and IL-21; IL-2, IL-12p70, and a STING GOF variant; IL-2, IL-21, and a STING GOF variant; IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα and a STING GOF variant; IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-15/IL-15Rα and IL-12p70; IL-15/IL-15Rα, and IL-21; IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; IL-15/IL-15Rα, IL-21, and a STING GOF variant; IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and IL-21; IL-12p70, IL-21 and a STING GOF variant; IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-12p70 and a STING GOF variant; IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); IL-12p70 and IL-18; IL-12p70, IL-18 and a STING GOF variant; and IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-2, and IL-12p70; a TGF-β decoy receptor, IL-2, and IL-21; a TGF-β decoy receptor, IL-2, IL-12p70, and a STING GOF variant; a TGF-β decoy receptor, IL-2, IL-21, and a STING GOF variant; a TGF-β decoy receptor, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a TGF-β decoy receptor, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, and a STING GOF variant; a TGF-β decoy receptor, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-12p70; a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-21; a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21,

and a STING GOF variant; a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-12p70, and IL-21; a TGF-β decoy receptor, IL-12p70, IL-21, and a STING GOF variant; a TGF-β decoy receptor, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor and IL-12p70; a TGF-β decoy receptor, IL-12p70, and a STING GOF variant; a TGF-β decoy receptor, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a TGF-β decoy receptor, IL-12p70, and IL-18; a TGF-β decoy receptor, IL-12p70, IL-18, and a STING GOF variant; a TGF-β decoy receptor, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a TGF-β decoy receptor and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, and IL-12p70; an anti-CTLA-4 antibody, IL-2, and IL-21; an anti-CTLA-4 antibody, IL-2, IL-12p70, and a STING GOF variant; an anti-CTLA-4 antibody, IL-2, IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-12p70; an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-21; an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-12p70, and IL-21; an anti-CTLA-4 antibody, IL-12p70, IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody and IL-12p70; an anti-CTLA-4 antibody, IL-12p70, and a STING GOF

variant; an anti-CTLA-4 antibody, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); an anti-CTLA-4 antibody, IL-12p70, and IL-18; an anti-CTLA-4 antibody, IL-12p70, IL-18, and a STING GOF variant; an anti-CTLA-4 antibody, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and an anti-CTLA-4 antibody and a STING GOF variant;

a CD40 agonist, IL-2, and IL-12p70; a CD40 agonist, IL-2, and IL-21; a CD40 agonist, IL-2, IL-12p70, and a STING GOF variant; a CD40 agonist, IL-2, IL-21, and a STING GOF variant; a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, and a STING GOF variant; a CD40 agonist, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-15/IL-15Rα, and IL-12p70; a CD40 agonist, IL-15/IL-15Rα, and IL-21; a CD40 agonist, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant; a CD40 agonist, IL-15/IL-15Rα, IL-21, and a STING GOF variant; a CD40 agonist, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a CD40 agonist, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and

a CD40 agonist, IL-12p70, and IL-21; a CD40 agonist, IL-12p70, IL-21, and a STING GOF variant; a CD40 agonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist and IL-12p70; a CD40 agonist, IL-12p70, and a STING GOF variant; a CD40 agonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); a CD40 agonist, IL-12p70, and IL-18; a CD40 agonist, IL-12p70, IL-18, and a STING GOF variant; a CD40 agonist, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt); and a CD40 agonist and a STING GOF variant.

The following table lists exemplary products that can be encoded in plasmids in the immunostimulatory bacteria, and some effects/characteristics of such products, including synergistic effects of the combination of the IL-15 or IL-15 complex, discussed and demonstrated in the Examples.

| Encoded Product | Effects/Characteristics |
|---|---|
| Engineered STING - to increase Type I IFN expression | Chemokine gradients recruit T-cells.<br>Induction of Type I IFN, T-cell activation, APC tumor antigen cross-presentation.<br>Constitutive STING variants validated in SAVI patients with Type I Interferonopathies. |
| IL-12 | Strong Th1 immune response driver.<br>Produced by activated APCs, particularly M1 Macrophages and DCs*<br>Data indicate that it is a strong driver of IFN-γ |
| IL-15 and/or IL-15 complex (IL-15Rα-IL-15sc) | Stimulates T-cell and NK cell proliferation.<br>Lacks $T_{reg}$ stimulation seen with IL-2. |
| IL-21 | Pleiotropic cytokine that stimulates T-cells and NK cells.<br>Converts M2 macrophages to M1 macrophages.<br>Produced upon TLR3 activation (RNA viral sensing). |
| IL-36γ | Promotes Th1 APCs and IFN-γ production by T-cells, NK cells and γδT-cells. |
| Modified 4-1BBL | Provides T-cell co-stimulation, improves cytotoxicity and memory phenotype of T-cells; modified with deletion or truncation of the cytoplasmic domain, and addition of extra positive residues to the N-terminal truncated cytoplasmic domain. |
| Anti-CTLA-4 scFV-Fc | Enhances T-cell priming and activation against weaker tumor antigens. |
| TGF-β Decoy Receptor Trap | Binds and removes TGF-beta, relieves immunosuppression of T-cells and Th1 APCs. |

*DC = Dendritic Cells

In any of the complementary combinations above, a TGF-β decoy receptor can be replaced with a TGF-β antagonizing polypeptide. As discussed above, TGF-β decoy receptors are any that act as decoys for binding TGF-β to remove it, or are TGF-β antagonizing polypeptides (*e.g.*, anti-TGF-beta antibodies or antibody fragments, and anti-TGF-beta receptor antibodies or antibody fragments). The STING protein or other DNA/RNA sensor that induces or activates type I IFN production, can be a GOF/constitutively active variant, or can be the wild-type protein, including the modified STING polypeptides and chimeric STING polypeptides described and provided herein. Any of the complementary combinations above also can be administered in combination with any one or more of: an anti-PD-1 antibody, an anti-CTLA-4 antibody, an anti-PD-L1 antibody, an anti-IL-6 antibody, an anti-Siglec-15

antibody, an anti-VEGF antibody, an anti-CD73 antibody, an anti-CD38 antibody, an anti-EGFR antibody, an anti-Her2 antibody, an anti-Mesothelin antibody, an anti-BCMA antibody, and antibody fragments thereof, as well as PARP inhibitors, HDAC inhibitors, or chemotherapy, and combinations thereof.

Plasmids and immunostimulatory bacteria provided herein encode combinations of therapeutic payloads. These include combinations of nucleic acid encoding any or all of the products listed in the table above. Combinations of complementary payloads were assessed, and exemplary combinations and their effects are described in the Examples. The effects of various combinations of payloads on the activation of antigen-specific T-cells, and on the secretion of CXCL10 by myeloid cells, a key chemokine involved in the recruitment of anti-tumor T-cells, were assessed. For example, combinations of the payloads can induce strong secretion of CXCL10 by bone marrow dendritic cells (BMDCs). Combining IL-36γ with IL-12p70 and STING R284G tazCTT led to higher secretion of CXCL10 and IFN-γ by BMDCs. Many of the combinations induce the activation of $CD8^+$ T-cell responses (*e.g.*, 4-1BB expression), and the secretion of IFN-γ. Particular cytokine combinations can activate T-cells. For example, the combinations of IL-12p70 + IL-15; IL-12p70 + IL-15 + IFN-α2; IL-12p70 + IL-15 + anti-4-1BB agonistic antibody; IL-12p70 + IL-15 + IL-36γ; IL-12p70 + IL-15 + IL-21; IL-12p70 + IL-21 + IL-36γ; IL-12p70 + IL-36γ + IFN-α2; IL-12p70 + IL-36γ + anti-4-1BB agonistic antibody; IL-15 + IL-36γ + IFN-α2; and IL-15 + IL-36γ + anti-4-1BB agonistic antibody, result in the secretion of high levels of IFN-γ, but relatively low levels of IL-6, from T-cells, making them ideal combinations for optimal T-cell activation, for the induction of anti-tumor immunity in the tumor microenvironment.

Additionally, several combinations of cytokines (IL-15/IL-15R alpha chain complex, IL12, IL-12p70, IL-15, IL-21, and IL-36γ) and 4-1BB engagement, activate T-cells to secrete high levels of IFN-γ, with and without TCR stimulation by an anti-CD3ε agonistic antibody, for $CD4^+$ and $CD8^+$ T-cells. STING variants described herein as well as IL-12 can increase antigen specific activation of human $CD8^+$ T-cells. Data also showed, in a mouse (mu) model of colorectal carcinoma, that immunostimulatory bacterial strains, expressing IL-15, or the combination of 4-1BBLΔcyt + IL-12 more potently inhibit tumor growth inhibition that the same strains

expressing 4-1BBL(Δcyt) or IL-12 alone, and result in a high complete response rate (50% cure rate). Other combinations also were tested and shown to have potent anti-tumor activity *in vivo*.

Combinations of payloads can include a co-stimulatory molecule, such as an OX40L polypeptide, or a 4-1BBL polypeptide, or one of the cytoplasmic deleted or truncated variants thereof, and/or the modified forms thereof described and exemplified herein; or an anti-immune checkpoint antibody or fragment thereof, such as an anti-CTLA-4 scFv-Fc or an anti-CTLA-4 scFv (see, Example 25 and SEQ ID NOs:428 and 429, respectively); one or more cytokines/chemokines, such as IL-12, IL-15, IL-18, IL-21, IL-23, IL-36γ, IFN-β, IFN-α2, and CXCL10; TGF-β binding decoy receptors and other TGF-beta polypeptide antagonists, such as, for example, a human soluble TGFβ receptor II fused with a human IgG1 Fc (hu sTGFβRII-Fc; SEQ ID NO:437), anti-TGF-beta antibodies or antibody fragments, anti-TGF-beta receptor antibodies or antibody fragments, and soluble TGF-beta antagonist polypeptides; and one or more of a STING protein or a modified and/or chimeric STING protein, as described and exemplified herein.

Payloads/products/polypeptides can be encoded as a polycistronic construct, under the control of a single promoter (*i.e.*, a single promoter system) and, as required, other regulatory sequences, and also can include 2A polypeptides or other such polypeptides that result in the translation of individual products. The payloads also can be expressed on plasmids containing two separate open reading frames (ORFs), each under the control of a different promoter (*i.e.*, a dual promoter system). Exemplary combinations of payloads, in the order they are encoded on a plasmid, and including the 2A peptide that is encoded in the polycistronic construct, are set forth in the following table. The table includes the 2A peptide, but other such peptides can be substitute and/or products can be separately encoded.

**Exemplary Combinations of Products and Exemplary Order on the Plasmids**

| 1st Encoded Product | 1st 2A Peptide | 2nd Encoded Product | 2nd 2A Peptide | 3rd Encoded Product | 3rd 2A Peptide | 4th Encoded Product |
|---|---|---|---|---|---|---|
| 4-1BBL* | T2A | IL-12p70 | | | | |
| 4-1BBL* | T2A | Chimeric STING** | | | | |
| 4-1BBL* | T2A | IL-12p70 | P2A | Chimeric STING** | | |

| 1st Encoded Product | 1st 2A Peptide | 2nd Encoded Product | 2nd 2A Peptide | 3rd Encoded Product | 3rd 2A Peptide | 4th Encoded Product |
|---|---|---|---|---|---|---|
| IL-12p70 | T2A | Chimeric STING** | | | | |
| IL-12p70 | T2A | IL-15 | | | | |
| IL-12p70 | T2A | IL-21 | | | | |
| IL-12p70 | T2A | IL-15 | | | | |
| IL-12p70 | T2A | IL-21 | | | | |
| IL-21 | T2A | IL-12p70 | | | | |
| IL-12p70 | T2A | IL-36γ | | | | |
| IL-36γ | T2A | IL-12p70 | | | | |
| 4-1BBL* | T2A | IL-12p70 | P2A | IL-15 | | |
| 4-1BBL* | T2A | IL-12p70 | P2A | IL-21 | | |
| 4-1BBL* | T2A | IL-12p70 | P2A | IL-36γ | | |
| 4-1BBL* | T2A | IL-12p70 | P2A | IL-15 | T2A | Chimeric STING** |
| 4-1BBL* | T2A | IL-12p70 | P2A | IL-21 | T2A | Chimeric STING** |
| 4-1BBL* | T2A | IL-12p70 | P2A | IL-36γ | T2A | Chimeric STING** |
| IL-12p70 | T2A | IL-21 | P2A | Chimeric STING** | | |
| IL-21 | T2A | IL-12p70 | P2A | Chimeric STING** | | |
| IL-12p70 | T2A | IL-15 | P2A | Chimeric STING** | | |
| IL-12p70 | T2A | IL-36γ | P2A | Chimeric STING** | | |
| IL-36γ | T2A | IL-12p70 | P2A | Chimeric STING** | | |
| Anti-CTLA-4 scFv-Fc | T2A | IL-12p70 | | | | |
| Anti-CTLA-4 scFv-Fc | T2A | IL-12p70 | P2A | Chimeric STING** | | |
| Anti-CTLA-4 scFv-Fc | T2A | IL-12p70 | P2A | IL-15 | T2A | |
| Anti-CTLA-4 scFv-Fc | T2A | IL-12p70 | P2A | IL-21 | T2A | |
| Anti-CTLA-4 scFv-Fc | T2A | IL-12p70 | P2A | IL-36γ | T2A | |
| 4-1BBL* | T2A | sTGFβRIIFc# | | | T2A | |
| sTGFβRIIFc# | T2A | IL-12p70 | P2A | Chimeric STING** | | |
| 4-1BBL* | T2A | sTGFβRIIFc# | P2A | IL-12p70 | T2A | |
| 4-1BBL* | T2A | IL-12p70 | P2A | sTGFβRIIFc# | T2A | |
| IL-36γ | T2A | IL-23 | P2A | OX40L | | |

*4-1BBL = the modified 4-1BBL with the cytoplasmic truncation and residue modifications to render the remaining cytoplasmic domain more positive to retain correct orientation with respect to the cell membrane.

**Chimeric STING = STING with the Tasmanian Devil CTT and the replacements R284G/N154S.

sTGFβRIIFc# = type II receptor betaglycan.

Other combinations of products are contemplated (see discussions above and Examples). Other such combinations of interest include any of the modified STING proteins and a cytokine, such as a chimeric STING, such as the human chimera with the Tasmanian Devil CTT, and particularly with one or more gain-of-function mutations, such as N154S/R284G. The complementary combinations, discussed herein, can provide synergistic results. For example, the combination of the chimeric STING polypeptide and IL-15/IL-15R alpha chain complex (IL-15Rα-IL-15sc), shown in the Examples, acts synergistically to improve anti-tumor effectiveness.

The properties of the immunostimulatory bacteria provided herein, such as the accumulation in tumor-resident myeloid cells, and in the TME, and the combinations of products/payloads that can be expressed, can be selected to cover the cancer immunity cycle. Each step in the cycle, and the role of the immunostimulatory bacteria and payloads is summarized as follows:

1) Release of cancer cell antigens – the immunostimulatory bacteria accumulate in the tumor-resident myeloid cells;

2) Cancer antigen presentation – the immunostimulatory bacteria provided herein encode and express immune stimulators, such as the STING polypeptides and variants thereof, and IL-12, leading to the expression of type I interferons, including IFN-α and IFN-β;

3) Priming and activation – the immunostimulatory bacteria encode the STING polypeptides and variants thereof, and the co-stimulatory proteins, such as 4-1BBL, and IL-12;

4) Trafficking of the T-cells to the tumor – the encoded STING variants are expressed, leading to the consequent expression of IFN-α and IFN-β;

5) Infiltration of T-cells into the tumor – vascular leakage and repolarization of immunosuppressive myeloid cells;

6) Cancer cell recognition by T-cells – Type I IFN, and IFNγ, and upregulation of MHC; and

7) Killing of cancer cells – the combination of encoded cytokines/chemokines, such as IL-12, IL-15, IL-15/IL-15R alpha chain complex (IL-15Rα-IL-15sc), IL-21, and/or IL-36γ, which induce T-cell proliferation and release of IFN-γ, and the expression of a soluble TGF-β decoy receptor.

A skilled person, based on the disclosure herein and their knowledge, can identify other product payload combinations and other orders of the products as encoded on a polycistronic construct, that have immune-activating and/or immune suppressing effects, to enhance the anti-tumor activities of the immunostimulatory bacteria provided herein.

## E. IMMUNOSTIMULATORY BACTERIA AS ANTIVIRAL THERAPEUTICS AND AS THERAPEUTICS AGAINST OTHER INFECTIOUS PATHOGENS

Immunosuppression of a host immune response plays a role in many infectious diseases, and particularly, in persistent viral infection, as well as in tumor immunosuppression. In persistent infections, the virus remains in specific cells of infected individuals. There are several types of persistent virus-host interactions: latent, chronic, and slow infections. In latent infections, there is no detectable infectious virus between episodes of recurrent disease; in chronic infections, there is continued presence of infectious virus following a primary infection, and there is chronic or recurrent disease. Slow infection is characterized by a prolonged incubation period, followed by progressive disease. During persistent infections, the viral genome can be stably integrated into the cellular DNA, or can be maintained episomally. Persistent infection occurs with numerous viruses and infectious agents, including, but not limited to, human T-cell leukemia viruses, Epstein-Barr virus, cytomegalovirus, herpesviruses, varicella zoster virus, measles virus, papovaviruses, prions, hepatitis viruses (including types A, B, C, D, and E), adenoviruses, parvoviruses, coronaviruses, human immunodeficiency virus (HIV), smallpox viruses, poliovirus, influenza viruses, rotaviruses, yellow fever virus, mumps virus, rubella virus, and papillomaviruses.

Bacteria have been used as delivery vehicles for vaccines and other anti-pathogen therapeutics, and have been used as vaccines for eliciting an immune response against a peptide that is foreign to the bacterial vector. For example, an *S. typhi* TY21a strain, containing a VEGFR2-encoding plasmid, induces VEGFR2-specific T-cell responses in humans (see, *e.g.*, Schmitz-Winnenthal *et al.* (2016) *Journal of Clinical Oncology* 34(15_suppl):3091-3091, DOI: 10.1200/JCO.2016.34.15_suppl.3091). The prior approaches using bacteria as vaccine vehicles, however, have had limited clinical success. The FDA-approved bacterially-

based vaccines only are for use against other bacterial diseases, and have not effectively induced anti-tumor or antiviral immunity in humans. As discussed herein, the immunostimulatory bacteria provided herein address these problems.

One way in which antiviral vaccines work is by delivering antigens that induce antibodies that are specific for the surface glycoproteins of enveloped viruses, or that are specific for the capsid proteins of non-enveloped viruses. Antibodies are the primary element of adaptive immunity, which is designed to pre-exist at protective levels, and to be present or induced during re-exposure to a viral pathogen. Pre-existing antibodies act with the speed of innate immunity, but with more specificity, higher avidity, and targeted functionality. An immunological goal for immunization is the induction of durable protective antibody responses. Protective antibody responses generally work best when they are neutralizing and inhibit infection. Neutralization can occur by three major mechanisms. A first mechanism, aggregation or immobilization of the virus, reduces the infectious inoculum by preventing the virus from reaching the target cell. A second mechanism involves an antibody directly blocking the attachment of the virus to a target cell, by blocking the receptor-binding domain. The third mechanism, neutralization, can occur post-attachment of the virus to a target cell, by preventing entry of the virus into the cell, or by uncoating through fusion inhibition. Neutralizing antibodies need to recognize oligomeric surface proteins in their native state, or in intermediate forms that may be transiently present before completion of the fusion process. These epitopes are generally conformational, and are often quaternary epitopes, and are not often present on monomeric forms of viral proteins.

Although antibodies are recognized as the primary mechanism of vaccine-induced protective immunity, virus-specific $CD8^+$ T-cell responses also are important for controlling virus infection, and for limiting the severity of disease (see, *e.g.*, Graham (2013) *Immunol. Rev.* 255(1):230-242). Antibody-mediated effector mechanisms are for preventing infection; $CD8^+$ T-cell-mediated effector mechanisms are for recognizing and clearing virus-infected cells. Since the immunostimulatory bacteria provided herein infect myeloid cells, the immunostimulatory bacteria can enhance antigen presentation to T-cells, inducing T-cell mediated responses. Antigen-specific cytotoxic T-lymphocytes (CTLs) are a sub-group of $CD8^+$ T-cells that are

activated by interaction with antigens that are presented on the cell surface in a complex with major histocompatibility complex class I (MHC I), which is the presentation pathway for cytosolic proteins of mammalian cells. Antigens are synthesized by bacterial pathogens, or bacterial vaccines release antigens into the cytoplasm, eliciting the cellular immune response by CTLs. Following this activation, CTLs are directed against infected cells that display the relevant antigenic peptides in complex with MHC I. Antigens also can be directly presented by MHC class II molecules on APCs (antigen-presenting cells) to $CD4^+$ helper cells, which can promote B-cell production of antibodies.

A limitation of prior bacterial vaccine and anti-pathogen strategies is that they rely on bacterial sensing pathways as adjuvants, which, as described herein, do not lead to strong adaptive antiviral or anti-tumor immunity. The immunostimulatory bacteria provided herein address these limitations. The immunostimulatory bacteria provided herein provide for the priming of heterologous proteins, including viral antigens, and other antiviral therapeutics, in the context of strong viral-sensing immune pathways, leading to effective antiviral vaccination, and also, to anti-tumor treatments. As detailed herein, the immunostimulatory bacteria provided herein lack many of the bacterial TLR sensing pathways, and eliminate the skewing of immunity towards innate antibacterial responses, while retaining antiviral pathways. During viral infection, as well as in subjects with cancer, innate immune signaling pathways, including toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and cytoplasmic DNA sensors, are activated for the synthesis of antiviral molecules, such as type I interferons (IFNs) and pro-inflammatory cytokines. The immunostimulatory bacteria provided herein are designed to stimulate such pathways by virtue of the genomic modifications, and also, the encoded therapeutic cargo(es), such as the STING proteins and modified STING proteins, cytokines, and chemokines.

The immunostimulatory bacteria provided herein advantageously, by virtue of genomic modifications, and, optional encoded therapeutic products, can direct the host immune response to be antiviral in nature. The immunostimulatory bacteria provided herein, as discussed, infect myeloid cells to deliver encoded anti-pathogen therapeutic products, such as antigens, antibodies, and other encoded therapeutics, and

can encode additional products, such as immunostimulatory proteins, to enhance a host's immune response. Thus, the immunostimulatory bacteria can provide a variety of attacks on pathogens.

The immunostimulatory bacteria provided herein, in subjects who have a tumor, accumulate in tumor-resident immune cells. In subjects who do not have a tumor, the bacteria primarily, or only, infect phagocytic antigen-presenting cells (APCs), which are cells that are very capable of transferring plasmids that can be read by the eukaryotic machinery, and produce proteins in their natural context. The plasmids encode single or multiple heterologous proteins, including, for example, viral antigens and other antiviral therapeutics, and also, can encode single or multiplexed immune payloads that induce antiviral immune-sensing pathways. The result is proper APC activation and antigen priming of T-cells, leading to durable humoral and cellular antiviral immunity, even in chronic infection environments, where suppressive myeloid cells prevent adaptive immunity.

Hence, provided herein are immunostimulatory bacteria that deliver protein antigens and antibodies, and other therapeutics for vaccination against pathogens, or for the treatment of resulting infections, including persistent viral infections. The pathogens include bacteria, protozoans, viruses, and prions, and other prion-like particles that cause diseases and disorders. Among the immunostimulatory bacteria, and exemplary of such bacteria, are strains/species of *Salmonella*, such as *S. typhimurium*. The immunostimulatory bacteria can encode the anti-pathogen products under control of a bacterial or a eukaryotic promoter. The promoter can be constitutive or can be inducible. Selection of the promoter, and other regulatory sequences, depends upon factors that include the particular product, the timing of expression of the product, and the use of the bacteria, such as whether they are for the treatment of an existing infection, or are for vaccination. The bacteria, which generally are *asd*$^-$, can encode *asd* on the plasmid to permit replication upon infection of the host cells with the bacteria, without any need for an antibiotic selection cassette. This permits the bacteria to replicate *in vivo*. As an alternative, a suicide system can be used to deliver payloads, (*i.e.*, therapeutic products) to tissue-resident myeloid cells. In this context, the bacteria are dosed *in vivo* using an *asd*$^-$ strain that lacks a plasmid containing a complementing *asd* gene cassette. *In vitro*,

diaminopimelic acid (DAP) is added to facilitate bacterial replication in the absence of a functional *asd* gene; such bacteria cannot grow *in vivo*. One bolus of the bacterial nucleic acid is delivered to tissue-resident myeloid cells after phagocytosis and intracellular destruction of the bacteria.

The immunostimulatory bacteria provided herein, including *Salmonella*, specifically target myeloid cells, including antigen-presenting cells (APCs), in the infected organism. APCs can migrate into lymph nodes, the spleen, and liver, providing presentation of expressed antigens to lymph node resident T-cells. The bacteria can be taken up by dendritic cells, which then express the antigens for the presentation pathway by MHC I and MHC II, and in turn, initiate specific T-cell responses. The immunostimulatory bacteria provided herein can exploit these pathways when used as vaccines, or to encode viral antigens and/or other proteins to protect against pathogens, such as influenza viruses, Ebola virus, Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19), *Porphyromonas gingivalis* (*P. gingivalis*), and others as set forth herein and/or as known to those of skill in the art. Targets include proteins, such as the spike protein of the coronaviruses (see, *e.g.*, SEQ ID NO:438), and ginigpain protease of *Porphyromonas gingivalis* (see, *e.g.*, SEQ ID NOs: 442-447), particularly the lysine-gingipain protease (Kgp or Lys-gingipain) from *P. gingivalis* strain ATCC 332277 (see, *e.g.*, SEQ ID NO:444), and lysine-gingipain proteases from other strains of *Porphyromonas gingivalis*, including strains 83, FDC381, and HG66 (see, *e.g.*, SEQ ID NOs: 445-447, respectively). Bacterial pathogens include, for example, species of *Escherichia, Klebsiella*, *Staphylococcus*, *Acinetobacter*, and *Pseudomonas*, particularly drug-resistant species and strains. Those of skill in the art readily can identify antigens to target. For anti-tumor applications, tumor antigens (see, *e.g.*, the Examples for exemplary tumor antigens), and proteases, reverse transcriptases (for RNA viruses), and DNA polymerases, and other replicative enzymes, can be targets. Exemplary species of pathogenic bacteria, include, but are not limited to: *E. coli*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *E. faecalis*, and *S. pneumoniae*.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel beta-coronavirus that is the causative agent of the coronavirus disease 2019 (COVID-19) pandemic. Coronavirus virions contain a self surface spike (S) glycoprotein that binds to host cell angiotensin-converting enzyme 2 (ACE2) receptors, and mediates entry via fusion of the host and viral membranes. The binding of the SARS-CoV-2 spike protein to the ACE2 receptor causes a larger conformational rearrangement of spike protein, from a metastable pre-fusion conformation, to a highly stable post-fusion conformation, which facilitates membrane fusion. Because it mediates cell attachment and entry, the spike (S) protein is essential for the viral life cycle, and is a primary target for neutralizing antibodies, and is a critical vaccine antigen (see, *e.g.*, Hsieh *et al.* (2020) *Science* 369:1501-1505).

SARS-COV2 virus proteins that draw the best or good $CD8^+$, include the following (see, Cohen *et al.* (2021) *Cell. Rep. Med. 2*:100354):

| **SARS-CoV-2 protein** | **CD8 T-cell response rate** | **SEQ ID NO** |
|---|---|---|
| S1 | 25% | 438 (full length spike protein); 439 (glycoprotein gene) |
| S2 | 12% | 438 (full length spike protein); 439 (glycoprotein gene) |
| Envelope (E) | 9% | 450 (gene); 451 (protein) |
| Membrane (M) | | 452 (gene); 453 (protein) |
| Nucleocapsid (N) | 57% | 462 (gene); 463 (protein) |
| ORF3a | 16% | 448 (gene); 449 (protein) |
| ORF6 | | 454 (gene); 455 (protein) |
| ORF7a | 25% | 456 (gene); 457 (protein) |
| ORF7b | | 458 (gene); 459 (protein) |
| ORF8 | | 460 (gene); 461 (protein) |

The receptor-binding domain (RBD; see, *e.g.*, SEQ ID NO:440) in the S1 subunit of the spike protein interacts directly with ACE2. The metastable pre-fusion conformation of the S protein may be referred to as "laying or down," while the conformational rearrangement into an ACE2-feasible conformation may be referred to as "up or standing." The "down" and "up" poses are differentiated based on the conformational rearrangement of the RBD; the down-to-up rearrangement facilitates receptor binding, while the rearrangement of up-to-down helps the virus to evade

immune surveillance (see, *e.g.*, Shah *et al.* (2020) *Computational and Structural Biotechnology Journal* 18:3401-3414).

Binding to the ACE2 receptor is followed by cleavage with transmembrane protease serine 2 (TMPRSS2). Both ACE2 and TMPRSS2 are abundantly expressed in the airways, lungs, and nasal/oral mucosa, as well as in the intestines. Naturally occurring mutations in the S protein have been identified in China (H49Y), Europe (V367F and D614G), and the United States (G476S and V483A), and have been studied for their effects on cell entry of SARS-CoV-2; in cells expressing ACE2 and TMPRSS2, the G476S mutation results in reduced cell entry, and the V483A mutation has no effect on cell entry, whereas the D614G, V367F, and H49Y mutations result in enhanced cell entry compared to that of the wild-type S protein. The D614G mutation displays a 3.5-fold higher level of entry activity compared to the wild-type S protein, an effect that is also seen in human small airway epithelial cells. The D614G mutation also results in increased binding affinity for the ACE2 receptor. Furthermore, the D614G mutant retains the neutralization sensitivity of the SARS-CoV-2 virus; anti-SARS-CoV-2 patient sera was able to effectively neutralize both wild-type SARS-CoV-2 and SARS-CoV-2 with the D614G mutation (see, *e.g.*, Ozono *et al.* (2021) *Nature Communications* 12:848). The S protein exists as a trimer, and structural analyses indicate that the D614G mutation stabilizes, and thus, prevents premature dissociation of the S trimer (see, *e.g.*, Zhang *et al.* (2021) *Science* 372:525-530). The V367F mutant also displays higher binding affinity to ACE2 compared to the reference strain Wuhan-Hu-1, as first isolated in Wuhan in December 2019, which may be due to stabilization of the RBD structure. Virus isolates also have been identified with both the V367F and D614G mutations. Phylogenetic analyses suggest that the V367F mutation evolved along with the D614G mutation, suggesting a synergistic effect of increased infectivity (see, *e.g.*, Ou *et al.* (2021) *bioRvix*, doi:10.1101/2020.03.15.991844).

The N501Y mutation has naturally emerged in SARS-CoV-2 lineages in the UK (B.1.1.7, or 20B/501Y.v1) and South Africa (B.1.351, or 20C/501Y.v2), and occurs at the human ACE2 binding site on the RBD of the SARS-CoV-2 S protein. Molecular dynamic stimulations reveal that N501 in the SARS-CoV-2 S protein RBD is in the proximity of hydrophobic residues of human ACE2; thus, the mutation of

hydrophilic N501 into a hydrophobic residue can improve interactions between the S protein and human ACE2. Experimental screens suggest that mutation of N501 into V, F, W, or Y can enhance binding of the RBD with human ACE2. These results have been validated in an *in vitro* study, showing that the mutations N501Y, N501F, N501W, and N501V result in enhanced binding affinity between the RBD of the spike protein and human ACE2 (see, *e.g.*, Luan *et al.* (2021) *FEBS Letters*, doi:10.1002/1873-3468.14076).

A mutant of the SARS-CoV-2 S-2P variant spike protein (see, *e.g.*, Hsieh *et al.* (2020) *Science* 369:1501-1505), with the proline substitutions F817P, A892P, A899P, A942P, K986P, and V987P, designated "HexaPro," displayed increased expression levels and stability compared to its parental construct. The high yield and increased stability of the HexaPro mutant should allow for the industrial production of subunit vaccines; it can also improve DNA- or mRNA-based vaccines by producing more antigen per nucleic acid molecule, improving the efficacy and/or reducing the dosage required (see, *e.g.*, Hsieh *et al.* (2020) *Science* 369:1501-1505).

Other mutations in the SARS-CoV-2 spike protein, that can enhance or improve its expression and/or its binding to the ACE2 receptor, include, for example, V417K (see, *e.g.*, Shah *et al.* (2020) *Computational and Structural Biotechnology Journal* 18:3402-3414); G502D, N501T, and Q498Y, as well as mutations in the residues N439/R426, L452/R426, T470/N457, E484/P470, Q498/Y484 and N501/T487 (see, *e.g.*, Verkhivker *et al.* (2021) bioRxiv preprint; doi: 10.1101/2021.02.21.432165); and W436R and D364Y (see, *e.g.*, Ou *et al.* (2021) *bioRvix*, doi:10.1101/2020.03.15.991844; and U.S.Patent No. 10, 973,908).

The immunostimulatory bacteria provided herein, when used as vaccines, or to encode viral antigens and/or other proteins to protect against pathogens, such as Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19), can encode the full length wild-type SARS-CoV-2 spike protein (see, *e.g.*, SEQ ID NO:438), or can encode the full length receptor binding domain (RBD) of the SARS-CoV-2 spike protein (see, SEQ ID NO:440), or can encode a portion of the spike protein RBD that is sufficient to induce or cause an immune response, and/or to immunize or vaccinate or protect a subject against SARS-CoV-2. The immunostimulatory bacteria also can encode mutants of the SARS-CoV-2 spike

protein, with mutations in the spike protein or a portion thereof, such as the RBD or a portion thereof, that increase or enhance the expression of the spike protein or RBD or portions thereof, and/or that increase or enhance the binding of the spike protein or RBD, or the portions thereof to the ACE2 receptor. The spike protein or spike protein RBD mutants include any described herein and known in the art, including, but not limited to, those comprising the mutations V367F, D614G, G476S, V483A, H49Y, N501Y, N501F, N501W, N501V, F817P, A892P, A899P, A942P, K986P, V987P, V417K, G502D, N501T, Q498Y, W436R and D364Y, and those comprising mutations at residues corresponding to residues N439/R426, L452/R426, T470/N457, E484/P470, Q498/Y484 and N501/T487 of the spike protein or portion thereof. The immunostimulatory bacteria can be used as vaccines against SARS-CoV-2 by encoding any of the antigenic sequences or modified forms of the spike protein or RBD, including portions thereof, described, for example, in U.S. Patent Nos. 10,973,908 and 10,702,600. The immunostimulatory bacteria also can be used as vaccines, to deliver the mRNA sequences utilized in the Pfizer-BioNTech COVID-19 vaccine, or in the Moderna COVID-19 vaccine, wherein:

1) the sequence of the mRNA in the Pfizer-BioNTech COVID-19 vaccine, as released by the World Health Organization, includes a 5'-capping structure, a 5' untranslated region (UTR), the extended signal sequence of the S glycoprotein, nucleic acid encoding the full-length spike (S) glycoprotein sequence containing the mutations K986P and V987P, and a poly(A) tail, and comprises the following sequence:

GAGAAΨAAAC ΨAGΨAΨΨCΨΨ CΨGGΨCCCCA CAGACΨCAGA GAGAACCCGC 50
CACCAΨGΨΨC GΨGΨΨCCΨGG ΨGCΨGCΨGCC ΨCΨGGΨGΨCC AGCCAGΨGΨG 100
ΨGAACCΨGAC CACCAGAACA CAGCΨGCCΨC CAGCCΨACAC CAACAGCΨΨΨ 150
ACCAGAGGCG ΨGΨACΨACCC CGACAAGGΨG ΨΨCAGAΨCCA GCGΨGCΨGCA 200
CΨCΨACCCAG GACCΨGΨΨCC ΨGCCΨΨΨCΨΨ CAGCAACGΨG ACCΨGGΨΨCC 250
ACGCCAΨCCA CGΨGΨCCGGC ACCAAΨGGCA CCAAGAGAΨΨ CGACAACCCC 300
GΨGCΨGCCCΨ ΨCAACGACGG GGΨGΨACΨΨΨ GCCAGCACCG AGAAGΨCCAA 350
CAΨCAΨCAGA GGCΨGGAΨCΨ ΨCGGCACCAC ACΨGGACAGC AAGACCCAGA 400
GCCΨGCΨGAΨ CGΨGAACAAC GCCACCAACG ΨGGΨCAΨCAA AGΨGΨGCGAG 450
ΨΨCCAGΨΨCΨ GCAACGACCC CΨΨCCΨGGGC GΨCΨACΨACC ACAAGAACAA 500
CAAGAGCΨGG AΨGGAAAGCG AGΨΨCCGGGΨ GΨACAGCAGC GCCAACAACΨ 550
GCACCΨΨCGA GΨACGΨGΨCC CAGCCΨΨΨCC ΨGAΨGGACCΨ GGAAGGCAAG 600
CAGGGCAACΨ ΨCAAGAACCΨ GCGCGAGΨΨC GΨGΨΨΨAAGA ACAΨCGACGG 650
CΨACΨΨCAAG AΨCΨACAGCA AGCACACCCC ΨAΨCAACCΨC GΨGCGGGAΨC 700
ΨGCCΨCAGGG CΨΨCΨCΨGCΨ CΨGGAACCCC ΨGGΨGGAΨCΨ GCCCAΨCGGC 750
AΨCAACAΨCA CCCGGΨΨΨCA GACACΨGCΨG GCCCΨGCACA GAAGCΨACCΨ 800

GACACCΨGGC GAΨAGCAGCA GCGGAΨGGAC AGCΨGGΨGCC GCCGCΨΨACΨ 850
AΨGΨGGGCΨA CCΨGCAGCCΨ AGAACCΨΨCC ΨGCΨGAAGΨA CAACGAGAAC 900
GGCACCAΨCA CCGACGCCGΨ GGAΨΨGΨGCΨ CΨGGAΨCCΨC ΨGAGCGAGAC 950
AAAGΨGCACC CΨGAAGΨCCΨ ΨCACCGΨGGA AAAGGGCAΨC ΨACCAGACCA 1000
GCAACΨΨCCG GGΨGCAGCCC ACCGAAΨCCA ΨCGΨGCGGΨΨ CCCCAAΨAΨC 1050
ACCAAΨCΨGΨ GCCCCΨΨCGG CGAGGΨGΨΨC AAΨGCCACCA GAΨΨCGCCΨC 1100
ΨGΨGΨACGCC ΨGGAACCGGA AGCGGAΨCAG CAAΨΨGCGΨG GCCGACΨACΨ 1150
CCGΨGCΨGΨA CAACΨCCGCC AGCΨΨCAGCA CCΨΨCAAGΨG CΨACGGCGΨG 1200
ΨCCCCΨACCA AGCΨGAACGA CCΨGΨGCΨΨC ACAAACGΨGΨ ACGCCGACAG 1250
CΨΨCGΨGAΨC CGGGGAGAΨG AAGΨGCGGCA GAΨΨGCCCCΨ GGACAGACAG 1300
GCAAGAΨCGC CGACΨACAAC ΨACAAGCΨGC CCGACGACΨΨ CACCGGCΨGΨ 1350
GΨGAΨΨGCCΨ GGAACAGCAA CAACCΨGGAC ΨCCAAAGΨCG GCGGCAACΨA 1400
CAAΨΨACCΨG ΨACCGGCΨGΨ ΨCCGGAAGΨC CAAΨCΨGAAG CCCΨΨCGAGC 1450
GGGACAΨCΨC CACCGAGAΨC ΨAΨCAGGCCG GCAGCACCCC ΨΨGΨAACGGC 1500
GΨGGAAGGCΨ ΨCAACΨGCΨA CΨΨCCCACΨG CAGΨCCΨACG GCΨΨΨCAGCC 1550
CACAAAΨGGC GΨGGGCΨAΨC AGCCCΨACAG AGΨGGΨGGΨG CΨGAGCΨΨCG 1600
AACΨGCΨGCA ΨGCCCCΨGCC ACAGΨGΨGCG GCCCΨAAGAA AAGCACCAAΨ 1650
CΨCGΨGAAGA ACAAAΨGCGΨ GAACΨΨCAAC ΨΨCAACGGCC ΨGACCGGCAC 1700
CGGCGΨGCΨG ACAGAGAGCA ACAAGAAGΨΨ CCΨGCCAΨΨC CAGCAGΨΨΨG 1750
GCCGGGAΨAΨ CGCCGAΨACC ACAGACGCCG ΨΨAGAGAΨCC CCAGACACΨG 1800
GAAAΨCCΨGG ACAΨCACCCC ΨΨGCAGCΨΨC GGCGGAGΨGΨ CΨGΨGAΨCAC 1850
CCCΨGGCACC AACACCAGCA AΨCAGGΨGGC AGΨGCΨGΨAC CAGGACGΨGA 1900
ACΨGΨACCGA AGΨGCCCGΨG GCCAΨΨCACG CCGAΨCAGCΨ GACACCΨACA 1950
ΨGGCGGGΨGΨ ACΨCCACCGG CAGCAAΨGΨG ΨΨΨCAGACCA GAGCCGGCΨG 2000
ΨCΨGAΨCGGA GCCGAGCACG ΨGAACAAΨAG CΨACGAGΨGC GACAΨCCCCA 2050
ΨCGGCGCΨGG AAΨCΨGCGCC AGCΨACCAGA CACAGACAAA CAGCCCΨCGG 2100
AGAGCCAGAA GCGΨGGCCAG CCAGAGCAΨC AΨΨGCCΨACA CAAΨGΨCΨCΨ 2150
GGGCGCCGAG AACAGCGΨGG CCΨACΨCCAA CAACΨCΨAΨC GCΨAΨCCCCA 2200
CCAACΨΨCAC CAΨCAGCGΨG ACCACAGAGA ΨCCΨGCCΨGΨ GΨCCAΨGACC 2250
AAGACCAGCG ΨGGACΨGCAC CAΨGΨACAΨC ΨGCGGCGAΨΨ CCACCGAGΨG 2300
CΨCCAACCΨG CΨGCΨGCAGΨ ACGGCAGCΨΨ CΨGCACCCAG CΨGAAΨAGAG 2350
CCCΨGACAGG GAΨCGCCGΨG GAACAGGACA AGAACACCCA AGAGGΨGΨΨC 2400
GCCCAAGΨGA AGCAGAΨCΨA CAAGACCCCΨ CCΨAΨCAAGG ACΨΨCGGCGG 2450
CΨΨCAAΨΨΨC AGCCAGAΨΨC ΨGCCCGAΨCC ΨAGCAAGCCC AGCAAGCGGA 2500
GCΨΨCAΨCGA GGACCΨGCΨG ΨΨCAACAAAG ΨGACACΨGGC CGACGCCGGC 2550
ΨΨCAΨCAAGC AGΨAΨGGCGA ΨΨGΨCΨGGGC GACAΨΨGCCG CCAGGGAΨCΨ 2600
GAΨΨΨGCGCC CAGAAGΨΨΨA ACGGACΨGAC AGΨGCΨGCCΨ CCΨCΨGCΨGA 2650
CCGAΨGAGAΨ GAΨCGCCCAG ΨACACAΨCΨG CCCΨGCΨGGC CGGCACAAΨC 2700
ACAAGCGGCΨ GGACAΨΨΨGG AGCAGGCGCC GCΨCΨGCAGA ΨCCCCΨΨΨGC 2750
ΨAΨGCAGAΨG GCCΨACCGGΨ ΨCAACGGCAΨ CGGAGΨGACC CAGAAΨGΨGC 2800
ΨGΨACGAGAA CCAGAAGCΨG AΨCGCCAACC AGΨΨCAACAG CGCCAΨCGGC 2850
AAGAΨCCAGG ACAGCCΨGAG CAGCACAGCA AGCGCCCΨGG GAAAGCΨGCA 2900
GGACGΨGGΨC AACCAGAAΨG CCCAGGCACΨ GAACACCCΨG GΨCAAGCAGC 2950
ΨGΨCCΨCCAA CΨΨCGGCGCC AΨCAGCΨCΨG ΨGCΨGAACGA ΨAΨCCΨGAGC 3000
AGACΨGGACC CΨCCΨGAGGC CGAGGΨGCAG AΨCGACAGAC ΨGAΨCACAGG 3050
CAGACΨGCAG AGCCΨCCAGA CAΨACGΨGAC CCAGCAGCΨG AΨCAGAGCCG 3100
CCGAGAΨΨAG AGCCΨCΨGCC AAΨCΨGGCCG CCACCAAGAΨ GΨCΨGAGΨGΨ 3150
GΨGCΨGGGCC AGAGCAAGAG AGΨGGACΨΨΨ ΨGCGGCAAGG GCΨACCACCΨ 3200
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CAΨAΨGΨGCC CGCΨCAAGAG AAGAAΨΨΨCA CCACCGCΨCC AGCCAΨCΨGC 3300
CACGACGGCA AAGCCCACΨΨ ΨCCΨAGAGAA GGCGΨGΨΨCG ΨGΨCCAACGG 3350
CACCCAΨΨGG ΨΨCGΨGACAC AGCGGAACΨΨ CΨACGAGCCC CAGAΨCAΨCA 3400
CCACCGACAA CACCΨΨCGΨG ΨCΨGGCAACΨ GCGACGΨCGΨ GAΨCGGCAΨΨ 3450

GΨGAACAAΨA CCGΨGΨACGA CCCΨCΨGCAG CCCGAGCΨGG ACAGCΨΨCAA 3500
AGAGGAACΨG GACAAGΨACΨ ΨΨAAGAACCA CACAAGCCCC GACGΨGGACC 3550
ΨGGGCGAΨAΨ CAGCGGAAΨC AAΨGCCAGCG ΨCGΨGAACAΨ CCAGAAAGAG 3600
AΨCGACCGGC ΨGAACGAGGΨ GGCCAAGAAΨ CΨGAACGAGA GCCΨGAΨCGA 3650
CCΨGCAAGAA CΨGGGGAAGΨ ACGAGCAGΨA CAΨCAAGΨGG CCCΨGGΨACA 3700
ΨCΨGGCΨGGG CΨΨΨAΨCGCC GGACΨGAΨΨG CCAΨCGΨGAΨ GGΨCACAAΨC 3750
AΨGCΨGΨGΨΨ GCAΨGACCAG CΨGCΨGΨAGC ΨGCCΨGAAGG GCΨGΨΨGΨAG 3800
CΨGΨGGCAGC ΨGCΨGCAAGΨ ΨCGACGAGGA CGAΨΨCΨGAG CCCGΨGCΨGA 3850
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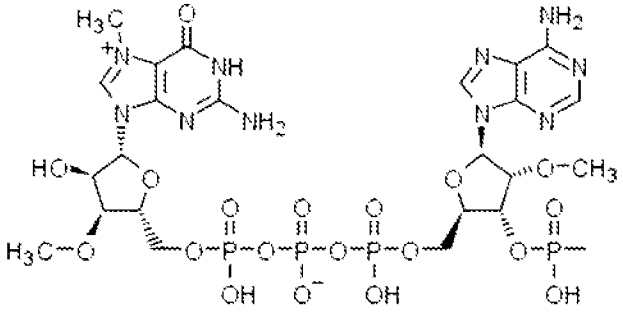
CGCAAΨGCΨA GCΨGCCCCΨΨ ΨCCCGΨCCΨG GGΨACCCCGA GΨCΨCCCCCG 3950
ACCΨCGGGΨC CCAGGΨAΨGC ΨCCCACCΨCC ACCΨGCCCCA CΨCACCACCΨ 4000
CΨGCΨAGΨΨC CAGACACCΨC CCAAGCACGC AGCAAΨGCAG CΨCAAAACGC 4050
ΨΨAGCCΨAGC CACACCCCCA CGGGAAACAG CAGΨGAΨΨAA CCΨΨΨAGCAA 4100
ΨAAACGAAAG ΨΨΨAACΨAAG CΨAΨACΨAAC CCCAGGGΨΨG GΨCAAΨΨΨCG 4150
ΨGCCAGCCAC ACCCΨGGAGC ΨAGCAAAAAA AAAAAAAAAA AAAAAAAAAA 4200
AAAAGCAΨAΨ GACΨAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 4250
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAA 4284

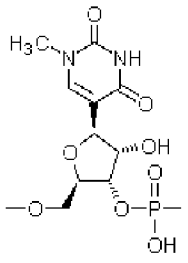
where Ψ = 1-methyl-3'-pseudouridylyl, and the sequence has the following features:

| Element | Description | Nucleotide Position |
|---|---|---|
| cap | A modified 5'-cap1 structure ($m^7G^+m^{3'}$-5'-ppp-5'-Am) | 1-2 |
| 5'-UTR | 5′-untranslated region derived from human alpha-globin RNA with an optimized Kozak sequence | 3-54 |
| sig | S glycoprotein signal peptide (extended leader sequence), which guides translocation of the nascent polypeptide chain into the endoplasmic reticulum. | 55-102 |
| S protein_mut | Codon-optimized sequence encoding full-length SARS-CoV-2 spike (S) glycoprotein containing mutations K986P and V987P to ensure the S glycoprotein remains in an antigenically optimal pre-fusion conformation; stop codons: 3874-3879 (underlined) | 103-3879 |
| 3'-UTR | The 3′ untranslated region comprises two sequence elements derived from the amino-terminal enhancer of split (AES) mRNA and the mitochondrial encoded 12S ribosomal RNA to confer RNA stability and high total protein expression. | 3880-4174 |
| poly(A) | A 110-nucleotide poly(A)-tail consisting of a stretch of 30 adenosine residues, followed by a 10-nucleotide linker sequence and another 70 adenosine residues. | 4175-4284 |

and the structure of the 5' capping structure is:



and the structure of m1Ψ = 1-methyl-3'-pseudouridylyl is:



; and

2) the sequence of the Moderna COVID-19 vaccine is available, from, *e.g.*, github.com/NAalytics/Assemblies-of-putative-SARS-CoV2-spike-encoding-mRNA-sequences-for-vaccines-BNT-162b2-and-mRNA-1273/blob/main/Assemblies%20of%20putative%20SARS-CoV2-spike-encoding%20mRNA%20sequences%20for%20vaccines%20BNT-162b2%20and%20mRNA-1273.docx.pdf.

Alternatively the sequence for the mRNA in the Pfizer-BioNTech COVID-19 vaccine, as available from github.com/NAalytics/Assemblies-of-putative-SARS-CoV2-spike-encoding-mRNA-sequences-for-vaccines-BNT-162b2-and-mRNA-1273/blob/main/Assemblies%20of%20putative%20SARS-CoV2-spike-encoding%20mRNA%20sequences%20for%20vaccines%20BNT-162b2%20and%20mRNA-1273.docx.pdf, can be used.

The immunostimulatory bacteria provided herein also can encode antigens to protect against and/or to treat tick-borne and other insect-borne diseases, and other infectious agents, such as prions and malarial pathogens, such as *Plasmodium falciparum*, as well as bacterial and viral pathogens. The immunostimulatory bacteria also can be used as vaccines against bacterial and protozoan pathogens, including *Plasmodium*, *C. difficile*, and *Listeria. Salmonella* and other bacteria have been used

for vaccines; any such known vaccine can be improved by encoding the antigen(s) in the immunostimulatory bacteria provided herein.

The immunostimulatory bacteria provided herein can induce immunity or an immune response to pathogens, particularly viral pathogens, resulting in effective antibody and T-cell responses. The immunostimulatory bacteria, such as the *Salmonella* bacteria, include genomic modifications so that they primarily or solely infect myeloid cells, and deliver plasmids that encode therapeutic products, such as antigens and antibodies, as well as additional immune-stimulating products that stimulate or induce an antiviral immune response. The immunostimulatory bacteria infect these antigen-presenting cells, where the encoded therapeutic product(s) are expressed.

The immunostimulatory bacteria can encode antigens, which result in the immune system of the host producing antibodies, including antibodies that recognize a virus or bacterium or other pathogen before it has infected a cell. The immunostimulatory bacteria for use as antivirals not only can encode antigens, but they can encode antibodies and immunostimulatory proteins to enhance a host's antiviral response. For use as vaccines, the immunostimulatory bacteria, by encoding antigens and other heterologous proteins, result in immunity or result in reduced severity of disease, or prevent the recurrence of persistent viral infections. Encoded antibodies include single-chain forms thereof, such as single-chain variable fragments (scFvs), nanobodies, and other such structures. The immunostimulatory properties of the bacteria, including those derived from encoding immunostimulatory proteins (described herein), such as STING, that enhance or promote an antiviral immune response, and also, the genomic modifications of the bacteria, such as the modifications that eliminate or reduce expression of asparaginase II, which enhance T-cell responses, result in a robust anti-pathogen therapeutic, including activation of type I IFN. As discussed herein, inactivated pathogen vaccines, such as the BCG vaccine for tuberculosis, can be improved by modifying the pathogen to inactivate or eliminate asparaginase activity. Asparaginase can reduce or inhibit the immune response to the vaccine; eliminating or reducing the activity of asparaginase can improve the effectiveness of a vaccine, such as the BCG vaccine.

## F. CONSTRUCTING EXEMPLARY PLASMIDS ENCODING THERAPEUTIC PRODUCTS FOR BACTERIAL DELIVERY

The immunostimulatory bacteria herein can be modified to encode one or more therapeutic products, including immunomodulatory proteins, that promote, or induce, or enhance an anti-tumor response. The therapeutic product can be encoded on a plasmid in the bacterium, under the control of a eukaryotic promoter, such as a promoter recognized by RNA polymerase II, for expression in a eukaryotic subject, particularly the subject for whom the immunostimulatory bacterium is to be administered, such as a human. The nucleic acid encoding the therapeutic product(s) can include, in addition to the eukaryotic promoter, other regulatory signals for expression or trafficking in the cells, such as for secretion or expression on the surface of a cell. Immunostimulatory proteins are those that, in the appropriate environment, such as a tumor microenvironment (TME), can promote, or participate in, or enhance an anti-tumor response in the subject to whom the immunostimulatory bacterium is administered. Immunostimulatory proteins include, but are not limited to, cytokines, chemokines, and co-stimulatory molecules. These include cytokines, such as, but not limited to, IL-2, IL-7, IL-12, IL-12p70 (IL-12p40 + IL-12p35), IL-15, IL-15/IL-15Rα chain complex, IL-18, IL-21, IL-23, IL-36γ, IL-2 that has attenuated binding to IL-2Ra, IL-2 that is modified so that it does not bind to IL-2Ra, IFN-α, and IFN-β; chemokines, such as, but not limited to, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11; proteins that are involved in or that effect or potentiate the recruitment and/or persistence of T-cells; and/or co-stimulatory molecules, such as, but not limited to, CD40, CD40L, OX40, OX40L, 4-1BB, 4-1BBL, 4-1BBL with a deletion in the cytoplasmic domain (4-1BBLΔcyt), ICOS, ICOS ligand, CD27, CD27 ligand, CD80, CD86, members of the TNF/TNFR superfamily and members of the B7-CD28 family. Other such immunostimulatory proteins that are used for treatment of tumors or that can promote, enhance, or otherwise increase or evoke an anti-tumor response, known to those of skill in the art, are contemplated for encoding in the immunostimulatory bacteria provided herein.

Other therapeutic products, encoded by the immunostimulatory bacteria herein, include cytosolic DNA/RNA sensors that induce or activate type I interferon production, including STING, MDA5, RIG-I, IRF3, and IRF7, as well as gain-of-

function and constitutively active variants thereof, and the chimeric STING polypeptides as described herein. For example, the constitutively active STING variants include those with the mutations V147L, N154S, V155M, C206Y, R281Q, and/or R284G, and combinations thereof, such as N154S/R284G, and others described herein and known in the art, while the constitutively active IRF3 variants include those with the mutations S396D, S398D, S402D, T404D, and/or S405D, and others described herein and known in the art. Other therapeutic products, encoded by the immunostimulatory bacteria herein, include antibodies and antibody fragments, including single-chain fragment variables (scFvs), Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fv fragments, disulfide-linked Fvs (dsFvs), Fd fragments, Fd' fragments, single-chain Fabs (scFabs), diabodies, anti-idiotypic (anti-Id) antibodies, synthetic antibodies, recombinantly produced antibodies, multi-specific antibodies (*e.g.*, bi-specific antibodies), human antibodies, non-human antibodies, humanized antibodies, chimeric antibodies, and intrabodies, or antigen-binding fragments of any of the above. The antibodies can be directed against immune checkpoints, such as PD-1, PD-L1, CTLA-4, IDO 1 and 2, CTNNB1 (β-catenin), SIRPα, VISTA, and TREX-1, and others known in the art or described herein, or can be directed against other targets, such as TGF-β, VEGF, HER2, EGFR, STAT3, and IL-6, and other such targets whose inhibition improves the anti-tumor response. The immunostimulatory bacteria also can encode RNAi, such as siRNA (shRNA and miRNA) against immune checkpoints, such as TREX1, and other targets whose inhibition, suppression, or disruption improves the anti-tumor response.

In some embodiments, the immunostimulatory bacteria herein are engineered to encode one or more cytokines to stimulate the immune system, including, but not limited to, IL-2, IL-7, IL-12 (IL-12p70 (IL-12p40 + IL-12p35)), IL-15 (and the IL-15:IL-15R alpha chain complex (IL-15/IL-Rα)), IL-18, IL-21, IL-23, IL-36 gamma, IFN-alpha and IFN-beta. Cytokines stimulate immune effector cells and stromal cells at the tumor site, and enhance tumor cell recognition by cytotoxic cells. In some embodiments, the immunostimulatory bacteria can be engineered to encode chemokines, such as, for example, one or more of CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11. Complementary combinations of any of the therapeutic products can be encoded and delivered to the tumor microenvironment, to enhance the

anti-tumor efficacy of the immunostimulatory bacteria. These modifications, and the immunostimulatory bacteria encoding them, are discussed above, and are exemplified below.

**1. Constitutive Promoters for Heterologous Expression of Proteins**

Plasmids provided herein are designed to encode a therapeutic product, such as an immunostimulatory protein, that, when expressed in a mammalian subject, confers or contributes to anti-tumor immunity in the tumor microenvironment; the immunostimulatory protein or other therapeutic product is encoded on a plasmid in the bacterium under control of a eukaryotic promoter, such as a promoter that is recognized by RNA polymerase II (RNAP II). Generally, the promoter is a constitutive promoter, such as a late eukaryotic virus promoter. Exemplary promoters include, but are not limited to, a cytomegalovirus (CMV) promoter, an elongation factor-1 alpha (EF-1α) promoter, a ubiquitin C (UBC) promoter, a simian virus 40 (SV40) early promoter, a phosphoglycerate kinase 1 (PGK) promoter, a chicken β-actin (CBA) promoter and its derivative promoters CAGG or CAG, a β-glucuronidase (GUSB) promoter, the MND promoter (a synthetic promoter that contains the U3 region of a modified MoMuLV (Moloney murine leukemia virus) LTR with myeloproliferative sarcoma virus enhancer and deleted negative control region), a eukaryotic initiation factor 4A-I (EIF4A1) promoter, a CD68 promoter, and a GAPDH promoter, among others (see, *e.g.*, Powell *et al.* (2015) *Discov. Med.* 19(102):49–57). The CAG promoter consists of: (C) the cytomegalovirus (CMV) early enhancer element; (A) the promoter, the first exon, and the first intron of chicken beta-actin gene; and (G) the splice acceptor of the rabbit beta-globin gene. MND is a synthetic promoter that contains the U3 region of a modified MoMuLV (Moloney murine leukemia virus) LTR with myeloproliferative sarcoma virus enhancer and deleted negative control region (murine leukemia virus-derived MND promoter (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted); see, *e.g.*, Li *et al.* (2010) *J. Neurosci. Methods* 189:56-64).

Two or more of these promoters can be encoded in multiple open reading frames (ORFs) on the plasmid. Certain promoters, including, but not limited to, CMV, contain multiple cAMP response element binding protein (CREB) sites. When

plasmids containing these elements are released to the cytosol, for example those contained within *S. typhimurium* that are released into the cytosol following bacterial destruction, they can be efficiently shuttled to the nucleus using the CREB-mediated host microtubule machinery (see, *e.g.*, Bai *et al.* (2017) *Biosci. Rep.* 37(6):BSR20160616).

The plasmids can include multiple promoters, including bacterial promoters, such as for expression of *asd*, and eukaryotic promoters for expression of therapeutic products. Various configurations of the promoters and other regulatory sequences have been assessed to improve expression of therapeutic products, and to improve bacterial growth and fitness. For example, among the configurations tested, reversing the orientation of the eukaryotic expression cassette on the plasmid, and inclusion of one or more bacterial terminators, can increase the efficiency of encoded payload expression, and can improve bacterial fitness.

**2. Multiple Therapeutic Product Expression Cassettes**

**a. Single Promoter Constructs**

Expression of multiple genes in the same cell from a single construct can be achieved, and is advantageous when the co-expression of several proteins is required to elicit a desired biological effect, such as an anti-tumor response. Internal ribosome entry site (IRES) sequences have been used to separate two coding sequences under control of a single promoter, however, the expression level of the second protein can be reduced compared to the first protein, and the length of the IRES sequence can be prohibitive in certain cases, such as when using viruses with small packaging capacities. The discovery of short (~18-22 amino acid long), virus-derived peptide sequences, known as 2A peptides, that mediate a ribosome-skipping event, enables the generation of multiple separate peptide products, at similar levels, from a single mRNA. The 2A peptide coding sequence is included between the polypeptide-encoding transgenes (see, *e.g.*, Daniels *et al.* (2014) *PLoS One* 9(6):e100637).

IRES elements and 2A peptides use different mechanisms for co-expression of multiple genes in one transcript. For example, when using an IRES element to express multiple genes in one mRNA, the gene directly downstream of the promoter is translated by the canonical cap-dependent mechanism, and those downstream of the IRES element are translated by a cap-independent mechanism, which has a lower

translation efficiency than the cap-dependent mechanism, resulting in unbalanced expression, with lower expression of the IRES-driven gene (see, *e.g.*, Chng *et al.* (2015) *mAbs* 7(2):403-412). 2A linked genes, on the other hand, are translated in one open reading frame (ORF). The cleavage of proteins separated by a 2A sequence occurs co-translationally, in an unconventional process, where a peptide bond often fails to form (*i.e.*, the peptide bond is "skipped") between the C-terminal glycine and proline in the 2A peptide. Despite this, translation proceeds, and two distinct proteins are produced in equal amounts. A short stretch, coding for approximately 20 amino acids, of the 2A peptide sequence, is sufficient to cause the bond-skipping. If the bond skipping does not occur, however, a fusion protein is generated that will not subsequently cleave (see, *e.g.*, Daniels *et al.* (2014) *PLoS One* 9(6):e100637).

Many of these 2A peptides have been described, including, but not limited to, T2A (see, *e.g.*, SEQ ID NO:327) from *Thosea asigna* virus, P2A (see, *e.g.*, SEQ ID NO:328) from porcine teschovirus-1, E2A (see, *e.g.*, SEQ ID NO:329) from equine rhinitis A virus, and F2A (see, *e.g.*, SEQ ID NO:330) from foot-and-mouth disease virus, among others. Different studies have reported conflicting cleavage efficiencies of the various 2A peptides, and the cleavage efficiency of a 2A peptide can be affected by the nature of the protein expressed, the order of genes flanking the 2A sequence, the length of the 2A peptide used, and the linker between the upstream protein and 2A peptide. Cleavage efficiency and enhanced protein expression can often be improved through the use of upstream viral cleavage sequences, such as, but not limited to, the peptide furin cleavage sequence, RRKR, as well as by inserting GSG and SGS peptide linkers, a V5 epitope tag (GKPUPNPLLGLDST), or a 3xFlag epitope tag immediately preceding the 2A (see, *e.g.*, Chng *et al.* (2015) *mAbs* 7(2):403-412).

The immunostimulatory bacteria herein, containing plasmids encoding therapeutic products, such as immunomodulatory proteins, with a single promoter and ORF, can express two or more proteins through the use of viral internal ribosomal entry sites (IRES), which are cap-independent, or through translational read-through of 2A peptides, and subsequent self-cleavage into equally expressed co-proteins. The plasmids can contain other regulatory elements, as discussed below and elsewhere herein. For example, an exemplary construct (see, Example 14) is CMV-muIL-2

CO_T2A_muIFN-α2-WPRE, where codon optimized murine IL-2 is co-expressed with murine IFN-α2, using a CMV promoter, and a T2A peptide. Additionally, a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) is included, to enhance expression. If, for example, a third therapeutic product is to be expressed by the plasmid, a 2A sequence is flanked by the first two proteins, which are expressed under the control of a first promoter, *e.g.*, CMV, and a third protein is encoded under the control of a second promoter, *e.g.*, EF-1α. Exemplary of such a construct is CMV-muIL-15Rα/IL-15sc_T2A_muSTING-R283G + EF-1α-muIL-18-WPRE, where murine 15Rα/IL-15sc, and murine STING with the replacement R283G or N153S/R283G (corresponding to R284G or N154S/R284G, respectively, in human STING) are co-expressed under control of a CMV promoter, using T2A, and murine IL-18 is expressed separately under control of an EF-1α promoter. This exemplary construct also includes a WPRE for enhanced expression.

As described herein, the immunostimulatory bacteria can be used as vaccines in which the encoded antigen(s) is/are expressed under control of a bacterial promoter for transcription in the bacterium, but include regulatory sequences, such as an IRES, that prevents translation in the bacterium, but facilitates or permits translation in the eukaryotic host, such as a human. Nucleic acid constructs encoding such antigens can optionally encode additional immune system/response enhancing proteins, such as a STING protein, and/or a cytokine, and/or other combinations, which in effect, act as adjuvants.

**b. Dual/Multiple Promoter Constructs**

Alternatively, the genetic payloads/therapeutic products can be expressed using dual or multiple promoter constructs, where each protein is expressed under the control of a separate promoter. Thus, plasmids encoding therapeutic products, such as immunomodulatory proteins, expressed in combinations, can contain multiple promoters, each controlling an individual intact ORF with proper stop codon processing (*i.e.*, dual/multiple promoter constructs); or multiple proteins can be expressed in a single ORF through the use of 2A peptides (*i.e.*, single promoter constructs); or the plasmid can contain a mixture of single and dual/multiple promoter constructs, to express three or more proteins, as described above.

**3. Regulatory Elements**

### a. Post-Transcriptional Regulatory Elements

In order to enhance expression of single and multiple therapeutic products/immunomodulatory proteins from a single plasmid, regulatory elements may be employed that enhance the transcription and translation of the protein(s) of interest. For example, the post-transcriptional regulatory element (PRE) of woodchuck hepatitis virus (WPRE), when inserted in the 3' untranslated region of the ORF, can enhance expression levels several fold (see, *e.g.*, Zufferey *et al.* (1999) *J. Virol.* 73(4):2886-2892). Similarly, other such elements, including, but not limited to, the Hepatitis B Virus PRE (HPRE), also can enhance expression. The combination of these can be used at the 3' ends of multiple ORFs to improve expression of multiple proteins on a single plasmid.

The PREs WPRE and HPRE are hepadnaviral *cis*-acting RNA elements that can increase the accumulation of cytoplasmic mRNA by promoting mRNA exportation from the nucleus, and can enhance post-transcriptional processing and stability.

### b. Polyadenylation Signal Sequences and Terminators

Other elements on the plasmid that can enhance protein expression include polyadenylation signal sequences and terminators. Polyadenylation is the post-transcriptional addition of a poly(A) tail to the 3' end of an mRNA transcript, which is part of the process that produces mature mRNA for translation. Polyadenylation signal sequences are important for nuclear export, mRNA stability, and efficient translation. A terminator is a sequence that defines the end of a transcript, creating a free 3' end, and initiates the release of the newly synthesized mRNA from the transcriptional machinery. The free 3' end is then available for the addition of the poly(A) tail. Terminators are found downstream of the gene to be transcribed, and typically occur directly after any 3' regulatory elements, such as the polyadenylation or poly(A) signal. Commonly used mammalian terminators in expression plasmids include the simian virus 40 (SV40), human growth hormone (hGH), bovine growth hormone (BGH or bGH), and rabbit beta-globin (rbGlob) polyA sequences, that include the sequence motif AAUAAA (SEQ ID NO:398), and promote both polyadenylation and termination.

When placed at the 3' end of the ORF, sequences such as the simian virus 40 poly A (SV40pA) or the bovine growth hormone poly A (bGHpA) signals, result in several-fold increased expression both *in vitro* and *in vivo* (see, *e.g.*, Powell *et al.* (2015) *Discov. Med.* 19(102):49–57). These and other such elements can further enhance the expression and translation of multiple therapeutic products, including immunomodulatory proteins, expressed from a single plasmid.

**c. Enhancers**

Promoters and enhancers are found upstream of the multiple cloning site (MCS) in a plasmid, and cooperate to determine the rate of transcription. Enhancers are sequences that bind activator proteins, in order to loop the DNA, and bring a specific promoter to the initiation complex, thus increasing the rate of transcription. They can be adjacent to, or far from the promoter they influence, and include CMV, EF-1α, SV40, and synthetic enhancers, or the MND promoter, which is a synthetic promoter that contains the U3 region of a modified MoMuLV (Moloney murine leukemia virus) LTR with myeloproliferative sarcoma virus enhancer. The immunostimulatory bacteria herein contain plasmids that can comprise enhancer(s) to enhance the expression of the therapeutic products/proteins encoded on the plasmids.

**d. Secretion Signals**

A secretion signal, also known as a signal sequence or peptide, a leader sequence or peptide, or a localization signal or sequence, is a short peptide at the N-terminus of a newly synthesized protein that is to be secreted. Signal peptides promote a cell to translocate a protein, usually to the cellular membrane. The efficiency of protein secretion is strongly determined by the signal peptide. Thus, the immunostimulatory bacteria herein contain plasmids that can comprise a signal peptide/secretion signal peptide, to facilitate and/or increase the expression or secretion of the encoded therapeutic product(s).

**e. Improving Bacterial Fitness**

The plasmids in the immunostimulatory bacteria that encode the therapeutic products, include genes and regulatory elements that are provided for expression of bacterial genes, and also, for expression of complex polycistronic eukaryotic payloads. The switch between such evolutionarily divergent organisms introduces

challenges for proper functioning in prokaryotes and eukaryotes. The bacteria are cultured *in vitro*, then administered to a eukaryotic subject, where the plasmids are delivered to cells, particularly to tumor-resident myeloid cells, in cancer subjects, where the payloads are expressed, processed and trafficked. Transcriptional leakiness from the eukaryotic promoter, such as the CMV promoter, in bacteria, combined with large eukaryotic genes and regulatory sequences, can result in reduced bacterial fitness that manifests as low injection stock viability, and reduced growth rate in broth culture.

**4. Origin of Replication and Plasmid Copy Number**

Plasmids are autonomously-replicating, extra-chromosomal circular double-stranded DNA molecules that are maintained within bacteria by means of a replication origin. Copy number influences the plasmid stability. High copy number generally results in greater stability of the plasmid when the random partitioning occurs at cell division. A high copy number of plasmids generally decreases the growth rate, thus possibly allowing for bacterial cells with few plasmids to dominate the culture, since they grow faster. This can be ameliorated by using gene attenuation and gene dosing strategies, that limit the expression of certain genes on the plasmid that can be toxic to the bacteria when present in high copy numbers. The origin of replication also determines the plasmid's compatibility, *i.e.*, its ability to replicate in conjunction with another plasmid within the same bacterial cell. Plasmids that utilize the same replication system cannot co-exist in the same bacterial cell; they are said to belong to the same compatibility group. The introduction of a new origin, in the form of a second plasmid from the same compatibility group, mimics the result of replication of the resident plasmid. Thus, any further replication is prevented until after the two plasmids have been segregated to different cells to create the correct pre-replication copy number.

Numerous bacterial origins of replication are known to those of skill in the art. The origin can be selected to achieve a desired copy number. Origins of replication contain sequences that are recognized as initiation sites of plasmid replication via DNA dependent DNA polymerases (see, *e.g.*, del Solar *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62(2):434-464). Different origins of replication provide for varying plasmid copy levels within each cell, and can range from one to hundreds of copies per cell.

Commonly used bacterial plasmid origins of replication include, but are not limited to, pMB1 derived origins, which have very high copy derivatives, such as pUC, and lower copy derivatives, such as pBR322, as well as ColE1, p15A, and pSC101, and other origins, which have low copy numbers. Such origins are well-known to those of skill in the art. For example, the pUC19 origin results in a copy number of 500-700 copies per cell. The pBR322 origin has a known copy number of 15-20 copies per cell. These origins only vary by a single base pair. The ColE1 origin copy number is 15-20, and derivatives, such as pBluescript, have copy numbers ranging from 300-500. The p15A origin that is in plasmid pACYC184, for example, results in a copy number of approximately 10. The pSC101 origins confer a copy number of approximately 5. Other low copy number vectors from which origins of replication can be obtained, include, for example, pWSK29, pWKS30, pWSK129, and pWKS130 (see, *e.g.*, Wang *et al.* (1991) *Gene 100*:195-199). Medium to low copy number is less than 150, or less than 100. Low copy number is less than 20, 25, or 30. Generally, less than medium copy number is less than 150 copies, and less than low copy number is less than about 25 or less than 25 copies, and generally, copy number refers to the average copies of plasmid per bacterium in a preparation. Those of skill in the art can identify plasmids with low, medium, or high copy numbers. For example, one method to determine experimentally if the copy number is high or low is to perform a miniprep. A high-copy plasmid should yield between 3-5 µg DNA per 1 ml LB culture; a low-copy plasmid will yield between 0.2-1 µg DNA per ml of LB culture. Sequences of bacterial plasmids, including identification of and sequence of the origin of replication, are well known (see, *e.g.*, snapgene.com/resources/plasmid_files/basic_cloning_vectors/pBR322/). Exemplary origins of replication, and their plasmid copy numbers, are summarized in the table below.

| Origin of Replication | Copy Number | SEQ ID NO. |
|---|---|---|
| pMB1 | Varies | 254 |
| p15A | 10-12 | 255 |
| pSC101 | ~5 | 256 |
| pBR322 | 15-20 | 243 |
| ColE1 | 15-20 | 257 |
| pPS10 | 15-20 | 258 |
| RK2 | ~5 | 259 |

| | | |
|---|---|---|
| R6K (alpha origin) | 15-20 | 260 |
| R6K (beta origin) | 15-20 | 261 |
| R6K (gamma origin) | 15-20 | 262 |
| P1 (oriR) | Low | 263 |
| R1 | Low | 264 |
| pWSK | Low | 265 |
| ColE2 | 10-15 | 266 |
| pUC (pMB1) | 500-700 | 267 |
| F1 | 300-500 | 268 |

High copy plasmids are selected for heterologous expression of proteins *in vitro*, because the gene dosage is increased relative to chromosomal genes, there are higher specific yields of protein, and for therapeutic bacteria, higher therapeutic dosages of encoded therapeutics. It is shown, herein, however, that for delivery of plasmids encoding therapeutic products (*e.g.*, immunomodulatory proteins), such as by *S. typhimurium*, in some embodiments, a high copy plasmid might be advantageous.

The requirement for bacteria to maintain the high copy plasmids can be a problem if the expressed molecule is toxic to the organism. The metabolic requirements for maintaining these plasmids can come at a cost of replicative fitness *in vivo*. Optimal plasmid copy number for delivery of therapeutic products can depend on the mechanism of attenuation of the strain engineered to deliver the plasmid. If needed, the skilled person, in view of the disclosure herein, can select an appropriate copy number for a particular immunostimulatory species and strain of bacteria.

For use as vaccines and/or to deliver RNA (such as mRNA) where the encoded products are expressed in the bacterium, the plasmid can be in higher or high copy number (greater than 150), to increase the amount of RNA or mRNA produced.

**5. CpG Motifs and CpG Islands**

Unmethylated cytidine-phosphate-guanosine (CpG) motifs are prevalent in bacterial, but not in vertebrate, genomic DNA. Pathogenic DNA and synthetic oligodeoxynucleotides (ODNs) containing CpG motifs activate host defense mechanisms, leading to innate and acquired immune responses. The unmethylated CpG motifs contain a central unmethylated CG dinucleotide plus flanking regions. In humans, four distinct classes of CpG ODNs have been identified, based on differences in structure, and in the nature of the immune response they induce. K-type ODNs (also referred to as B-type) contain from 1 to 5 CpG motifs, typically on a

phosphorothioate backbone. D-type ODNs (also referred to as A-type) have a mixed phosphodiester/phosphorothioate backbone and have a single CpG motif, flanked by palindromic sequences that permit the formation of a stem-loop structure, as well as poly G motifs at the 3' and 5' ends. C-type ODNs have a phosphorothioate backbone and contain multiple palindromic CpG motifs that can form stem loop structures or dimers. P-Class CpG ODNs have a phosphorothioate backbone, and contain multiple CpG motifs with double palindromes that can form hairpins at their GC-rich 3' ends (see, *e.g.*, Scheiermann *et al.* (2014) *Vaccine* 32(48):6377-6389). For purposes herein, the CpGs are encoded in the plasmid DNA; they can be introduced as a motif, or in a gene.

Toll-like receptors (TLRs) are key receptors for sensing pathogen-associated molecular patterns (PAMPs) and activating innate immunity against pathogens (see, *e.g.*, Akira *et al.* (2001) *Nat. Immunol.* 2(8):675-680). TLR9 recognizes hypomethylated CpG motifs in the DNA of prokaryotes that do not occur naturally in mammalian DNA (see, *e.g.*, McKelvey *et al.* (2011) *J. Autoimmun.* 36:76-86). Recognition of CpG motifs, upon phagocytosis of pathogens into endosomes in immune cell subsets, and activates innate and adaptive immunity.

Immunostimulatory bacteria, such as *Salmonella* species, such as *S. typhimurium* strains, carrying plasmids containing CpG islands or motifs, are provided herein. These bacteria can activate TLR9. As exemplified herein, bacterial plasmids that contain hypomethylated CpG islands can elicit innate and adaptive anti-tumor immune responses that, in combination with the therapeutic products encoded on the plasmid, such as immunostimulatory proteins and constitutively active variants of STING, IRF3, and other cytosolic DNA/RNA sensors, can have synergistic or enhanced anti-tumor activity. For example, the *asd* gene (see, *e.g.*, SEQ ID NO:48) encodes a high frequency of hypomethylated CpG islands. CpG motifs can be included in combination with any of the therapeutic products, described or apparent from the description herein, in the immunostimulatory bacteria, to thereby enhance or improve anti-tumor immune responses in a treated subject.

Immunostimulatory CpGs can be included in the plasmids, by including a nucleic acid, typically from a bacterial gene, that encodes a gene product, and also by adding a nucleic acid that encodes CpG motifs. The plasmids herein can include CpG

motifs. Exemplary CpG motifs are known (see, *e.g.*, U.S. Patent Nos. 8,232,259, 8,426,375, and 8,241,844). These include, for example, synthetic immunostimulatory oligonucleotides, that are between 10 and 100, 10 and 20, 10 and 30, 10 and 40, 10 and 50, or 10 and 75, base pairs long, with the general formula: $(CpG)_n$, where n is the number of repeats. Generally, at least one or two repeats are used; non-CG bases can be interspersed. Those of skill in the art are very familiar with the general use of CpG motifs for inducing an immune response by modulating TLRs, particularly TLR9.

**6. Plasmid Maintenance/Selection Components**

The maintenance of plasmids in laboratory settings is usually ensured by the inclusion of an antibiotic resistance gene on the plasmid, and the use of antibiotics in the growth media. As described above, the use of an *asd* deletion mutant, complemented with a functional *asd* gene on the plasmid, allows for plasmid selection *in vitro* without the use of antibiotics, and allows for plasmid maintenance *in vivo*. The *asd* gene complementation system provides for such selection/maintenance (see, *e.g.*, Galán *et al.* (1990) *Gene* 94(1):29-35). The use of the *asd* gene complementation system to maintain plasmids in the tumor microenvironment increases the potency of *S. typhimurium* and other immunostimulatory bacterial strains, engineered to deliver plasmids encoding therapeutic products, such as immunostimulatory proteins, constitutively active cytosolic DNA/RNA sensors, antibodies, antibody fragments, or other such products as discussed herein.

**7. DNA Nuclear Targeting Sequences**

DNA nuclear targeting sequences (DTS), such as the SV40 DTS, mediate the translocation of DNA sequences through the nuclear pore complex. The mechanism of this transport is reported to be dependent on the binding of DNA binding proteins that contain nuclear localization sequences. The inclusion of a DTS on a plasmid to increase nuclear transport and expression has been demonstrated (see, *e.g.*, Dean, D.A. *et al.* (1999) *Exp. Cell Res.* 253(2):713-722), and has been used to increase gene expression from plasmids delivered by *S. typhimurium* (see, *e.g.*, Kong *et al.* (2012) *Proc. Natl. Acad. Sci. U.S.A.* 109(47):19414–19419).

Rho-independent or class I transcriptional terminators, such as the T1 terminator of the *rrnB* gene of *E. coli*, contain sequences of DNA that form secondary structures that cause dissociation of the transcription elongation complex.

Transcriptional terminators are included in the plasmid in order to prevent expression of heterologous proteins by the *S. typhimurium* transcriptional machinery. This ensures that expression of the therapeutic products is confined to the host cell transcriptional machinery.

Plasmids used for transformation of *Salmonella*, such as *S. typhimurium*, as a cancer therapy described herein, contain all or some of the following attributes: 1) one or more constitutive promoters for heterologous expression of proteins; 2) one or more human immunomodulatory expression cassettes; 3) a bacterial origin of replication and optimized plasmid copy number; 4) immunostimulatory CpG islands; 5) an *asd* gene selectable marker for plasmid maintenance and selection; 6) DNA nuclear targeting sequences; and 7) transcriptional terminators.

## G. EXEMPLARY BACTERIAL STRAINS AND MECHANISM OF ACTION FOR USE AS VACCINES AND THERAPEUTICS

As described throughout, provided are immunostimulatory bacteria that contain genome modifications that improve their properties for use as anti-cancer therapeutics, as vaccines against pathogens, and as delivery vehicles for RNA, which can be used for as therapeutic products, or, that, can be translated to provide immunostimulatory products, antigens, and combinations thereof for the various uses. The immunostimulatory bacteria include genomic modifications discussed throughout that improve their properties for use as anti-cancer therapeutics, for vaccines, and RNA delivery platforms, and also that reduce the inflammatory properties of bacteria,.also include payloads, including combinations of payload products, that enhance or act with the properties of the bacteria for these applications. Payloads and genomic modifications are discussed and described throughout the disclosure herein, and are exemplified in the working examples below. The following is a discussion of exemplary bacteria, combinations of genomic modifications and payloads, and particularly advantageous for their use as vaccines. Disclosure throughout describes properties, payloads, and modifications for use as cancer therapetuics, and as thereapeutics that are generally useful for appropriate immune stimulation or modulation for use for treating cancer and other diseases and conditions.

It is understood that the skilled artisan can effect similar genomic modifications in other bacterial species and strains, such as *E. coli*, and *Listeria*, to

achieve similar effects and results. Additionally, as discussed above non-*Salmonell*a species can be modified to encode a *rck* gene, such the *rck* gene from *Salmonella*, to increase resistance to complement.

For vaccination, the use and general mechanism of action for the bacteria provided herein includes administration, particularly direct tissue administration, such as by intramuscular injection, inhalation, intradermal administration, vaginal administration, and other such routes, to generate an *in situ* immune response to the antigen or product, which can be for prevention (immunization to develop an immmunoprotective response) or treatment, for a pathogen or for cancer. The bacteria induce durable adaptive antiviral immunity. The bacteria are suitably formulated for the selected route. This can include formulation, for example, as an aerosol, tablet, emulsion, powder, as appropriate. The so-formulated administered bacteria are taken up into tissue-resident phagocytes, by phagocytosis. For these purposes, the bacteria are modified so that they do no replicate *in vivo*, but deliver their payloads into the cells, such as tissue-resident phagocytic antigen-presenting cells (APCs). The encoded products include, for example antigens for presentation, STING to induce type I IFN. The encoded products can go into the lymph nodes resulting in antibodies via CD4+ and CD8+ priming, and the APCs present for priming and activation of antigen-specific CD8+ cells, which stimulate IFN-gamma, and also participate in direct killing of virally infected cells; macrophages phagocytose apoptotic infected cells, thereby spreading epitopes, which results in further T-cell activation

**1. Exemplary Immunostimulatory Bacteria — *In Situ* Cancer Vaccination Mechanism of Action (MOA)**

Among the immunostimulatory bacteria provided herein are immunostimulatory bacteria that are designed to accumulate in tumor-resident myeloid cells, such as macrophage. These bacteria contain a plasmid that encodes immunostimulatory protein and combinations thereof that induce an anti-viral/anti-tumor immune response. The genomic modifications include the elimination of flagella, alterations of the LPS, and elimination of curli fimbriae. These or modifications in bacterial strains with similar effects, reduce TLR2/4/5 responses/signaling, and result in accumulation in the tumor microenvironment and myeloid cells. Other modifiations include auxotrophies, such as *asd*$^-$ or *ThyA*$^-$ render

the bacteria unable to growh *in vivo*, and other auxotrophies, such as including *purI*-, that render the bacteria auxotrophic for adenosine, increase accumulation in the tumor microenvironment, which can have elevated adenosine, which immunosuppressive, and genomic modifications to eliminate or inactive asparaginase, which can inhibit T cells. The combinaiont of some or all of these bacterial modifications result in bacteria that accumulate in the macrophage in the tumor microenvironment and tumors. Exemplary of such immunostimulatory bacteria is the strain designated YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI strain. The strain can be used, for example, for *in situ* cancer antigen vaccination contains a plasmid expressing either STING, eSTING (referring to engineered STING, such as those provided herein, particularly those that constitutively induce type I interferon), IL-15 (or IL-15 receptor complex) + eSTING, and other type I IFN producing combinations of payloads, exptressed under control of a eukaryotic promoter, such as the CMV promoter. The plasmid additionally contains sequences encoding the open reading frames of full-length tumor antigens or peptide antigen fragments. The route of administration is IV, whereby the strain traffics to the tumor and is taken up by tumor-resident macrophages, resulting in plasmid transfer to the nucleus and expression of payloads. In the presence of type I IFN, the macrophages process and present the cancer antigens for $CD8^+$ T-cell priming within the tumor, as well as trafficking to the lymph nodes to prime $CD4^+$ and $CD8^+$ T-cells. Primed and activated $CD8^+$ T-cells eliminate tumor cells bearing the primed antigen, induce long-lived tissue-resident memory CD8+ T-cells, as well as circulating as memory T-cells after the tumor is cleared to prevent tumor recurrence.

**2. Exemplary immunostimulatory bacteria for Peripheral Cancer Vaccination and Mechanism of Action**

The YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA strain containing an additional thymidine auxotrophy is used for peripheral cancer antigen vaccination. The strain cannot proliferate in the absence of thymidine supplementation, and, upon administration, as by intramuscular injection into an extremity, it is phagocytosed by tissue-resident macrophages, such as following IM injection in the arm. The strain contains a plasmid that encodes STING, eSTING, IL-15 + eSTING, or other type I IFN producing combination, as well as sequences of

full-length tumor antigens or peptides, from either a bacterial promoter and IRES sequence or a eukaryotic promoter. For the bacterial promoter containing strains, the bacteria will then transcribe the RNA of these encoded payloads, and deliver them upon phagocytosis and destroyed by tissue-resident macrophages. For the eukaryotic promoter containing strains, the phagocyte transcribes the RNA. The resulting mRNA the is translated into proteins, and the encoded antigens are presented by the macrophages in the presence of type I IFN for $CD8^+$ T-cell priming. The cancer antigen-primed $CD8^+$ T-cells circulate and eliminate cancerous cells, such as those that remain after surgical tumor resection. Strains such as this also are used as a prophylactic vaccine to reduce the risk of developing a particular cancer, such as for vaccination against familial genetic mutations that could lead to tumor formation.

**3. Exemplary Immunostimulatory Bacteria for Pathogen Vaccination and Mechanism of Action (MOA)**

Also provided are bacterial strains that include additional auxotrophy, such as *thyA*$^-$ strains. Exemplary of such include YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA strain, which has an additional thymidine auxotrophy. Such srains cannot proliferate in the absence of thymidine supplementation. These strains can be used for vacciniation against pathogens. They are administered by route that infection by the particular pathogen occurs naturally. Following administration, such as by intramuscular, intranasal, inhalation, or oral delivery, or combinations thereof, depending on the site or sites where the infection naturally occurs, they are cleared from tissue-resident macrophages, leaving plasmids. These strains contain a plasmid encoding for expression of an immunostimulatory protein or combination thereof, such as STING, engineered STING (eSTING), IL-15 + eSTING, or other type I IFN producing proteins or combinations of proteins, and also pathogen sequences of antigens and/or peptides, under transcriptional control of a bacterial promoter, but including eukaryotic sequences, such as an IRES, for translation by eukarotic ribosomes. The bacteria produce RNA encoding these payloads, and, after administration, upon phagocytosis and destruction by tissue-resident macrophages, deliver the encoded mRNA. The mRNA is translated into proteins by the eukaryotic host cells, and the antigens are presented by the macrophages in the presence of type I IFN for $CD8^+$ T-cell priming. The type I IFN can be stimulated by the encoded

immunostimulatory protein, such as a STING or modified STING. Additionally, the bacteria can provide the type I IFN and pathogen antigens as proteins using a plasmid with a bacterial promoter and sequences for bacterial transcription and translation. The macrophages then prime $CD8^+$ T-cells in the tissue, as well as traffic to the lymph nodes to prime $CD4^+$ and $CD8^+$ T-cells. Primed and activated $CD8^+$ T-cells then circulate as patrolling memory T-cells, as well as traffic back to the tissue of origin to remain as tissue-resident memory $CD8^+$ T-cells. The primed $CD4^+$ T-cells induce B-cell activation to produce high titers of durable neutralizing antibodies. This vaccination strategy mimics the highly successful smallpox, polio, measles, chicken pox, and other live-attenuated vaccines that are administered in the tissues where disease would naturally occur.

## H. PHARMACEUTICAL PRODUCTION, COMPOSITIONS, AND FORMULATIONS

Provided herein are methods for manufacturing, and pharmaceutical compositions and formulations, containing any of the immunostimulatory bacteria provided herein and pharmaceutically acceptable excipients or additives. The pharmaceutical compositions can be used in the treatment of diseases, such as hyperproliferative diseases or conditions, such as a tumor or cancer. The immunostimulatory bacteria can be administered as a single agent therapy, or can be administered in a combination therapy with a further agent(s) or treatment(s). Combination therapy includes combining therapy with the immunostimulatory bacteria and/or other delivery vehicles provided herein, with any other anti-cancer therapy or treatment, including, but not limited to, immunotherapies, such as CAR-T therapy and checkpoint inhibitors, radiation, surgery, chemotherapeutic agents, such as nucleoside analogs and platinum compounds, and cellular therapies. The compositions can be formulated for single dosage administration, or for multiple dosage administration. The agents can be formulated for direct administration. The compositions can be provided as a liquid or dried formulation.

### 1. Manufacturing

#### a. Cell Bank Manufacturing

As the active ingredient of the immunotherapeutic described herein is composed of engineered self-replicating bacteria, the selected composition will be

expanded into a series of cell banks that will be maintained for long-term storage and as the starting material for manufacturing of the drug substance. Cell banks are produced under current good manufacturing practices (cGMP) in an appropriate manufacturing facility per the Code of Federal Regulations (CFR) 21 part 211, or other relevant regulatory authority. As the active agent of the immunotherapeutic is a live bacterium, the products described herein are, by definition, non-sterile and cannot be terminally sterilized. Care must be taken to ensure that aseptic procedures are used throughout the manufacturing process to prevent contamination. As such, all raw materials and solutions must be sterilized prior to use in the manufacturing process.

A master cell bank (MCB) is produced by sequential serial single colony isolation of the selected bacterial strain, to ensure no contaminants are present in the starting material. A sterile culture vessel containing sterile media (can be complex media, *e.g.*, LB or MSB, or defined media, *e.g.*, M9 supplemented with appropriate nutrients) is inoculated with a single well-isolated bacterial colony and the bacteria are allowed to replicate, *e.g.*, by incubation at 37 °C with shaking. The bacteria are then prepared for cryopreservation by suspension in a solution containing a cryoprotective agent or agents.

Examples of cryoprotective agents include: proteins, such as human or bovine serum albumin, gelatin, and immunoglobulins; carbohydrates, including monosaccharides (*e.g.*, galactose, D-mannose, sorbose, *etc.*) and their non-reducing derivatives (*e.g.*, methylglucoside), disaccharides (*e.g.*, trehalose, sucrose, and others), cyclodextrins, and polysaccharides (*e.g.*, raffinose, maltodextrins, dextrans, *etc.*); amino-acids (*e.g.*, glutamate, glycine, alanine, arginine or histidine, tryptophan, tyrosine, leucine, phenylalanine, *etc.*); methylamines, such as betaine; polyols, such as trihydric or higher sugar alcohols, *e.g.*, glycerin, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; surfactants, *e.g.*, Pluronic®; or organo-sulfur compounds, such as dimethyl sulfoxide (DMSO), and combinations thereof. Cryopreservation solutions can include one or more cryoprotective agents in a solution that also can contain salts (*e.g.*, sodium chloride, potassium chloride, magnesium sulfate), and/or buffering agents, such as sodium phosphate, tris(hydroxymethyl)aminomethane (TRIS), 4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid (HEPES), and other such buffering agents known to those of skill in the art.

Suspension of the bacteria in cryopreservation solution can be achieved either by addition of a concentrated cryoprotective agent or agents to the culture material to achieve a final concentration that preserves viability of the bacteria during the freezing and thawing process (*e.g.*, 0.5% to 20% final concentration of glycerol), or by harvesting the bacteria (*e.g.*, by centrifugation) and suspending in a cryopreservative solution containing the appropriate final concentration of cryoprotective agent(s). The suspension of bacteria in cryopreservation solution is then filled into appropriate sterile vials (plastic or glass) with a container closure system that is capable of maintaining closure integrity under frozen conditions (*e.g.*, butyl stoppers and crimp seals). The vials of master cell bank are then frozen (either slowly by means of a controlled rate freezer, or quickly by means of placing directly into a freezer). The MCB is then stored frozen at a temperature that preserves long-term viability (*e.g.*, at or below -60 °C). Thawed master cell bank material is thoroughly characterized to ensure identity, purity, and activity per regulation by the appropriate authorities.

Working cell banks (WCBs) are produced much the same way as the master cell bank, but the starting material is derived from the MCB. MCB material can be directly transferred into a fermentation vessel containing sterile media and expanded as above. The bacteria are then suspended in a cryopreservation solution, filled into containers, sealed, and frozen at or below -20 °C. Multiple WCBs can be produced from MCB material, and WCB material can be used to make additional cell banks (*e.g.*, a manufacturer's working cell bank (MWCB)). WCBs are stored frozen, and are characterized to ensure identity, purity, and activity. WCB material is typically the starting material used in the production of the drug substance of biologics such as engineered bacteria.

**b. Drug Substance Manufacturing**

Drug substance is manufactured using aseptic processes under cGMP, as described above. Working cell bank material is typically used as starting material for manufacturing of drug substance under cGMP, however, other cell banks can be used (*e.g.*, MCB or MWCB). Aseptic processing is used for production of all cell therapies

including bacterial cell-based therapies. The bacteria from the cell bank are expanded by fermentation; this can be achieved by production of a pre-culture (*e.g.*, in a shake flask), or by direct inoculation of a fermenter. Fermentation is accomplished in a sterile bioreactor or flask that can be single-use disposable, or re-usable. Bacteria are harvested by concentration (*e.g.*, by centrifugation, continuous centrifugation, or tangential flow filtration). Concentrated bacteria are purified from media components and bacterial metabolites by exchange of the media with buffer (*e.g.*, by diafiltration). The bulk drug product is formulated and preserved as an intermediate (*e.g.*, by freezing or drying), or is processed directly into a drug product. Drug substance is tested for identity, strength, purity, potency, and quality.

**c. Drug Product Manufacturing**

Drug product is defined as the final formulation of the active substance contained in its final container. Drug product is manufactured using aseptic processes under cGMP. Drug product is produced from drug substance. Drug substance is thawed or reconstituted if necessary, then formulated at the appropriate target strength. Because the active component of the drug product is live, engineered bacteria, the strength is determined by the number of colony forming units (CFUs) contained within the suspension. The bulk product is diluted in a final formulation appropriate for storage and use, as described below. Containers are filled and sealed with a container closure system, and the drug product is labeled. The drug product is stored at an appropriate temperature to preserve stability and is tested for identity, strength, purity, potency, and quality, and released for human use if it meets specified acceptance criteria.

**2. Compositions**

Pharmaceutically acceptable compositions are prepared in view of approvals for a regulatory agency or other agency, and/or prepared in accordance with generally recognized pharmacopeia for use in animals and in humans. The compositions can be prepared as solutions, suspensions, powders, or sustained release formulations. Typically, the compounds are formulated into pharmaceutical compositions using techniques and procedures well-known in the art (see, *e.g.*, Ansel, *Introduction to Pharmaceutical Dosage Forms*, Fourth Edition, 1985, page 126). The formulation should suit the mode of administration.

Compositions can be formulated for administration by any route known to those of skill in the art, including intramuscular, intravenous, intradermal, intralesional, intraperitoneal, subcutaneous, intratumoral, epidural, nasal, oral, vaginal, rectal, topical, local, otic, inhalational, buccal (*e.g.*, sublingual), and transdermal administration, or by any suitable route. Other modes of administration also are contemplated. Administration can be local, topical, or systemic, depending upon the locus of treatment. Local administration to an area in need of treatment can be achieved by, for example, but not limited to, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant. Compositions also can be administered with other biologically active agents, either sequentially, intermittently, or in the same composition. Administration also can include controlled release systems, including controlled release formulations and device controlled release, such as by means of a pump.

The most suitable route in any given case depends on a variety of factors, such as the nature of the disease, the progress of the disease, the severity of the disease, and the particular composition which is used. Pharmaceutical compositions can be formulated in dosage forms appropriate for each route of administration. In particular, the compositions can be formulated into any suitable pharmaceutical preparations for systemic, local, intraperitoneal, oral, or direct administration. For example, the compositions can be formulated for administration subcutaneously, intramuscularly, intratumorally, intravenously, or intradermally. Administration methods can be employed to decrease the exposure of the active agent to degradative processes, such as immunological intervention via antigenic and immunogenic responses. Examples of such methods include local administration at the site of treatment, or continuous infusion.

The immunostimulatory bacteria can be formulated into suitable pharmaceutical preparations, such as solutions, suspensions, tablets, dispersible tablets, pills, capsules, powders, sustained release formulations, or elixirs, for oral administrations, as well as transdermal patch preparations, and dry powder inhalers. Typically, the compounds are formulated into pharmaceutical compositions using techniques and procedures well-known in the art (see, *e.g.*, Ansel, *Introduction to*

*Pharmaceutical Dosage Forms*, Fourth Edition, 1985, page 126). Generally, the mode of formulation is a function of the route of administration. The compositions can be formulated in dried (lyophilized or other forms of vitrification) or liquid form. Where the compositions are provided in dried form, they can be reconstituted just prior to use by addition of an appropriate buffer, for example, a sterile saline solution.

### 3. Formulations

#### a. Liquids, Injectables, Emulsions

The formulation generally is made to suit the route of administration. Parenteral administration, generally characterized by injection or infusion, either subcutaneously, intramuscularly, intratumorally, intravenously, or intradermally is contemplated herein. Preparations of bacteria for parenteral administration include suspensions ready for injection (direct administration), frozen suspensions that are thawed prior to use, dry soluble products, such as lyophilized powders, ready to be combined with a resuspension solution just prior to use, and emulsions. Dried thermostable formulations, such as lyophilized formulations, can be used for storage of unit doses for later use.

The pharmaceutical preparation can be in a frozen liquid form, for example, a suspension. If provided in frozen liquid form, the drug product can be provided as a concentrated preparation to be thawed and diluted to a therapeutically effective concentration before use.

The pharmaceutical preparations also can be provided in a dosage form that does not require thawing or dilution for use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives, as appropriate, such as suspending agents (*e.g.*, sorbitol, cellulose derivatives, or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives suitable for use with microbial therapeutics. The pharmaceutical preparations can be presented in dried form, such as lyophilized or spray-dried, for reconstitution with water or other sterile suitable vehicle before use.

Suitable excipients are, for example, water, saline, dextrose, or glycerol. The solutions can be either aqueous or non-aqueous. If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and

other buffered solutions used for intravenous hydration. For intratumoral administration, solutions containing thickening agents, such as glucose, polyethylene glycol, and polypropylene glycol, oil emulsions, and mixtures thereof can be appropriate to maintain localization of the injectant.

Pharmaceutical compositions can include carriers or other excipients. For example, pharmaceutical compositions provided herein can contain any one or more of a diluents(s), adjuvant(s), antiadherent(s), binder(s), coating(s), filler(s), flavor(s), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), or sorbent(s), and a combination thereof, or a vehicle with which a modified therapeutic bacteria is administered. For example, pharmaceutically acceptable carriers or excipients used in parenteral preparations include aqueous vehicles, non-aqueous vehicles, isotonic agents, buffers, antioxidants, local anesthetics, suspending and dispersing agents, emulsifying agents, sequestering or chelating agents, and other pharmaceutically acceptable substances. Formulations, including liquid preparations, can be prepared by conventional means with pharmaceutically acceptable additives or excipients.

Pharmaceutical compositions can include carriers, such as a diluent, adjuvant, excipient, or vehicle, with which the compositions are administered. Examples of suitable pharmaceutical carriers are described in “Remington’s Pharmaceutical Sciences” by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound or agent, generally in purified form or partially purified form, together with a suitable amount of carrier, so as to provide the form for proper administration to the patient. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, and sesame oil. Water is a typical carrier. Saline solutions and aqueous dextrose and glycerol solutions also can be employed as liquid carriers, particularly for injectable solutions. Compositions can contain along with an active ingredient: a diluent, such as lactose, sucrose, dicalcium phosphate, or carboxymethylcellulose; a lubricant, such as magnesium stearate, calcium stearate, and talc; and a binder, such as starch, natural gums, such as gum acacia, gelatin, glucose, molasses, polyvinylpyrrolidine, celluloses and derivatives thereof, povidone, crospovidone, and other such binders known to those of skill in the art. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice,

flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, and ethanol. For example, suitable excipients are, for example, water, saline, dextrose, glycerol, or ethanol. A composition, if desired, also can contain other minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, stabilizers, solubility enhancers, and other such agents, such as, for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, and cyclodextrins.

Pharmaceutically acceptable carriers used in parenteral preparations include aqueous vehicles, non-aqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, local anesthetics, suspending and dispersing agents, emulsifying agents, sequestering or chelating agents and other pharmaceutically acceptable substances. Examples of aqueous vehicles include Sodium Chloride Injection, Ringer's Injection, Isotonic Dextrose Injection, Sterile Water Injection, and Dextrose and Lactated Ringer's Injection. Non-aqueous parenteral vehicles include fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil, and peanut oil. Isotonic agents include sodium chloride and dextrose. Buffers include phosphate and citrate. Antioxidants include sodium bisulfate. Local anesthetics include procaine hydrochloride. Suspending and dispersing agents include sodium carboxymethylcellulose, hydroxypropyl methylcellulose, and polyvinylpyrrolidone. Emulsifying agents include, for example, polysorbates, such Polysorbate 80 (TWEEN 80). Sequestering or chelating agents of metal ions, such as EDTA, can be included. Pharmaceutical carriers also include polyethylene glycol, and propylene glycol for water miscible vehicles, and sodium hydroxide, hydrochloric acid, citric acid, or lactic acid, for pH adjustment. Non-anti-microbial preservatives can be included.

The pharmaceutical compositions also can contain other minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, stabilizers, solubility enhancers, and other such agents, such as, for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, and cyclodextrins. Implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained (see, *e.g.*, U.S. Patent No. 3,710,795), also is contemplated herein. The percentage of active compound contained in such parenteral compositions

is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject.

**b. Dried Thermostable Formulations**

The bacteria can be dried. Dried thermostable formulations, such as lyophilized or spray dried powders and vitrified glass, can be reconstituted for administration as solutions, emulsions and other mixtures. The dried thermostable formulations can be prepared from any of the liquid formulations, such as the suspensions, described above. The pharmaceutical preparations can be presented in lyophilized or vitrified form, for reconstitution with water or other suitable vehicle, before use.

The thermostable formulation is prepared for administration by reconstituting the dried compound with a sterile solution. The solution can contain an excipient which improves the stability or other pharmacological attribute of the active substance or reconstituted solution, prepared from the powder. The thermostable formulation is prepared by dissolving an excipient, such as dextrose, sorbitol, fructose, corn syrup, xylitol, glycerin, glucose, sucrose, or other suitable agent, in a suitable buffer, such as citrate, sodium or potassium phosphate, or other such buffer known to those of skill in the art. Then, the drug substance is added to the resulting mixture, and stirred until it is mixed. The resulting mixture is apportioned into vials for drying. Each vial will contain a single dosage, containing $1x10^5$ to $1x10^{11}$ CFUs per vial. After drying, the product vial is sealed with a container closure system that prevents moisture or contaminants from entering the sealed vial. The dried product can be stored under appropriate conditions, such as at -20 °C, 4 °C, or room temperature. Reconstitution of this dried formulation with water or a buffer solution provides a formulation for use in parenteral administration. The precise amount depends upon the indication treated and selected compound. Such amount can be empirically determined.

**4. Compositions for Other Routes of Administration**

Depending upon the condition treated, other routes of administration in addition to parenteral, such as topical application, transdermal patches, and oral and rectal administration, also are contemplated herein. The suspensions and powders described above can be administered orally, or can be reconstituted for oral administration. Pharmaceutical dosage forms for rectal administration are rectal

suppositories, capsules, and tablets and gel capsules for systemic effect. Rectal suppositories include solid bodies for insertion into the rectum which melt or soften at body temperature, releasing one or more pharmacologically or therapeutically active ingredients. Pharmaceutically acceptable substances in rectal suppositories are bases or vehicles and agents to raise the melting point. Examples of bases include cocoa butter (theobroma oil), glycerin-gelatin, CARBOWAX® (polyethylene glycol), and appropriate mixtures of mono-, di-, and triglycerides of fatty acids. Combinations of the various bases can be used. Agents to raise the melting point of suppositories include spermaceti and wax. Rectal suppositories can be prepared either by the compressed method, or by molding. The typical weight of a rectal suppository is about 2 to 3 grams. Tablets and capsules for rectal administration are manufactured using the same pharmaceutically acceptable substance and by the same methods as for formulations for oral administration. Formulations suitable for rectal administration can be provided as unit dose suppositories. These can be prepared by admixing the drug substance with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

For oral administration, pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients, such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone, or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose, or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc, or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulfate). The tablets can be coated by methods well-known in the art.

Formulations suitable for buccal (sublingual) administration include, for example, lozenges containing the active compound in a flavored base, usually sucrose and acacia or tragacanth; and pastilles containing the compound in an inert base, such as gelatin and glycerin, or sucrose and acacia.

Topical mixtures are prepared as described for local and systemic administration. The resulting mixtures can be solutions, suspensions, emulsions or the like, and are formulated as creams, gels, ointments, emulsions, solutions, elixirs, lotions, suspensions, tinctures, pastes, foams, aerosols, irrigations, sprays,

suppositories, bandages, dermal patches, or any other formulations suitable for topical administration.

The compositions can be formulated as aerosols for topical application, such as by inhalation (see, *e.g.*, U.S. Patent Nos. 4,044,126, 4,414,209, and 4,364,923, which describe aerosols for the delivery of a steroid useful for treatment of lung diseases). These formulations, for administration to the respiratory tract, can be in the form of an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case, the particles of the formulation will typically have diameters of less than 50 microns, or less than 10 microns.

The compounds can be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions, and for application to the eye, or for intracisternal or intraspinal application. Topical administration is contemplated for transdermal delivery, and also for administration to the eyes or mucosa, or for inhalation therapies. Nasal solutions of the active compound alone, or in combination with other pharmaceutically acceptable excipients, also can be administered.

Formulations suitable for transdermal administration are provided. They can be provided in any suitable format, such as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches contain the active compound in an optionally buffered aqueous solution of, for example, 0.1 to 0.2 M concentration, with respect to the active compound. Formulations suitable for transdermal administration also can be delivered by iontophoresis (see, *e.g.*, Tyle, P. (1986) *Pharmaceutical Research* 3(6):318-326), and typically take the form of an optionally buffered aqueous solution of the active compound.

Pharmaceutical compositions also can be administered by controlled release formulations and/or delivery devices (see *e.g.*, U.S. Patent Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,916,899; 4,008,719; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,591,767; 5,639,476; 5,674,533; and 5,733,566).

## 5. Dosages and Administration

The compositions can be formulated as pharmaceutical compositions for single dosage or multiple dosage administration. The immunostimulatory bacteria can be included in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. For example, the concentration of the pharmaceutically active compound is adjusted so that an injection provides an effective amount to produce the desired pharmacological effect. The therapeutically effective concentration can be determined empirically by testing the immunostimulatory bacteria in known *in vitro* and *in vivo* systems, such as by using the assays described herein or known in the art. For example, standard clinical techniques can be employed. *In vitro* assays and animal models can be employed to help identify optimal dosage ranges. The precise dose, which can be determinied empirically, can depend on the age, weight, body surface area, and condition of the patient or animal, the particular immunostimulatory bacteria administered, the route of administration, the type of disease to be treated, and the seriousness of the disease.

Hence, it is understood that the precise dosage and duration of treatment is a function of the disease being treated, and can be determined empirically using known testing protocols, or by extrapolation from *in vivo* or *in vitro* test data. Concentrations and dosage values also can vary with the severity of the condition to be alleviated. It is to be further understood that, for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or use of compositions and combinations containing them. The compositions can be administered hourly, daily, weekly, monthly, yearly, or once. Generally, dosage regimens are chosen to limit toxicity. It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust therapy to lower dosage due to toxicity, or bone marrow, liver, or kidney, or other tissue dysfunctions. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

The immunostimulatory bacteria are included in the composition in an amount sufficient to exert a therapeutically useful effect. For example, the amount is one that achieves a therapeutic effect in the treatment of a hyperproliferative disease or condition, such as cancer, or an amount that is effective as a vaccine, or to deliver mRNA. An exemplary dose can be about 1 x $10^9$ CFUs/$m^2$, but depends upon the route of administration. As shown in the table below, and noted above, higher doses can be administered. The data below, from an experiment in a mouse model, show that strains with the genome modifications as described herein have significantly improved tolerability, at least about 15-fold, compared to strain VNP20009, and, thus, can be dosed in higher amounts.

| Strain Name | Strain Genotype | $LD_{50}$ in BALB/c Mice | Fold Attenuated | Fold Improved vs, VNP2009 | Human Equivalent Dose (CFU/$m^2$) |
|---|---|---|---|---|---|
| VNP20009 | Δ*purI* + Δ*msbB* | 4.4E6 | ~44,000 | - | 6.33E+08 |
| ΔFLG | Δ*purI* + Δ*msbB* + Δ*asdA* + Δ*FLG* | 2.0E7 | 200,000 | 4.5 | 2.88E+09 |
| Δ*pagP* | Δ*purI* + Δ*msbB* + Δ*asdA* + Δ*pagP* | 1.4E7 | 139,000 | 3.2 | 2.00E+09 |
| ΔFLG/Δ*pagP* | Δ*purI* + Δ*msbB* + Δ*asdA* + Δ*FLG* + Δ*pagP* | >6.2E7 | >620,000 | >14 | >8.91E+09 |

The immunostimulatory bacteria described and provided herein can be administered by any suitable route, including, but not limited to, intravenously (IV), and mucosally, such as by nasal/intranasal administration, and by inhalation into the lung. This is particularly adantageous in embodiments in which the bacteria are used as vaccines against pathogens, such as bacterial and viral pathogens, including, for example, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The bacteria can be provided as a tablet, powder, or liquid, or other suitable formulation; they can be stored frozen, or refrigerated, or stored at room temperature, depending upon the formulation. Thus, the bacteria that provide mRNA can be administered via a variety of routes, and can be conveniently stored and formulated.

Pharmaceutically and therapeutically active compounds and derivatives thereof are typically formulated and administered in unit dosage forms or multiple dosage forms. Each unit dose contains a predetermined quantity of therapeutically active compound sufficient to produce the desired therapeutic effect, in association

with the required pharmaceutical carrier, vehicle, or diluent. Unit dosage forms, include, but are not limited to, tablets, capsules, pills, powders, granules, parenteral suspensions, oral solutions or suspensions, and oil-in-water emulsions, containing suitable quantities of the compounds or pharmaceutically acceptable derivatives thereof. Unit dose forms can be contained in vials, ampoules and syringes, or individually packaged tablets or capsules. Unit dose forms can be administered in fractions or multiples thereof. A multiple dose form is a plurality of identical unit dosage forms packaged in a single container to be administered in segregated unit dose form. Examples of multiple dose forms include vials, bottles of tablets or capsules, or bottles of pints or gallons. Hence, multiple dose form is a multiple of unit doses that are not segregated in packaging. Generally, dosage forms or compositions containing active ingredient in the range of 0.005% to 100%, with the balance made up from non-toxic carrier, can be prepared. Pharmaceutical compositions can be formulated in dosage forms appropriate for each route of administration.

The unit-dose parenteral preparations are packaged in an ampoule, a vial, or a syringe with a needle. The volume of liquid solution or reconstituted powder preparation, containing the pharmaceutically active compound, is a function of the disease to be treated and the particular article of manufacture chosen for package. All preparations for parenteral administration must be sterile, as is known and practiced in the art.

As indicated, compositions provided herein can be formulated for any route known to those of skill in the art, including, but not limited to, subcutaneous, intramuscular, intravenous, intradermal, intralesional, intraperitoneal, epidural, vaginal, rectal, local, otic, or transdermal administration, or any route of administration. Formulations suited for such routes are known to one of skill in the art. Compositions also can be administered with other biologically active agents, either sequentially, intermittently, or in the same composition.

Pharmaceutical compositions can be administered by controlled release formulations and/or delivery devices (see, *e.g.*, U.S. Patent Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,847,770; 3,916,899; 4,008,719; 4,687,660; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,354,556; 5,591,767; 5,639,476; 5,674,533; and 5,733,566). Various delivery systems are known and can be used to

administer selected compositions, are contemplated for use herein, and such particles can be easily made.

### 6. Packaging and Articles of Manufacture

Also provided are articles of manufacture containing packaging materials, any pharmaceutical composition provided herein, and a label that indicates that the compositions are to be used for treatment of diseases or conditions as described herein. For example, the label can indicate that the treatment is for a tumor or for cancer.

Combinations of immunostimulatory bacteria described herein and another therapeutic agent also can be packaged in an article of manufacture. In one example, the article of manufacture contains a pharmaceutical composition containing the immunostimulatory bacteria composition and no further agent or treatment. In other examples, the article of manufacture contains another further therapeutic agent, such as a different anti-cancer agent. In this example, the agents can be provided together or separately, for packaging as articles of manufacture.

The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well-known to those of skill in the art. See, for example, U.S. Patent Nos. 5,323,907, 5,052,558, and 5,033,252, each of which is incorporated herein in its entirety. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment. Exemplary of articles of manufacture are containers, including single chamber and dual chamber containers. The containers include, but are not limited to, tubes, bottles, and syringes. The containers can further include a needle for intravenous administration.

The choice of package depends on the agents, and whether such compositions will be packaged together or separately. In general, the packaging is non-reactive with the compositions contained therein. In other examples, some of the components can be packaged as a mixture. In other examples, all components are packaged separately. Thus, for example, the components can be packaged as separate compositions that, upon mixing just prior to administration, can be directly administered together.

Alternatively, the components can be packaged as separate compositions for administration separately.

Selected compositions including articles of manufacture thereof also can be provided as kits. Kits can include a pharmaceutical composition described herein, and an item for administration provided as an article of manufacture. The compositions can be contained in the item for administration or can be provided separately to be added later. The kit can, optionally, include instructions for application, including dosages, dosing regimens, and instructions for modes of administration. Kits also can include a pharmaceutical composition described herein and an item for diagnosis.

## I. METHODS OF TREATMENT AND USES

The methods provided herein include methods of administering or using the immunostimulatory bacteria, for treating subjects having a disease or condition whose symptoms can be ameliorated or lessened by administration of such bacteria, such as cancer. In particular examples, the disease or condition is a tumor or a cancer. Bacteria provided herein also can be used as vaccines and other such applications, discussed in sections above. In those instances, not only can the bacteria be administered as described in this section, as described with reference to their use as anti-cancer agents, but also by other routes that mimic the natural routes of entry of various pathogens. For example, as discussed in the above sections, for vaccination, such as against viruses, such as respiratory viruses, they can be administered by inhalation into the nose or lungs. These bacteria elicit T cell responses and immune responses in the tissues in which they are administered, such as the nose, esophagus, and lungs, to promote *in situ* immune responses, resulting in memory T cells that are antigen-specific, as well as B cells, to provide long term responses.

Additionally, methods of combination therapies with one or more additional agents for treatment, such as an anti-cancer agent or an anti-hyaluronan agent, also are provided. The bacteria can be administered by any suitable route, including, but not limited to, parenteral, systemic, topical, and local, such as intra-tumoral, intravenous, rectal, oral, intramuscular, mucosal, and other routes. Because of the modifications of the bacteria described herein, problems associated with systemic administration are solved. Formulations suitable for each route of administration are provided. The

skilled person can establish suitable regimens and doses, and select routes of administration.

**1. Diagnostics for Patient Selection for Treatment and for Monitoring Treatment**

**a. Patient Selection**

Biomarkers can be used to identify patients who are likely to respond to therapy with the immunostimulatory bacteria provided herein. For example, the Adenosine Signature and the Myeloid Signature can be assessed by NanoString gene expression panels, and T-cell infiltration of tumors can be assessed by the Immunoscore® test, which is an *in vitro* diagnostic test used for predicting the risk of relapse in early stage colon cancer patients, by measuring the host immune response at a tumor site. Patients whose tumors or body fluids indicate an immune responsiveness or an immune response are more likely to respond to the treatment with the immunostimulatory bacteria provided herein.

Other biomarkers include tumor-infiltrating lymphocytes (TILs), CD73, CD39, TNAP (tissue-nonspecific alkaline phosphatase), CD38, CD68, PD-L1, and FoxP3. For example, tumors that can be treated with the immunostimulatory bacteria provided herein are T-cell excluded, exhibit high levels of purines/adenosine, and are unresponsive to PD-1/PD-L1 targeted therapies.

Gene expression profiles (GEPs), which can be determined using various NanoString gene expression panels, can be analyzed, for example, to identify the "adenosine signature" of tumors. High concentrations of adenosine are found in certain tumors, including colorectal carcinoma (CRC), non-small cell lung cancer (NSCLC), and pancreatic cancer, among others. Patients with tumors that exhibit high concentrations of purines/adenosine are likely to respond to therapy, since the immunostimulatory bacteria herein accumulate and replicate in purine/adenosine rich tumor microenvironments. Thus, the identification of tumors that express an "Adenosine Signature" can be used to predict patient response to therapy. Additionally, the immunostimulatory bacteria herein preferentially accumulate in and infect tumor-resident myeloid cells. Thus, the "Myeloid Signature" also can be used to predict patient response to therapy with the immunostimulatory bacteria. For example, it has been shown that the "Adenosine Signature" is nearly identical to the

"Myeloid Signature" that is associated with poor response to atezolizumab (anti-PD-L1) monotherapy in renal cell carcinoma (RCC) patients, which is indicative of the role of adenosine in tumor escape from anti-PD-L1 therapy (see, *e.g.*, McDermott *et al.* (2018) *Nature Medicine* 24:749-757). Tumor-myeloid and tumor-adenosine NanoString signature panels are available and can be used for the selection of patients.

Macrophages limit T-cell infiltration into solid tumors and suppress their function, for example, in triple negative breast cancer (see, *e.g.*, Keren *et al.* (2018) *Cell* 174:1373-1387). In certain cancers, such as CRC, macrophages dominate the intratumoral immune population and promote T-cell exclusion, and as a result, tumor-associated macrophages are associated with poor prognosis in CRC (see, *e.g.*, Bindea *et al.* (2013) *Immunity* 39:782-795). Immunoscore®, a method to estimate the prognosis of cancer patients, based on the immune cells that infiltrate the cancer and surround it, can be used to measure T-cell exclusion or T-cell infiltration. Immunoscore® incorporates the effects of the host immune response into cancer classification and improves prognostic accuracy. It measures the density of two T lymphocyte populations (CD3/CD8, CD3/CD45RO, or CD8/CD45RO) in the center and at the periphery of the tumor, and provides a score ranging from 0 (I0), when low densities of both cell types are found in both regions, to Immunoscore 4 (I4), when high densities are found in both regions. Low infiltration of T lymphocytes results in a low Immunoscore®, which correlates with high risk, while high infiltration of T lymphocytes results in a high Immunoscore®, which correlates with low risk. Immunoscore®, thus, can be evaluated as a prospective biomarker to identify patients that will respond to therapy with the immunostimulatory bacteria provided herein. For example, T-cell poor/uninflamed tumors can be treated, because the immunostimulatory bacteria provided herein induce T-cell infiltration in cold tumors. Such tumors represent a high, unmet need population that is refractory to checkpoint inhibition.

Extracellular adenosine is produced by the sequential activities of membrane associated ectoenzymes, CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1, or NTPDase1) and CD73 (ecto-5'-nucleotidase), which are expressed on tumor stromal cells, together producing adenosine by phosphohydrolysis of ATP or ADP

that is produced from dead or dying cells. CD39 converts extracellular ATP (or ADP) to 5'-AMP, which is converted to adenosine by CD73. Expression of CD39 and CD73 on endothelial cells is increased under the hypoxic conditions of the tumor microenvironment, thereby increasing levels of adenosine. Thus, CD39 and CD73 can be used as biomarkers that indicate adenosine-rich tumors that can be targeted with the immunostimulatory bacteria provided herein.

CD38, also known as cyclic ADP ribose hydrolase, is a glycoprotein that is found on the surface of many immune cells, including $CD4^+$ T-cells, $CD8^+$ T-cells, B lymphocytes, and natural killer cells. The loss of CD38, which is a marker of cell activation, is associated with impaired immune responses, and has been linked to leukemias, myelomas, and solid tumors. Additionally, increased expression of CD38 is an unfavorable diagnostic marker in chronic lymphocytic leukemia and is associated with increased disease progression. CD38 also is used as a target for daratumumab (Darzalex®), which has been approved for the treatment of multiple myeloma. CD68 is highly expressed by monocytes, circulating macrophages and by tissue macrophages (*e.g.*, Kupffer cells, microglia). FoxP3 is involved in immune system responses, and acts as a regulator in the development and function of regulatory T-cells (or Tregs), which are immunosuppressive. In cancer, an excess of regulatory T-cell activity can prevent the immune system from destroying cancer cells. Thus, CD38, CD68 and FoxP3 also can be used as biomarkers for the selection of patients that are likely, to respond to therapy with the immunostimulatory bacteria herein.

**b. Diagnostics to Assess or Detect Activity of the Immunostimulatory Bacteria are Indicative of the Effectiveness of Treatment**

Biomarkers can be used to monitor the immunostimulatory bacteria following treatment. Biomarkers occur in tumor samples and/or in body fluid samples, such as blood, plasma, urine, saliva, and other fluids. Validated, peripheral blood biomarkers are used to evaluate the immune status of patients prior to and during treatment, to determine changes in the immune status, which correlate with the effectiveness of treatment. A change to, or an increase in, anti-tumor immune response status indicates that treatment with the immunostimulatory bacteria is having an effect.

Immunomodulatory activity of the immunostimulatory bacteria provided herein, for example, in dose escalation and expansion studies, can be assessed. Examination of biomarkers reveals prognostic and predictive factors relating to disease (*e.g.*, a tumor) status and its treatment, which can aid in monitoring treatment. Evaluating the tumor microenvironment, for example, provides insights into the mechanism of tumor responses to immunotherapies. Serum biomarkers to detect immunomodulatory activity of the immunostimulatory bacteria include, but are not limited to, CXCL10 (IP-10), CXCL9, interferon-β, interferon-γ, proinflammatory serum cytokines (*e.g.*, IL-6, TNF-α, MCP-1/CCL1), and IL-18 binding protein.

CXCL10 and CXCL9 are chemokines that are necessary for $CD8^+$ T-cell activation and trafficking to tumors, for example, in response to immunotherapies. In a phase 3 trial of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for the treatment of previously treated patients with metastatic renal cell carcinoma (mRCC), immune pharmacodynamic effects that were shared by the majority of patients, irrespective of the dose administered, were identified. Assessment of the IFN-γ regulated serum chemokines CXCL9 and CXCL10 was performed using a multiplex panel based on Luminex technology (Myriad® Rules-Based Medicine (RBM)), and the results demonstrated that increased CXCL9 and CXCL10 serum levels, as well as increased transcription in the tumor, correlated with clinical response. Median increases in chemokine levels after treatment with nivolumab from baseline were 101% for CXCL9 and 37% for CXCL10 in peripheral blood (see, *e.g.*, Choueiri *et al.* (2016) *Clin. Cancer Res.* 22(22):5461-5471). Additionally, treatment of patients with advanced solid tumors or lymphomas with the MK-1454 STING agonist (Merck), resulted in a dose-dependent increase in serum CXCL10 after intratumoral dosing (see, *e.g.*, Harrington *et al.* ESMO Annual Meeting (2018)).

Dose-dependent increases in serum levels of IFN-β were observed following intratumoral dosing of the ADU-S100 STING agonist (Aduro) (see, *e.g.*, Meric-Bernstam *et al.* ASCO Annual Meeting (2019)). Additionally, intravenous administration of VNP20009 induced a dose-dependent increase in the serum levels of the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, and IL-12 (see, *e.g.*, Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152).

IL-18 participates in protective immune responses to intracellular bacteria, fungi and viruses, and has demonstrated anti-tumor activity in preclinical models of lung cancer, breast cancer, sarcoma, and melanoma. The biological activity of IL-18 is modulated in a negative feedback loop by IL-18 binding protein (IL-18BP), induced through IFN-γ. Thus, serum levels of IL-18BP are predictive of clinical IFN-γ activity. The intravenous administration of recombinant human IL-18 (rhIL-18) to patients with advanced cancers resulted in increased serum concentrations of IL-18 binding protein in a dose-dependent manner, as well as increases in IFN-γ, GM-CSF, and soluble Fas ligand (see, *e.g.*, Robertson *et al.* (2006) *Clin. Cancer Res.* 12(14):4265-4273). Additionally, the levels of IL-18BP in urine and serum were observed to correlate with tumor status in patients with prostate cancer; significant differences in urinary IL-18BP levels were found between cases with and without prostate cancer, and increased serum IL-18BP levels correlated with increasing prostate cancer Gleason score, demonstrating that elevated IL-18BP secretion from prostate cancer cells can be indicative of an attempt by cancer to escape immune surveillance (see, *e.g.*, Fujita *et al.* (2011) *Int. J. Cancer* 129(2):424-432). Thus, IL-18BP can be used as a biomarker for tumor immune responses.

**2. Tumors**

The immunostimulatory bacteria, combinations, uses and methods provided herein are applicable to treating all types of tumors, including cancers, particularly solid tumors, including lung cancer, bladder cancer, non-small cell lung cancer, gastric cancers, head and neck cancers, ovarian cancer, liver cancer, pancreatic cancer, kidney cancer, breast cancer, colorectal cancer, and prostate cancer. The methods also can be used for treating hematological cancers.

Tumors and cancers subject to treatment by the immunostimulatory bacteria, compositions, combinations, uses and methods provided herein include, but are not limited to, those that originate in the immune system, skeletal system, muscles and heart, breast, pancreas, gastrointestinal tract, central and peripheral nervous system, renal system, reproductive system, respiratory system, skin, connective tissue systems, including joints, fatty tissues, and the circulatory system, including blood vessel walls. Examples of tumors that can be treated with the immunostimulatory bacteria provided herein include carcinomas, gliomas, sarcomas (including

liposarcoma), adenocarcinomas, adenosarcomas, and adenomas. Such tumors can occur in virtually all parts of the body, including, for example, the breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head and neck, ovary, prostate, brain, pancreas, skin, bone, bone marrow, blood, thymus, uterus, testicles, cervix, or liver.

Tumors of the skeletal system include, for example, sarcomas and blastomas such as osteosarcoma, chondrosarcoma, and chondroblastoma. Muscle and heart tumors include tumors of both skeletal and smooth muscles, *e.g.*, leiomyomas (benign tumors of smooth muscle), leiomyosarcomas, rhabdomyomas (benign tumors of skeletal muscle), rhabdomyosarcomas, and cardiac sarcomas. Tumors of the gastrointestinal tract include, *e.g.*, tumors of the mouth, esophagus, stomach, small intestine, and colon, and colorectal tumors, as well as tumors of gastrointestinal secretory organs, such as the salivary glands, liver, pancreas, and the biliary tract. Tumors of the central nervous system (CNS) include tumors of the brain, retina, and spinal cord, and can also originate in associated connective tissue, bone, blood vessels, or nervous tissue. Treatment of tumors of the peripheral nervous system are also contemplated. Tumors of the peripheral nervous system include malignant peripheral nerve sheath tumors. Tumors of the renal system include those of the kidneys, *e.g.*, renal cell carcinoma, as well as tumors of the ureters and bladder. Tumors of the reproductive system include tumors of the cervix, uterus, ovary, prostate, testes, and related secretory glands. Tumors of the immune system include both blood-based and solid tumors, including lymphomas, *e.g.*, both Hodgkin's and non-Hodgkin's lymphomas. Tumors of the respiratory system include tumors of the nasal passages, bronchi, and lungs. Tumors of the breast include, *e.g.*, both lobular and ductal carcinomas.

Other examples of tumors that can be treated by the immunostimulatory bacteria and methods provided herein include Kaposi's sarcoma, CNS neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas and cerebral metastases, melanoma, gastrointestinal and renal carcinomas and sarcomas, rhabdomyosarcoma, glioblastoma (such as glioblastoma multiforme), and leiomyosarcoma. Examples of other cancers that can be treated as provided herein include, but are not limited to, lymphoma, blastoma, neuroendocrine tumors, mesothelioma, schwannoma, meningioma, melanoma, and leukemia or lymphoid malignancies. Examples of such

cancers include hematologic malignancies, such as Hodgkin's lymphoma, non-Hodgkin's lymphomas (Burkitt's lymphoma, small lymphocytic lymphoma, chronic lymphocytic leukemia, mycosis fungoides, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, marginal zone lymphoma, hairy cell leukemia, and lymphoplasmacytic leukemia), tumors of lymphocyte precursor cells, including B-cell acute lymphoblastic leukemia/lymphoma, and T-cell acute lymphoblastic leukemia/lymphoma, thymoma, tumors of the mature T and NK cells, including peripheral T-cell leukemias, adult T-cell leukemia/T-cell lymphomas and large granular lymphocytic leukemia, Langerhans cell histiocytosis, myeloid neoplasias such as acute myelogenous leukemias, including acute myeloid leukemia (AML) with maturation, AML without differentiation, acute promyelocytic leukemia, acute myelomonocytic leukemia, and acute monocytic leukemias, myelodysplastic syndromes, and chronic myeloproliferative disorders, including chronic myelogenous leukemia; tumors of the central nervous system such as glioma, glioblastoma, neuroblastoma, astrocytoma, medulloblastoma, ependymoma, and retinoblastoma; solid tumors of the head and neck (*e.g.*, nasopharyngeal cancer, salivary gland carcinoma, and esophageal cancer), lung (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung), digestive system (*e.g.*, gastric or stomach cancer, including gastrointestinal cancer, cancer of the bile duct or biliary tract, colon cancer, rectal cancer, colorectal cancer, and anal carcinoma), reproductive system (*e.g.*, testicular, penile, prostate, uterine, vaginal, vulval, cervical, ovarian, and endometrial cancers), skin (*e.g.*, melanoma, basal cell carcinoma, squamous cell cancer, actinic keratosis, and cutaneous melanoma), liver (*e.g.*, liver cancer, hepatic carcinoma, hepatocellular cancer, and hepatoma), bone (*e.g.*, osteoclastoma, and osteolytic bone cancers), additional tissues and organs (*e.g.*, pancreatic cancer, bladder cancer, kidney or renal cancer, thyroid cancer, breast cancer, cancer of the peritoneum, and Kaposi's sarcoma), tumors of the vascular system (*e.g.*, angiosarcoma and hemangiopericytoma), Wilms' tumor, retinoblastoma, osteosarcoma, and Ewing's sarcoma.

### 3. Administration

In practicing the uses and methods herein, immunostimulatory bacteria provided herein can be administered to a subject, including a subject having a tumor

or having neoplastic cells, or a subject to be immunized. One or more steps can be performed prior to, simultaneously with, or after administration of the immunostimulatory bacteria to the subject, including, but not limited to, diagnosing the subject with a condition appropriate for administering immunostimulatory bacteria, determining the immunocompetence of the subject, immunizing the subject, treating the subject with a chemotherapeutic agent, treating the subject with radiation, or surgically treating the subject.

For embodiments that include administering immunostimulatory bacteria to a tumor-bearing subject for therapeutic purposes, the subject typically has previously been diagnosed with a neoplastic condition. Diagnostic methods also can include determining the type of neoplastic condition, determining the stage of the neoplastic condition, determining the size of one or more tumors in the subject, determining the presence or absence of metastatic or neoplastic cells in the lymph nodes of the subject, or determining the presence of metastases in the subject.

Some embodiments of the therapeutic methods for administering immunostimulatory bacteria to a subject can include a step of determining the size of the primary tumor or the stage of the neoplastic disease, and, if the size of the primary tumor is equal to or above a threshold volume, or if the stage of the neoplastic disease is at or above a threshold stage, an immunostimulatory bacterium is administered to the subject. In a similar embodiment, if the size of the primary tumor is below a threshold volume, or if the stage of the neoplastic disease is at or below a threshold stage, the immunostimulatory bacterium is not yet administered to the subject; such methods can include monitoring the subject until the tumor size or neoplastic disease stage reaches a threshold amount, and then administering the immunostimulatory bacterium to the subject. Threshold sizes can vary according to several factors, including rate of growth of the tumor, ability of the immunostimulatory bacterium to infect a tumor, and immunocompetence of the subject. Generally, the threshold size will be a size sufficient for an immunostimulatory bacterium to accumulate and replicate in or near the tumor, without being completely removed by the host's immune system, and will typically also be a size sufficient to sustain a bacterial infection for a time long enough for the host to mount an immune response against the tumor cells, typically about one week or more, about ten days or more, or about two

weeks or more. Exemplary threshold stages are any stage beyond the lowest stage (*e.g.*, Stage I or equivalent), or any stage where the primary tumor is larger than a threshold size, or any stage where metastatic cells are detected.

Any mode of administration of a microorganism to a subject can be used, provided the mode of administration permits the immunostimulatory bacteria to enter a tumor or metastasis. Modes of administration can include, but are not limited to, intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intratumoral, multipuncture, inhalation, intranasal, oral, intracavity (*e.g.*, administering to the bladder via a catheter, or administering to the gut by suppository or enema), aural, rectal, and ocular administration.

One skilled in the art can select any mode of administration compatible with the subject and the bacteria, and that also is likely to result in the bacteria reaching tumors and/or metastases. The route of administration can be selected by one skilled in the art according to any of a variety of factors, including the nature of the disease, the kind of tumor, and the particular bacteria contained in the pharmaceutical composition. Administration to the target site can be performed, for example, by ballistic delivery, or as a colloidal dispersion system, or systemic administration can be performed by injection into an artery.

The dosage regimen can be any of a variety of methods and amounts, and can be determined by one skilled in the art according to known clinical factors. A single dose can be therapeutically effective for treating a disease or disorder in which immune stimulation effects treatment. Exemplary of such stimulation is an immune response, that includes, but is not limited to, one or both of a specific immune response and non-specific immune response, both specific and non-specific responses, innate response, primary immune response, adaptive immunity, secondary immune response, memory immune response, immune cell activation, immune cell proliferation, immune cell differentiation, and cytokine expression.

As is known in the medical arts, dosages for a subject can depend on many factors, including the subject's species, size, body surface area, age, sex, immunocompetence, and general health, the particular bacteria to be administered, the duration and route of administration, the kind and stage of the disease, for example, the tumor size, and other compounds, such as drugs, being administered concurrently.

In addition to the above factors, such levels can be affected by the infectivity of the bacteria and the nature of the bacteria, as can be determined by one skilled in the art. In the present methods, appropriate minimum dosage levels of bacteria can be levels sufficient for the bacteria to survive, grow and replicate in a tumor or metastasis. Exemplary minimum levels for administering a bacterium to a 65 kg human can include at least about $5 \times 10^6$ colony forming units (CFUs), at least about $1 \times 10^7$ CFUs, at least about $5 \times 10^7$ CFUs, at least about $1 \times 10^8$ CFUs, or at least about $1 \times 10^9$ CFUs. In the present methods, appropriate maximum dosage levels of bacteria can be levels that are not toxic to the host, levels that do not cause splenomegaly of 3x or more, or levels that do not result in colonies or plaques in normal tissues or organs after about 1 day, or after about 3 days, or after about 7 days. Exemplary maximum levels for administering a bacterium to a 65 kg human can include no more than about $5 \times 10^{11}$ CFUs, no more than about $1 \times 10^{11}$ CFUs, no more than about $5 \times 10^{10}$ CFUs, no more than about $1 \times 10^{10}$ CFUs, or no more than about $1 \times 10^9$ CFUs.

The methods and uses provided herein can include a single administration of immunostimulatory bacteria to a subject, or multiple administrations of immunostimulatory bacteria to a subject, or others of a variety of regimens, including combination therapies with other anti-tumor therapeutics and/or treatments. These include, for example, cellular therapies, such as administration of modified immune cells; CAR-T therapy; CRISPR therapy; checkpoint inhibitors, such as antibodies (*e.g.*, anti-PD-1 antibodies, anti-PD-L1 antibodies, and anti-CTLA-4 antibodies, and other such immunotherapies); chemotherapeutic compounds, such as nucleoside analogs; surgery; and radiotherapy. Other cancer therapies also include anti-VEGF, anti-VEGFR, anti-VEGFR2, anti-TGF-β, or anti-IL-6 antibodies, or fragments thereof, cancer vaccines, and oncolytic viruses.

In some embodiments, a single administration is sufficient to establish immunostimulatory bacteria in a tumor, where the bacteria can colonize, and can cause or enhance an anti-tumor response in the subject. In other embodiments, the immunostimulatory bacteria provided for use in the methods herein can be administered on different occasions, separated in time, typically by at least one day. Separate administrations can increase the likelihood of delivering a bacterium to a tumor or metastasis, where a previous administration may have been ineffective in

delivering the bacterium to a tumor or metastasis. In embodiments, separate administrations can increase the locations on a tumor or metastasis where bacterial colonization/proliferation can occur, or can otherwise increase the titer of bacteria accumulated in the tumor, which can increase eliciting or enhancing a host's anti-tumor immune response.

When separate administrations are performed, each administration can be a dosage amount that is the same or different relative to other administration dosage amounts. In one embodiment, all administration dosage amounts are the same. In other embodiments, a first dosage amount can be a larger dosage amount than one or more subsequent dosage amounts, for example, at least 10x larger, at least 100x larger, or at least 1000x larger, than subsequent dosage amounts. In one example of a method of separate administrations, in which the first dosage amount is greater than one or more subsequent dosage amounts, all subsequent dosage amounts can be the same, smaller amount, relative to the first administration.

Separate administrations can include any number of two or more administrations, including two, three, four, five, or six administrations. One skilled in the art readily can determine the number of administrations to perform, or the desirability of performing one or more additional administrations, according to methods known in the art for monitoring therapeutic methods, and other monitoring methods provided herein. Accordingly, the methods provided herein include methods of providing to the subject one or more administrations of immunostimulatory bacteria, where the number of administrations can be determined by monitoring the subject, and, based on the results of the monitoring, determining whether or not to provide one or more additional administrations. Deciding whether or not to provide one or more additional administrations can be based on a variety of monitoring results, including, but not limited to, indication of tumor growth or inhibition of tumor growth, appearance of new metastases or inhibition of metastasis, the subject's anti-bacterial antibody titer, the subject's anti-tumor antibody titer, the overall health of the subject, and the weight of the subject.

The time period between administrations can be any of a variety of time periods. The time period between administrations can be a function of any of a variety of factors, including monitoring steps, as described in relation to the number of

administrations, the time period for a subject to mount an immune response, the time period for a subject to clear bacteria from normal tissue, or the time period for bacterial colonization/proliferation in the tumor or metastasis. In one example, the time period can be a function of the time period for a subject to mount an immune response; for example, the time period can be more than the time period for a subject to mount an immune response, such as more than about one week, more than about ten days, more than about two weeks, or more than about a month. In another example, the time period can be less than the time period for a subject to mount an immune response, such as less than about one week, less than about ten days, less than about two weeks, or less than about a month. In another example, the time period can be a function of the time period for bacterial colonization/proliferation in the tumor or metastasis; for example, the time period can be more than the amount of time for a detectable signal to arise in a tumor or metastasis after administration of a microorganism expressing a detectable marker, such as about 3 days, about 5 days, about a week, about ten days, about two weeks, or about a month.

The methods used herein also can be performed by administering compositions, such as suspensions and other formulations, containing the immunostimulatory bacteria provided herein. Such compositions contain the bacteria and a pharmaceutically acceptable excipient or vehicle, as provided herein or known to those of skill in the art.

As discussed above, the uses and methods provided herein also can include administering one or more therapeutic compounds, such as anti-tumor compounds or other cancer therapeutics, to a subject, in addition to administering the immunostimulatory bacteria to the subject. The therapeutic compounds can act independently, or in conjunction with the immunostimulatory bacteria, for tumor therapeutic effects. Therapeutic compounds that can act independently include any of a variety of known chemotherapeutic compounds that can inhibit tumor growth, inhibit metastasis growth and/or formation, decrease the size of a tumor or metastasis, or eliminate a tumor or metastasis, without reducing the ability of the immunostimulatory bacteria to accumulate in a tumor, replicate in the tumor, and cause or enhance an anti-tumor immune response in the subject. Examples of such chemotherapeutic agents include, but are not limited to, alkylating agents, such as

thiotepa and cyclophosphamide; alkyl sulfonates, such as busulfan, improsulfan, and piposulfan; androgens, such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, and testolactone; anti-adrenals, such as aminoglutethimide, mitotane, and trilostane; anti-androgens, such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; antibiotics, such as aclacinomycin, actinomycin, anthramycin, azaserine, bleomycin, cactinomycin, calicheamicin, carubicin, carminomycin, carzinophilin, chromomycin, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; anti-estrogens, including, for example, tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (Fareston®); anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, methotrexate, pteropterin, and trimetrexate; aziridines, such as benzodepa, carboquone, meturedepa, and uredepa; ethylenimines and methylmelamines, including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide, and trimethylol melamine; folic acid replenishers, such as folinic acid; nitrogen mustards, such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitrosoureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; platinum analogs, such as cisplatin and carboplatin; vinblastine; platinum; proteins, such as arginine deiminase and asparaginase; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; pyrimidine analogs, such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, and 5-FU; taxanes, such as paclitaxel and docetaxel, and albuminated forms thereof (*i.e.*, *nab*-paclitaxel and *nab*-docetaxel); topoisomerase inhibitors, such as RFS-2000; thymidylate synthase inhibitors, such as Tomudex®; and additional chemotherapeutics, including aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatrexate; defosfamide; demecolcine;

diaziquone; difluoromethylornithine (DFMO); eflornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; Navelbine; Novantrone; teniposide; daunomycin; aminopterin; Xeloda®; ibandronate; CPT-11; retinoic acid; esperamycins; capecitabine; and topoisomerase inhibitors, such as irinotecan. Pharmaceutically acceptable salts, acids, or derivatives of any of the above also can be used.

Therapeutic compounds that act in conjunction with the immunostimulatory bacteria include, for example, compounds that increase the immune response eliciting properties of the bacteria, *e.g.*, by increasing expression of encoded therapeutic products, such as cytokines, chemokines, co-stimulatory molecules, proteins that constitutively induce type I IFNs, RNAi molecules that inhibit, suppress, or disrupt expression of checkpoint gene(s), a checkpoint inhibitor antibody and antibodies or fragments thereof against other targets, or compounds that can further augment bacterial colonization/proliferation. For example, a gene expression-altering compound can induce or increase transcription of a gene in a bacterium, such as an exogenous gene encoded on the plasmid, thereby provoking an immune response. Any of a wide variety of compounds that can alter gene expression are known in the art, including IPTG and RU486. Exemplary genes whose expression can be up-regulated include those encoding proteins and RNA molecules, including toxins, enzymes that can convert a prodrug to an anti-tumor drug, cytokines, transcription regulating proteins, shRNA, siRNA, and ribozymes. In other embodiments, therapeutic compounds that can act in conjunction with the immunostimulatory bacteria to increase the colonization/proliferation or immune response eliciting properties of the bacteria, are compounds that can interact with a bacterially-encoded gene product, and such interaction can result in an increased killing of tumor cells or

an increased anti-tumor immune response in the subject. A therapeutic compound that can interact with a bacterially-encoded gene product can include, for example, a prodrug or other compound that has little or no toxicity, or other biological activity in its subject-administered form, but after interaction with a bacterially-encoded gene product, the compound can develop a property that results in tumor cell death, including but not limited to, cytotoxicity, the ability to induce apoptosis, or the ability to trigger an immune response. A variety of prodrug-like substances are known in the art, including ganciclovir, 5-fluorouracil, 6-methylpurine deoxyriboside, cephalosporin-doxorubicin, 4-[(2-chloroethyl)(2-mesuloxyethyl)amino]benzoyl-L-glutamic acid, acetaminophen, indole-3-acetic acid, CB1954, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, bis-(2-chloroethyl)amino-4-hydroxyphenylaminomethanone 28, 1-chloromethyl-5-hydroxy-1,2-dihyro-3H-benz[e]indole, epirubicin-glucuronide, 5'-deoxy5-fluorouridine, cytosine arabinoside, and linamarin.

**4. Monitoring**

The methods provided herein can further include one or more steps of monitoring the subject, monitoring the tumor, and/or monitoring the immunostimulatory bacteria administered to the subject. Any of a variety of monitoring steps can be included in the methods provided herein, including, but not limited to, monitoring tumor size, monitoring the presence and/or size of metastases, monitoring the subject's lymph nodes, monitoring the subject's weight or other health indicators, including blood or urine markers, monitoring anti-bacterial antibody titer, monitoring bacterial expression of a detectable gene product, and directly monitoring bacterial titer in a tumor, tissue, or organ of a subject.

The purpose of the monitoring can be simply for assessing the health state of the subject, or the progress of therapeutic treatment of the subject, or can be for determining whether or not further administration of the same or a different immunostimulatory bacterium is warranted, or for determining when or whether or not to administer a compound to the subject where the compound can act to increase the efficacy of the therapeutic method, or the compound can act to decrease the pathogenicity of the bacteria administered to the subject.

In some embodiments, the methods provided herein can include monitoring one or more bacterially-expressed genes. Bacteria, such as those provided herein or otherwise known in the art, can express one or more detectable gene products, including but not limited to, detectable proteins.

As provided herein, measurement of a detectable gene product expressed in a bacterium can provide an accurate determination of the level of bacteria present in the subject. As further provided herein, measurement of the location of the detectable gene product, for example, by imaging methods, including tomographic methods, can determine the localization of the bacteria in the subject. Accordingly, the methods provided herein that include monitoring a detectable bacterial gene product can be used to determine the presence or absence of the bacteria in one or more organs or tissues of a subject, and/or the presence or absence of the bacteria in a tumor or metastases of a subject. Further, the methods provided herein that include monitoring a detectable bacterial gene product can be used to determine the titer of bacteria present in one or more organs, tissues, tumors, or metastases. Methods that include monitoring the localization and/or titer of bacteria in a subject can be used for determining the pathogenicity of bacteria, since bacterial infection, and particularly the level of infection, of normal tissues and organs can indicate the pathogenicity of the bacteria. The methods that include monitoring the localization and/or titer of the immunostimulatory bacteria in a subject can be performed at multiple time points and, accordingly, can determine the rate of bacterial replication in a subject, including the rate of bacterial replication in one or more organs or tissues of a subject; accordingly, methods that include monitoring a bacterial gene product can be used for determining the replication competence of the bacteria. The methods provided herein also can be used to quantitate the amount of immunostimulatory bacteria present in a variety of organs or tissues, and tumors or metastases, and can thereby indicate the degree of preferential accumulation of the bacteria in a subject; accordingly, the bacterial gene product monitoring can be used in methods of determining the ability of the bacteria to accumulate in tumors or metastases, in preference to normal tissues or organs. Since the immunostimulatory bacteria used in the methods provided herein can accumulate in an entire tumor or can accumulate at multiple sites in a tumor, and can also accumulate in metastases, the methods provided herein for monitoring a

bacterial gene product can be used to determine the size of a tumor, or the number of metastases present in a subject. Monitoring such presence of a bacterial gene product in a tumor or metastasis over a range of time can be used to assess changes in the tumor or metastasis, including growth or shrinking of a tumor, or development of new metastases, or disappearance of metastases, and also can be used to determine the rate of growth or shrinking of a tumor, or the rate of development of new metastases or disappearance of metastases, or the change in the rate of growth or shrinking of a tumor, or the change in the rate of development of new metastases or disappearance of metastases. Accordingly, monitoring a bacterial gene product can be used for monitoring a neoplastic disease in a subject, or for determining the efficacy of treatment of a neoplastic disease, by determining the rate of growth or shrinking of a tumor, or the development of new metastases or disappearance of metastases, or the change in the rate of growth or shrinking of a tumor, or the development of new metastases or disappearance of metastases.

Any of a variety of detectable proteins can be detected by monitoring, exemplary of which are any of a variety of fluorescent proteins (*e.g.*, green fluorescent proteins), any of a variety of luciferases, transferrin, or other iron-binding proteins; or receptors, binding proteins, and antibodies, where a compound that specifically binds the receptor, binding protein or antibody can be a detectable agent, or can be labeled with a detectable substance (*e.g.*, a radionuclide or imaging agent).

Tumor and/or metastasis size can be monitored by any of a variety of methods known in the art, including external assessment methods, or tomographic or magnetic imaging methods. In addition to the methods known in the art, methods provided herein, for example, monitoring bacterial gene expression, can be used for monitoring tumor and/or metastasis size.

Monitoring size over several time points can provide information regarding the increase or decrease in the size of a tumor or metastasis, and can also provide information regarding the presence of additional tumors and/or metastases in the subject. Monitoring tumor size over several time points can provide information regarding the development of a neoplastic disease in a subject, including the efficacy of treatment of a neoplastic disease in a subject.

The methods provided herein also can include monitoring the antibody titer in a subject, including antibodies produced in response to administration of the immunostimulatory bacteria to a subject. The bacteria administered in the methods provided herein can elicit an immune response to endogenous bacterial antigens. The bacteria administered in the methods provided herein also can elicit an immune response to exogenous genes expressed by the bacteria. The bacteria administered in the methods provided herein also can elicit an immune response to tumor antigens. Monitoring antibody titer against bacterial antigens, bacterially-expressed exogenous gene products, or tumor antigens can be used to monitor the toxicity of the bacteria, to monitor the efficacy of treatment methods, or to monitor the level of gene product(s) or antibodies for production and/or harvesting.

Monitoring antibody titer can be used to monitor the toxicity of the bacteria. Antibody titer against a bacteria can vary over the time period after administration of the bacteria to the subject, where at some particular time points, a low anti-(bacterial antigen) antibody titer can indicate a lower toxicity, while at other time points, a high anti-(bacterial antigen) antibody titer can indicate a higher toxicity. The bacteria used in the methods provided herein can be immunogenic, and can, therefore, elicit an immune response soon after administering the bacteria to the subject. Generally, immunostimulatory bacteria against which the immune system of a subject can mount a strong immune response can be bacteria that have low toxicity when the subject's immune system can remove the bacteria from all normal organs or tissues. Thus, in some embodiments, a high antibody titer against bacterial antigens soon after administering the bacteria to a subject can indicate low toxicity of the bacteria.

In other embodiments, monitoring antibody titer can be used to monitor the efficacy of treatment methods. In the methods provided herein, antibody titer, such as anti-(tumor antigen) antibody titer, can indicate the efficacy of a therapeutic method, such as a therapeutic method to treat neoplastic disease. Therapeutic methods provided herein can include causing or enhancing an immune response against a tumor and/or metastasis. Thus, by monitoring the anti-(tumor antigen) antibody titer, it is possible to monitor the efficacy of a therapeutic method in causing or enhancing an immune response against a tumor and/or metastasis.

In other embodiments, monitoring antibody titer can be used for monitoring the level of gene product(s) or antibodies for production and/or harvesting. As provided herein, methods can be used for producing proteins, RNA molecules or other compounds, by expressing an exogenous gene in a microorganism that has accumulated in a tumor, in the tumor microenvironment, and/or in tumor-resident immune cells. Monitoring antibody titer against the protein, RNA molecule, or other compound can indicate the level of production of the protein, RNA molecule, or other compound by the tumor-accumulated microorganism, and also, can directly indicate the level of antibodies specific for such a protein, RNA molecule, or other compound.

The methods provided herein also can include methods of monitoring the health of a subject. Some of the methods provided herein are therapeutic methods, including neoplastic disease therapeutic methods. Monitoring the health of a subject can be used to determine the efficacy of the therapeutic method, as is known in the art. The methods provided herein also can include a step of administering to a subject an immunostimulatory bacterium, as provided herein. Monitoring the health of a subject can be used to determine the pathogenicity of an immunostimulatory bacterium administered to a subject. Any of a variety of health diagnostic methods for monitoring disease, such as neoplastic disease, infectious disease, or immune-related disease, can be monitored, as is known in the art. For example, the weight, blood pressure, pulse, breathing, color, temperature, or other observable state of a subject can indicate the health of a subject. In addition, the presence, or absence, or level of one or more components in a sample from a subject can indicate the health of a subject. Typical samples can include blood and urine samples, where the presence, or absence, or level of one or more components can be determined by performing, for example, a blood panel or a urine panel diagnostic test. Exemplary components indicative of a subject's health include, but are not limited to, white blood cell count, hematocrit, and c-reactive protein concentration.

The methods provided herein can include monitoring a therapy, where therapeutic decisions can be based on the results of the monitoring. Therapeutic methods provided herein can include administering to a subject immunostimulatory bacteria, where the bacteria can preferentially accumulate in a tumor, the tumor microenvironment, or in tumor-resident immune cells, and/or in metastases, and

where the bacteria can cause or enhance an anti-tumor immune response. Such therapeutic methods can include a variety of steps, including multiple administrations of a particular immunostimulatory bacterium, administration of a second immunostimulatory bacterium, or administration of a therapeutic compound. Determination of the amount, timing, or type of immunostimulatory bacteria or compound to administer to the subject can be based on one or more results from monitoring the subject. For example, the antibody titer in a subject can be used to determine whether or not it is desirable to administer an immunostimulatory bacterium and, optionally, a compound, the quantity of bacteria and/or compound to administer, and the type of bacteria and/or compound to administer, where, for example, a low antibody titer can indicate the desirability of administering an additional immunostimulatory bacterium, a different immunostimulatory bacterium, and/or a therapeutic compound, such as a compound that induces bacterial gene expression, or a therapeutic compound that is effective independent of the immunostimulatory bacteria.

In another example, the overall health state of a subject can be used to determine whether or not it is desirable to administer an immunostimulatory bacterium and, optionally, a compound, the quantity of bacterium and/or compound to administer, and the type of bacterium and/or compound to administer, where, for example, determining that the subject is healthy can indicate the desirability of administering additional bacteria, different bacteria, or a therapeutic compound, such as a compound that induces bacterial gene/genetic payload/therapeutic product expression. In another example, monitoring a detectable bacterially-expressed gene product can be used to determine whether it is desirable to administer an immunostimulatory bacterium and, optionally, a compound, the quantity of bacterium and/or compound to administer, and the type of bacterium and/or compound to administer, where, for example, determining that the subject is healthy can indicate the desirability of administering additional bacteria, different bacteria, or a therapeutic compound, such as a compound that induces bacterial gene/genetic payload/therapeutic product expression. Such monitoring methods can be used to determine whether or not the therapeutic method is effective, whether or not the therapeutic method is pathogenic to the subject, whether or not the bacteria have

accumulated in a tumor or metastasis, and whether or not the bacteria have accumulated in normal tissues or organs. Based on such determinations, the desirability and form of further therapeutic methods can be derived.

In another example, monitoring can determine whether or not immunostimulatory bacteria have accumulated in a tumor or metastasis of a subject. Upon such a determination, a decision can be made to further administer additional bacteria, a different immunostimulatory bacterium, and, optionally, a compound to the subject.

## J. EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

### Example 1

### Auxotrophic Strains of *S. typhimurium*

### The *Salmonella* Strain YS1646 is Auxotrophic for Adenosine

Strains provided herein are engineered to be auxotrophic for adenosine. As a result, they are attenuated *in vivo* because they are unable to replicate in the low adenosine concentrations of normal tissue, and colonization occurs primarily in the solid tumor microenvironment (TME), where adenosine levels are high. The *Salmonella* strain YS1646 is a derivative of the wild-type strain ATCC 14028, and was engineered to be auxotrophic for purines due to disruption of the *purI* gene (synonymous with *purM*) (see, *e.g.*, Low *et al.* (2004) *Methods Mol. Med.* 90:47-60). Subsequent analysis of the entire genome of YS1646 demonstrated that the *purI* gene was not in fact deleted, but was instead disrupted by a chromosomal inversion (see, *e.g.*, Broadway *et al.* (2014) *J. Biotechnol.* 192:177-178), and that the entire gene is still contained within two parts of the YS1646 chromosome that is flanked by insertion sequences, one of which has an active transposase. The presence of the complete genetic sequence of the *purI* gene, disrupted by means of a chromosomal reengagement, leaves open the possibility of reversion to a wild-type gene. While it has previously been demonstrated that the purine auxotrophy of YS1646 was stable after >140 serial passages *in vitro*, it was not clear what the reversion rate is (see, *e.g.*, Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002).

It is shown herein that, when provided with adenosine, strain YS1646 is able to replicate in minimal medium, whereas the wild-type parental strain, ATCC 14028 can grow in minimal media that is not supplemented with adenosine. Strain YS1646 was grown overnight in lysogeny broth (LB) medium, washed with M9 minimal medium, and diluted into M9 minimal medium containing no adenosine, or increasing concentrations of adenosine. Growth was measured using a SpectraMax® M3 Spectrophotometer (Molecular Devices) at 37 °C, reading the $OD_{600}$ every 15 minutes.

The results showed that, unlike a wild-type strain (ATCC 14028), which was able to grow in all concentrations of adenosine, strain YS1646 only was able to replicate when adenosine was provided at concentrations ranging from 11 to 300 micromolar, and was completely unable to replicate in M9 alone, or in M9 supplemented with 130 nanomolar adenosine. These data demonstrate that *purI* mutants are able to replicate at concentrations of adenosine that are found in the tumor microenvironment, but not at concentrations found in normal tissues. Engineered adenosine auxotrophic strains exemplified herein include strains in which all or portions of the *purI* open reading frame are deleted from the chromosome to prevent reversion to wild-type. Such gene deletions can be achieved by any method known to one of skill in the art, including the lambda red system, as described below.

**The *Salmonella* Strain YS1646 is Auxotrophic for ATP**

In addition to the purine and adenosine auxotrophy, it was determined whether the *purI*-deleted strain also can scavenge ATP. ATP accumulates to high levels in the tumor microenvironment, due to leakage from dying tumor cells. It is shown herein that, when provided with ATP, strain YS1646 is able to replicate in minimal media, but is unable to grow when not supplemented with ATP. To demonstrate this, strain YS1646 was grown overnight in LB medium, washed with M9 minimal medium, and diluted into M9 minimal medium containing no ATP, or increasing concentrations of ATP (Fisher). Growth was measured using a SpectraMax® M3 Spectrophotometer (Molecular Devices) at 37 °C, reading the $OD_{600}$ every 15 minutes. The results demonstrated that strain YS1646 is able to replicate when ATP is provided at concentrations of 0.012 mM, but not in M9 alone.

**Example 2**

**Defects in Intracellular Replication are Attributed to the *msbB* Mutation**

The YS1646 strain contains mutations in *purI*, which limits replication to sites containing high concentrations of purines, adenosine, or ATP, and mutations in *msbB*, which alters the lipopolysaccharide (LPS) surface coat in order to reduce TLR4-mediated pro-inflammatory signaling. It also has been established that, unlike wild-type *Salmonella*, strain YS1646 is unable to replicate in macrophages. Experiments were performed to determine which of these genetic mutations is responsible for conferring that phenotype within the wild-type strain, ATCC 14028.

In this assay, mouse RAW macrophage cells (InvivoGen, San Diego, Ca.) were infected with wild-type *Salmonella* strains containing deletions in *purI*, *msbB*, or both, at a multiplicity of infection (MOI) of approximately 5 bacteria per cell for 30 minutes, then the cells were washed with PBS, and medium containing gentamicin was added to kill extracellular bacteria. Intracellular bacteria are not killed by gentamicin, as it cannot cross the cell membrane. At various time points after infection, cell monolayers were lysed by osmotic shock with water, and the cell lysates were diluted and plated on LB agar to enumerate surviving colony forming units (CFUs).

As shown in the table below, wild-type *Salmonella* strains containing only the *purI*$^-$ mutation still were able to replicate. This explains why there is only a modest improvement in tolerability observed with the *purI* deletion alone, while achieving a high degree of specificity to the tumor microenvironment. Strains containing only the *msbB*$^-$ mutation, as well as strains containing the *purI*$^-$ and *msbB*$^-$ mutations, were unable to replicate, and were rapidly cleared from cells within 48 hours.

| | **CFUs/Well** | | | | | |
|---|---|---|---|---|---|---|
| **Hours** | **ATCC 14028 Δ*purI*** | | **ATCC 14028 Δ*purI*/Δ*msbB*** | | **ATCC 14028 Δ*msbB*** | |
| **1** | 104000 | 108000 | 68000 | 68000 | 88000 | 40000 |
| **2.5** | 5600 | 6000 | 760 | 960 | 3200 | 3200 |
| **5** | 5600 | 4000 | 1120 | 880 | 800 | 680 |
| **27** | 11200 | 5600 | 4 | 4 | 20 | 4 |

## Example 3

### *Salmonella asd* Gene Knockout Strain Engineering and Characterization

Strain YS1646Δ*asd* was prepared. It is an attenuated *Salmonella typhimurium* strain derived from strain YS1646 (which can be purchased from ATCC, Catalog # 202165) that has been engineered to have a deletion in the *asd* gene. In this example, the *Salmonella typhimurium* strain YS1646Δ*asd* was engineered using modifications of the method of Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)), as described below.

**Introduction of the Lambda Red Helper Plasmid into Strain YS1646**

The YS1646 strain was prepared to be electrocompetent as described previously (Sambrook J. (1998) *Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory*), by growing a culture in LB and concentrating 100-fold, and then washing three times with ice-cold 10% glycerol. The electrocompetent strain was electroporated with the Lambda red helper plasmid pKD46 (SEQ ID NO:218), using a 0.2 cm gap cuvette at the following settings: 2.5 kV, 186 ohms, and 50 μF. Transformants carrying pKD46 were grown in 5 mL SOC medium with ampicillin and 1 mM L-arabinose at 30 °C, and selected on LB agar plates containing ampicillin. A YS1646 clone containing the lambda red helper plasmid pKD46 then was made electrocompetent, as described above for strain YS1646.

**Construction of *asd* Gene Knockout Cassette**

The *asd* gene from the genome of strain YS1646 (Broadway *et al.* (2014) *J. Biotechnology* 192:177-178) was used for designing the *asd* gene knockout cassette. A plasmid containing 204 and 203 base pairs (bps) of homology to the left hand and right hand regions, respectively, of the *asd* gene, was transformed into DH5-alpha competent cells (Thermo Fisher Scientific). A kanamycin gene cassette flanked by loxP sites was cloned into this plasmid. The *asd* gene knockout cassette then was PCR amplified using primers asd-1 and asd-2 (see, Table 1), and gel purified.

**Deletion of *asd* gene**

The YS1646 strain carrying plasmid pKD46 was electroporated with the gel-purified linear *asd* gene knock-out cassette. Electroporated cells were recovered in SOC medium and plated onto LB agar plates supplemented with kanamycin (20

μg/mL) and diaminopimelic acid (DAP, 50 μg/mL). During this step, lambda red recombinase induces homologous recombination of the chromosomal *asd* gene with the *kan* cassette (due to the presence of homologous flanking sequences upstream and downstream of the chromosomal *asd* gene), and knockout of the chromosomal copy of the *asd* gene occurs. The presence of the disrupted *asd* gene in the selected kanamycin-resistant clones was confirmed by PCR amplification, with primers from the YS1646 genome flanking the sites of disruption (primer asd-3), and from the multi-cloning site (primer scFv-3) (see, Table 1). Colonies were also replica plated onto LB plates, with and without supplemental DAP, to demonstrate DAP auxotrophy. All clones with the *asd* gene deletion were unable to grow in the absence of supplemental DAP, demonstrating DAP auxotrophy.

**Table 1. Primer Information**

| Primer Name | Primer Sequence | SEQ ID NO. |
|---|---|---|
| asd-1 | ccttcctaacgcaaattccctg | 219 |
| asd-2 | ccaatgctctgcttaactcctg | 220 |
| asd-3 | gcctcgccatgtttcagtacg | 221 |
| asd-4 | ggtctggtgcattccgagtac | 222 |
| scFv-3 | cataatctgggtccttggtctgc | 223 |
| APR-001 | aaaaaagcttgcagctctggcccgtg | 226 |
| APR-002 | aaaaaagcttttagaaaaactcatcgagcatcaaatga | 227 |
| APR-003 | acactagaaggacagtatttggtatctg | 228 |
| APR-004 | agccgtagttaggccacc | 229 |
| flic-1 | cgttatcggcaatctggaggc | 232 |
| flic-2 | ccagcccttacaacagtggtc | 233 |
| flic-3 | gtctgtcaacaactggtctaacgg | 234 |
| flic-4 | agacggtcctcatccagataagg | 235 |
| fljb-1 | ttccagacgacaagagtatcgc | 236 |
| fljb-2 | cctttaggtttatccgaagccagaatc | 237 |
| fljb-3 | caccaggtttttcacgctgc | 238 |
| fljb-4 | acacgcatttacgcctgtcg | 239 |
| pagp-1 | gcgtgacggttctgagtgct | 315 |
| pagp-2 | cgtctttgctgccatcttccg | 316 |
| pagp-3 | acaataacgacgactccgataagg | 317 |
| pagp-4 | ctgctgaatgtgctgattaacctg | 318 |
| ansb-1 | accttagaagatagccgcaaagc | 319 |
| ansb-2 | cagagacatgacacccacgattatc | 320 |
| ansb-3 | gcaaaccgctatccagaacga | 321 |
| ansb-4 | agtttaagtatgccgtggtactgc | 322 |
| csgd-1 | cacttgctttaagatttgtaatggctag | 323 |

| Primer Name | Primer Sequence | SEQ ID NO. |
|---|---|---|
| csgd-2 | ggtgtattcgctttcccatttgtc | 324 |
| csgd-3 | tgtgctgtccaggttaatgcc | 325 |
| csgd-4 | gacgacggttttctcgaagtctc | 326 |

**Kanamycin Gene Cassette Removal**

The *kan* selectable marker was removed by using the Cre/loxP site-specific recombination system. The YS1646Δ*asd* gene $Kan^R$ mutant was transformed with pJW168 (SEQ ID NO:224), a temperature-sensitive plasmid expressing the Cre recombinase. $Amp^R$ colonies were selected at 30 °C; pJW168 was subsequently eliminated by growth at 42 °C. A selected clone was tested for loss of *kan* by replica plating on LB agar plates with and without kanamycin, and confirmed by PCR verification using primers from the YS1646 genome flanking the sites of disruption (primers asd-3 and asd-4; for primer sequences, see Table 1).

**Confirmation of Functional *asd* Deletion Mutant Strain YS1646Δ*asd* (also designated AST-101)**

The Δ*asd* mutant was unable to grow on LB agar plates at 37 °C, but was able to grow on LB plates containing 50 µg/mL diaminopimelic acid (DAP). The Δ*asd* mutant growth rate was evaluated in LB liquid media; it was unable to grow in liquid LB, but was able to grow in LB supplemented with 50 µg/mL DAP, as determined by measuring absorbance at 600 nM.

**Sequence Confirmation of the *asd* Locus Sequence in Strain YS1646Δ*asd* After *asd* Gene Deletion**

The *asd* gene deletion strain was verified by DNA sequencing using primers asd-3 and asd-4 (see, Table 1). Sequencing of the region flanking the *asd* locus was performed, and the sequence confirmed that the *asd* gene was deleted from the YS1646 chromosome.

**Complementation of *asd* Deletion by *asd* Expression from Plasmids**

A plasmid, pATIU6 (SEQ ID NO:225), was chemically synthesized and assembled. The plasmid contained the following features: a high copy (pUC19) origin of replication, a U6 promoter for driving expression of a short hairpin, an ampicillin resistance gene flanked by HindIII restriction sites for subsequent removal, and the *asd* gene containing 85 base pairs of sequence upstream of the start codon (SEQ ID

NO:246). Into this vector, shRNAs targeting murine TREX1 were introduced by restriction digestion with SpeI and XhoI, and ligation and cloning into *E. coli* DH5-alpha cells. The resulting plasmid was designated pATI-shTREX1.

**Electroporation of Plasmids into Immunostimulatory Bacterial Strains**

Selected plasmids, containing expression cassettes encoding immunostimulatory proteins and a functional *asd* gene, were electroporated into *S. typhimurium* strains lacking the *asd* gene with a BTX® ECM600 electroporator, using a 0.2 cm gap cuvette (BTX, San Diego, Calif.) at the following settings: 2.5 kV, 186 ohms, and 50 μF. Electroporated cells were added to 1 mL SOC supplemented with 50 μM diaminopimelic acid (DAP), incubated for 1 hour at 37 °C, and then spread onto agar plates that do not contain DAP, to select for strains that received plasmids with a functional *asd* gene. After single colony isolation, cell banks were produced by inoculating a flask of sterile lysogeny broth (LB) with a single well isolated colony of *S. typhimurium*, and incubating at 37 °C with agitation at 250 RPM. After the culture was grown to stationary phase, the bacteria were washed in PBS containing 10% glycerol, and stored in aliquots frozen at less than -60 °C.

The plasmid pATI-shTREX1 was amplified in *E. coli* and purified for transformation into the YS1646Δ*asd* strain by electroporation and clonal selection on LB Amp plates, to produce strain YS1646Δ*asd*-shTREX1. The YS1646Δ*asd* mutants complemented with pATIU6-derived plasmids were able to grow on LB agar and liquid media in the absence of DAP.

In a subsequent iteration, the ampicillin resistance gene ($Amp^R$) from pATI-shTREX1 was replaced with a kanamycin resistance gene. This was accomplished by digestion of the pATI-shTREX1 plasmid with HindIII, followed by gel purification to remove the $Amp^R$ gene. The kanamycin resistance ($Kan^R$) gene was amplified by PCR using primers APR-001 and APR-002 (SEQ ID NO:226 and SEQ ID NO:227, respectively), followed by digestion with HindIII, and ligation into the gel purified, digested pATIU6 plasmid.

In subsequent iterations, a single point mutation was introduced into the pATIKan plasmid at the pUC19 origin of replication, using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and the primers APR-003 (SEQ ID NO:228) and APR-004 (SEQ ID NO:229), to change the nucleotide T at position 148 to a C.

This mutation makes the origin of replication homologous to the pBR322 origin of replication, which is a low copy origin of replication, in order to reduce the plasmid copy number.

**Plasmid Maintenance Demonstrated *In Vivo* Using *asd* Complementation System**

In this example, CT26 tumor-bearing mice were treated with strain YS1646 containing a plasmid that expresses an shRNA targeting TREX1 (YS1646-shTREX1), or with an *asd*-deleted strain of YS1646, containing a plasmid with a functional *asd* gene and an shRNA targeting TREX1 (YS1646Δ*asd*-shTREX1).

CT26 (Colon Tumor #26) is a tumor model that originated from exposing BALB/c mice to N-nitro-N-methylurethane (NMU), resulting in a highly metastatic carcinoma that recapitulates the aggressive, undifferentiated and checkpoint-refractory human colorectal carcinoma (see, *e.g.*, Castle *et al.* (2014) *BMC Genomics* 15(1):190). When implanted subcutaneously in the flank, as opposed to orthotopically in the colon, the tumor immunophenotype is much more immunosuppressive and checkpoint refractory. While largely lacking in T-cell infiltration, the tumor is rich in myeloid cells, such as macrophages and myeloid-derived suppressor cells (MDSCs) (see, *e.g.*, Zhao *et al.* (2017) *Oncotarget* 8(33):54775-54787). As this model more closely resembles human microsatellite stable (MSS) colorectal cancer, it is an ideal model to evaluate the therapeutic approach provided herein.

For this experiment, 6-8 week-old female BALB/c mice (3 mice per group) were inoculated subcutaneously (SC) in the right flank with CT26 (purchased from ATCC) tumor cells ($2x10^5$ cells in 100 µL PBS). Mice bearing 8 day-old established flank tumors were intravenously (IV) injected with three doses of $5 x10^6$ CFUs of the YS1646Δ*asd*-shTREX1 strain, or the parental YS1646-shTREX1 strain, on days 8, 15, and 23. The plasmid encodes shTREX1 as an exemplary therapeutic product; any other desired therapeutic product or products can be substituted.

Body weights and tumors were measured twice weekly. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula, $1/2(\text{length} \times \text{width}^2)$. Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations.

At 12 days after the final *Salmonella* injection, tumors were homogenized, and homogenates were serially diluted and plated on LB agar plates, to enumerate the total number of colony forming units (CFUs) present, or on LB plates containing kanamycin, to enumerate the number of kanamycin resistant colonies.

The results demonstrated that *S. typhimurium* strain YS1646-shTREX1 did not have selective pressure to maintain the shRNA plasmid, and demonstrated significant plasmid loss, as the percent of kanamycin resistant ($Kan^R$) colonies was less than 10%. The strain that used the *asd* gene complementation system for plasmid maintenance, YS1646Δ*asd*-shTREX1, had nearly identical numbers of kanamycin resistant and kanamycin sensitive CFUs. These data demonstrate that the *asd* gene complementation system is sufficient to maintain the plasmid in the context of the tumor microenvironment in mice.

**Enhanced Anti-Tumor Efficacy Using *asd* Complementation System**

The *asd* complementation system is designed to prevent plasmid loss and potentiate the anti-tumor efficacy of the therapeutic product delivery by *S. typhimurium* strains *in vivo*. To test this, YS1646Δ*asd* strains containing the shTREX1 plasmid (YS1646Δ*asd*-shTREX1), or scrambled control (YS1646Δ*asd*-shSCR), that contain a functional *asd* gene cassette, were compared for anti-tumor efficacy in a murine colon carcinoma model, to strain YS1646 containing plasmid pEQU6-shTREX1 (YS1646-shTREX1), a plasmid that lacks an *asd* gene cassette, and therefore, does not have a mechanism for plasmid maintenance. shTREX1 is an exemplary therapeutic product.

For this experiment, 6-8 week-old female BALB/c mice (8 mice per group) were inoculated SC in the right flank with CT26 cells ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected twice, on day 8 and on day 18, with $5x10^6$ CFUs of YS1646Δ*asd*-shTREX1, or YS1646-shTREX1, and compared to PBS control.

The YS1646-shTREX1 strain demonstrated enhanced tumor control compared to PBS (70% tumor growth inhibition (TGI), day 28) despite its demonstrated plasmid loss over time. The Δ*asd* strain containing the plasmid with the *asd* gene complementation system and shTREX1 (YS1646Δ*asd*-shTREX1) demonstrated

superior tumor growth inhibition compared to PBS (82% TGI, $p = 0.002$, day 25). These data demonstrate that improved potency is achieved by preventing plasmid loss, using the *asd* complementation system, and delivery of shTREX1, as compared to YS1646 containing plasmids without the *asd* gene complementation system. Thus, strains with *asd* complementation systems are superior anti-cancer therapeutics.

**Example 4**

***S. typhimurium* Flagellin Knockout by Deletion of the *fliC* and *fljB* Genes**

**Strain Engineering and Characterization**

In the example herein, the live attenuated *S. typhimurium* YS1646 strain containing the *asd* gene deletion was further engineered to delete the *fliC* and *fljB* genes, in order to remove both flagellin subunits. This eliminates pro-inflammatory TLR5 activation, in order to reduce pro-inflammatory signaling and improve anti-tumor adaptive immunity.

**Deletion of *fliC* Gene**

In this example, *fliC* was deleted from the chromosome of the YS1646Δ*asd* strain using modifications of the method of Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)) as described in detail in the previous example. Briefly, synthetic *fliC* gene homology arm sequences, that contained 224 and 245 bases of homologous sequence flanking the *fliC* gene, were cloned into a plasmid called pSL0147 (SEQ ID NO:230). A kanamycin gene cassette flanked by cre/loxP sites then was cloned into plasmid pSL0147, and the *fliC* gene knockout cassette was then PCR amplified with primers flic-1 (SEQ ID NO:232) and flic-2 (SEQ ID NO:233), gel purified, and then introduced into the YS1646Δ*asd* strain carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. Electroporated cells were recovered in SOC + DAP medium, and plated onto LB agar plates supplemented with kanamycin (20 μg/mL) and diaminopimelic acid (DAP, 50 μg/mL). Colonies were selected and screened for insertion of the knockout fragment by PCR using primers flic-3 (SEQ ID NO:234) and flic-4 (SEQ ID NO:235). pKD46 then was cured by culturing the selected kanamycin resistant strain at 42 °C and screening for loss of ampicillin resistance. The kanamycin resistance marker then was cured by electroporation of a temperature-sensitive plasmid expressing the Cre recombinase (pJW168), and $Amp^R$ colonies were selected at 30 °C; pJW168 was

subsequently eliminated by growing cultures at 42 °C. Selected *fliC* knockout clones were then tested for loss of the kanamycin marker by PCR, using primers flanking the sites of disruption (flic-3 and flic-4), and evaluation of the electrophoretic mobility on agarose gels.

**Deletion of *fljB* Gene**

The *fljB* gene was then deleted from the YS1646Δ*asd*/Δ*fliC* strain using modifications of the methods described above. Synthetic *fljB* gene homology arm sequences that contained 249 and 213 bases of the left hand and right hand sequence, respectively, flanking the *fljB* gene, were synthesized and cloned into a plasmid called pSL0148 (SEQ ID NO:231). A kanamycin gene cassette flanked by cre/loxP sites then was cloned into pSL0148, and the *fljB* gene knockout cassette was PCR amplified with primers fljb-1 (SEQ ID NO:236) and fljb-2 (SEQ ID NO:237) (see, Table 1), gel purified, and introduced into strain YS1646Δ*asd*/Δ*fliC* carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination, as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *fliC* and *fljB* gene knockout sequences were amplified by PCR using primers flic-3 and flic-4, or fljb-3 (SEQ ID NO:238) and fljb-4 (SEQ ID NO:239), respectively, and verified by DNA sequencing. This mutant derivative of strain YS1646 was designated YS1646Δ*asd*/Δ*fliC*/Δ*fljB*, or YS1646Δ*asd*/ΔFLG for short.

***In Vitro* Characterization of Engineered *S. typhimurium* Flagellin Knockout Strain**

The YS1646-derived *asd*⁻ mutant strain harboring the deletions of both *fliC* and *fljB*, herein referred to as YS1646Δ*asd*/ΔFLG, was evaluated for swimming motility by spotting 10 microliters of overnight cultures onto swimming plates (LB containing 0.3% agar and 50 mg/mL DAP). While motility was observed for the YS1646Δ*asd* strain, no motility was evident with the YS1646Δ*asd*/ΔFLG strain. The YS1646Δ*asd*/ΔFLG strain then was electroporated with a plasmid containing an *asd* gene, and its growth rate in the absence of DAP was assessed. The YS1646Δ*asd*/ΔFLG strain, with an *asd* complemented plasmid was able to replicate in LB in the absence of supplemental DAP, and grew at a rate comparable to the

YS1646Δ*asd* strain containing an *asd* complemented plasmid. These data demonstrate that the elimination of flagellin does not decrease the fitness of *S. typhimurium in vitro*.

**Elimination of Flagella Decreases Pyroptosis in Murine Macrophages**

$5x10^5$ mouse RAW macrophage cells (InvivoGen, San Diego, Ca.) were infected with the YS1646Δ*asd*/ΔFLG strain, or the parental YS1646Δ*asd* strain, both harboring an *asd* complemented plasmid, at an MOI of approximately 100 in a gentamicin protection assay. After 24 hours of infection, culture supernatants were collected and assessed for lactate dehydrogenase release as a marker of macrophage cell death, using a Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, Ma.). The YS1646Δ*asd* strain induced 75% maximal LDH release, while the YS1646Δ*asd*/ΔFLG strain induced 54% maximal LDH release, demonstrating that deletion of the flagellin genes reduces the *S. typhimurium*-induced pyroptosis of infected macrophages.

**Flagella-Deleted Mutants Lead to Less Pyroptosis in Infected Human Monocytes**

To demonstrate that the YS1646Δ*asd*/ΔFLG strains are reduced in their ability to cause cell death in macrophages, THP-1 human macrophage cells (ATCC Catalog # 202165) were infected with the *S. typhimurium* strains YS1646 and YS1646Δ*asd*/ΔFLG, with the Δ*asd* strain containing plasmids encoding a functional *asd* gene to ensure plasmid maintenance. $5x10^4$ cells were placed in a 96-well dish with DMEM and 10% FBS. Cells were infected with washed log-phase cultures of *S. typhimurium* for 1 hour at an MOI of 100 CFUs per cell, then the cells were washed with PBS, and the media was replaced with media containing 50 μg/mL gentamicin to kill extracellular bacteria, and 50 ng/mL of IFNγ to convert the monocytes into a macrophage phenotype. After 24 hours, the THP-1 cells were stained with CellTiter-Glo® reagent (Promega), and the percentage of viable cells was determined using a luminescent cell viability assay using a SpectraMax® MR plate reader (Molecular Devices) to quantify the luminescence. The cells infected with the YS1646 strain had only 38% viability, while the cells infected with the YS1646Δ*asd*/ΔFLG strain had

51% viability, indicating that the deletion of the flagellin genes induced less cell death of human macrophages, despite a very high and supraphysiological MOI.

**Flagella is not Required for Tumor Colonization After Systemic Administration**

To assess the impact of the flagellin knockout strains, administered in a murine model of colon carcinoma, 6-8 week-old female BALB/c mice (5 mice per group) were inoculated SC in the right flank with CT26 cells ($2x10^5$ cells in 100 µL PBS). Mice bearing 10-day established flank tumors were IV injected with a single dose of 3 $x10^5$ CFUs of the YS1646Δ*asd*/ΔFLG-shTREX1 strain, or the parental YS1646Δ*asd*-shTREX1 strain. At day 35 post tumor implantation, mice were euthanized, and tumors were homogenized and plated on LB plates to enumerate the number of colony forming units (CFUs) per gram of tumor tissue. The YS1646Δ*asd*-shTREX1 strain colonized tumors at a mean of 5.9 $x10^7$ CFUs per gram of tumor tissue, while the flagella-deleted YS1646Δ*asd*/ΔFLG-shTREX1 strain colonized the tumors with almost a 2-fold increased mean of 1.1 x $10^8$ CFUs/g of tumor tissue. The splenic colonization of the YS1646Δ*asd*-shTREX1 strain was calculated as a mean of 1.5 x $10^3$ CFUs/g of spleen tissue, whereas splenic colonization of the flagella-deleted YS1646Δ*asd*/ΔFLG-shTREX1 strain was slightly lower, at a mean of 1.2 x $10^3$ CFUs/g of spleen tissue.

These data demonstrate that the absence of flagella not only does not negatively impact tumor colonization after IV administration, but it enhances tumor colonization compared to the flagella-intact strain. Importantly, deletion of the flagella slightly reduces splenic colonization, giving a tumor to spleen ratio of 100,000-fold. These data demonstrate that, contrary to the expectation from the art, not only are the flagella not required for tumor colonization, but their elimination enhances tumor colonization, while reducing splenic colonization.

**The Flagella-Deleted Strain Demonstrates Enhanced Anti-Tumor Activity in Mice**

To assess the impact of the flagellin knockout strains, administered in a murine model of colon carcinoma, 6-8 week-old female BALB/c mice (5 mice per group) were inoculated SC in the right flank with CT26 cells ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected with a single dose of 3 $x10^5$ CFUs of the YS1646Δ*asd*/ΔFLG-shTREX1 strain, or the YS1646Δ*asd*-

shTREX1 strain, and compared to PBS control. Mice were monitored by caliper measurements for tumor growth.

The results demonstrated that the YS1646Δ*asd*/ΔFLG-shTREX1 strain, incapable of making flagella, showed enhanced tumor control compared to the parental YS1646Δ*asd*-shTREX1 strain (27% TGI, day 24), and significant tumor control compared to the PBS control (73% TGI, $p = 0.04$, day 24). These data demonstrate that, not only is the flagella not required for tumor colonization, but its loss can enhance anti-tumor efficacy.

**Flagella-Deleted Strains Demonstrate Enhanced Adaptive Immunity in a Murine Tumor Model**

The impact of deletion of the flagellin on the immune response, and whether STING activation from tumor myeloid cell-delivery of shRNA to the STING checkpoint gene TREX1 would promote an adaptive type I IFN immune signature, was assessed. The CT26 murine model of colon carcinoma was used, where 6-8 week-old female BALB/c mice (5 mice per group) were inoculated SC in the right flank with CT26 cells ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected 11 days post tumor implantation with $5 x10^6$ CFUs of the YS1646Δ*asd*/ΔFLG-shTREX1 strain, or the parental YS1646Δ*asd*-shTREX1, or the scrambled plasmid control strain, YS1646Δ*asd*-shSCR, and compared to PBS control.

Mice were bled 7 days post-dosing on Sodium Heparin coated tubes (Becton Dickinson). Non-coagulated blood was then diluted in the same volume of PBS and peripheral blood mononuclear cells (PBMCs) were separated from the interphase layer of whole blood using Lympholyte®-M cell separation reagent (Cedarlane). Isolated PBMCs were washed with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes at room temperature, and resuspended in flow buffer. One million PBMCs were seeded per well of a V-bottom 96-well plate. Cells were centrifuged at 1300 RPM for 3 minutes at room temperature (RT) and resuspended in 100 µL of flow buffer containing fluorochrome-conjugated AH1 peptide:MHC class I tetramers (MBL International), and the cell surface flow cytometry antibodies CD4 FITC clone RM4-5; CD8a BV421 clone 53-6.7; F4/80 APC clone BM8; CD11b PE-Cy7 clone M1/70; CD45 BV570 clone 30-F11; CD3 PE clone 145-2C11; Ly6C BV785 clone HK1.4; I-A/I-E APC-Cy7 clone M5/114.15.2; Ly6G BV605 clone 1A8; and CD24

PercP-Cy5.5 clone M1/69 (all from BioLegend), for 45 minutes at room temperature and in the dark. After 45 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1200 RPM for 3 minutes. The cells were then resuspended in PBS + 2% FBS containing DAPI (4',6-diamino-2-phenylindole; dead/live stain), and data were immediately acquired using the NovoCyte® flow cytometer (ACEA Biosciences, Inc.) and analyzed using FlowJo™ software (Tree Star, Inc.).

The following cell types were enumerated as a percentage of total live cells: $CD11b^+$ $Gr1^+$ neutrophils (possibly MDSCs, although further phenotyping in an *ex vivo* functional assay would be required), $CD11b^+$ $F4/80^+$ macrophages, $CD8^+$ T-cells, and $CD8^+$ T-cells that recognize the CT26 tumor rejection antigen gp70 (AH1), the product of the envelope gene of murine leukemia virus (MuLV)-related cell surface antigen (see, *e.g.*, Castle *et al.* (2014) *BMC Genomics* 15(1):190).

The results, summarized in the table below, show that the YS1646Δ*asd*-shSCR strain, containing a plasmid encoding a non-specific scrambled shRNA, elicits the typical anti-bacterial immune profile of significantly increased neutrophils, as compared to PBS ($p = 0.02$), to the flagella-intact YS1646Δ*asd*-shTREX1 strain ($p = 0.02$), and to the flagella-deleted strain YS1646Δ*asd*/ΔFLG-shTREX1 ($p = 0.01$), which had the lowest levels of circulating neutrophils. Similarly, bacterially-induced macrophages also were significantly elevated in the YS1646Δ*asd*-shSCR strain, as compared to PBS ($p = 0.01$), to the YS1646Δ*asd*-shTREX1 strain ($p = 0.01$), and to YS1646Δ*asd*/ΔFLG-shTREX1 strain ($p = 0.01$). Thus, both strains carrying type I IFN-inducing payloads were capable of overwriting the normal anti-bacterial immune response, which clears bacterial infections through neutrophils and macrophages, and does not induce adaptive T-cell-mediate immunity. However, while the overall circulating levels of $CD8^+$ T-cells were similar across all groups, the flagella-deleted YS1646Δ*asd*/ΔFLG-shTREX1 strain demonstrated significantly increased percentages of AH1-$tetramer^+$ $CD8^+$ T-cells, as compared to PBS ($p = 0.04$).

These data demonstrate the feasibility of engineering a bacteria to deliver viral-like type I IFN-inducing plasmids to tumor-resident myeloid cells. This results in a dramatic reprogramming of the immune response towards a more viral, and less bacterial, immune profile. Deletion of the flagella further enhanced the shift away from bacterially-recruited neutrophils and macrophages, and towards significantly

increased tumor antigen-specific $CD8^+$ T-cells. Thus, eliminating bacterial TLR5-mediated inflammation can enhance adaptive immunity.

| Immune Cells | % Live Cells Mean ± SD | | | |
|---|---|---|---|---|
| | **PBS** | **YS1646Δ*asd*-shSCR** | **YS1646Δ*asd*-shTREX1** | **YS1646Δ*asd*/ΔFLG-shTREX1** |
| **Neutrophils** | 6.27 ± 2.62 | 19.21 ± 9.46 | 5.87 ± 3.94 | 4.01 ± 1.65 |
| **Macrophages** | 10.08 ± 2.11 | 23.14 ± 9.04 | 9.12 ± 3.84 | 7.39 ± 2.11 |
| **$CD8^+$ T-Cells** | 6.64 ± 0.56 | 7.17 ± 0.60 | 7.14 ± 2.30 | 6.44 + 1.43 |
| **$AH1^+$ $CD8^+$ T-Cells** | 0.83 ± 0.12 | 1.06 ± 1.11 | 2.27 ± 1.44 | 4.12 ± 3.08 |

SD = Standard deviation

**Flagella-Deleted Strains are Restricted to the Phagocytic Myeloid Immune Cell Compartment *In Vivo***

According to the literature, Δ*fljB*/Δ*fliC* strains demonstrate suppression of many downstream genes associated with SPI-1-mediated entry into non-phagocytic cells. In order to determine whether the YS1646Δ*asd*/ΔFLG strain also is deficient for non-phagocytic cell uptake, a YS1646Δ*asd*/ΔFLG strain, constitutively expressing mCherry (a red fluorescent protein) under the bacterial *rpsM* promoter, was IV administered to MC38 subcutaneous flank tumor-bearing mice.

The MC38 (murine colon adenocarcinoma #38) model was derived similarly as the CT26 model using mutagenesis, but with dimethylhydralazine, and in a C57BL/6 mouse strain (see, *e.g.*, Corbett *et al.* (1975) *Cancer Res.* 35(9):2434-2439). Similarly to CT26, subcutaneous implantation results in a more T-cell excluded and immunosuppressive tumor microenvironment than when implanted orthotopically in the colon (see, *e.g.*, Zhao *et al.* (2017) *Oncotarget* 8(33):54775-54787). MC38 has a higher mutational burden than CT26, and a similar viral-derived gp70 antigen (p15E) that can be detected by $CD8^+$ T-cells, although it is not considered a rejection antigen. While variants of MC38 have been found to be partially responsive to checkpoint therapy, most variants of the cell line are considered checkpoint refractory and T-cell excluded (see, *e.g.*, Mariathasan *et al.* (2018) *Nature* 555:544-548), including the MC38 cells used herein.

For this experiment, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 cells ($5x10^5$ cells in 100 µL PBS).

Mice bearing large established flank tumors were IV injected on day 34 with $1x10^6$ CFUs of the YS1646Δ*asd*/ΔFLG-mCherry strain. Tumors were resected 7 days post-IV dosing, and cut into 2-3 mm pieces into gentleMACS™ C tubes (Miltenyi Biotec) filled with 2.5 mL enzyme mix (RPMI-1640 containing 10% FBS with 1 mg/mL Collagenase IV and 20 µg/mL DNase I). The tumor pieces were dissociated using OctoMACS™ (Miltenyi Biotec) specific dissociation program (mouse implanted tumors), and the whole cell preparation was incubated with agitation for 45 minutes at 37 °C. After the 45 minute incubation, a second round of dissociation was performed using the OctoMACS™ (mouse implanted tumor) program, and the resulting single cell suspensions were filtered through a 70 µM nylon mesh into a 50 mL tube. The nylon mesh was washed once with 5 mL of RPMI-1640 + 10% FBS, and the cells were filtered a second time using a new 70 µM nylon mesh into a new 50 mL tube. The nylon mesh was washed with 5 mL of RPMI-1640 + 10% FBS, and the filtered cells were then centrifuged at 1000 RPM for 7 minutes. The resulting dissociated cells were resuspended in PBS and kept on ice before the staining process.

For the flow-cytometry staining, 100 µL of the single cell suspensions were seeded in wells of a V-bottom 96-well plate. PBS containing a dead/live stain (Zombie Aqua™, BioLegend), and Fc Blocking reagents (BD Biosciences), were added at 100 µL per well and the cells were incubated on ice for 30 minutes in the dark. After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. Cells were then resuspended in PBS + 2% FBS containing fluorochrome-conjugated antibodies (CD4 FITC clone RM4-5; CD8a BV421 clone 53-6.7; F4/80 APC clone BM8; CD11b PE-Cy7 clone M1/70; CD45 BV570 clone 30-F11; CD3 PE clone 145-2C11; Ly6C BV785 clone HK1.4; I-A/I-E APC-Cy7 clone M5/114.15.2; Ly6G BV605 clone 1A8; and CD24 PercP-Cy5.5 clone M1/69, all from BioLegend), and incubated on ice for 30 minutes in the dark. After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes and resuspended in flow cytometry fixation buffer (Thermo Fisher Scientific). Flow cytometry data were acquired using the NovoCyte® Flow Cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

The results demonstrated that 7.27% of tumor-infiltrating monocytes had taken up the flagella-deleted mCherry strain in the tumor microenvironment. Similarly, 8.96% of the tumor-associated macrophage (TAM) population, and 3.33% of the tumor-infiltrating dendritic cells (DCs) had taken up the flagella-deleted mCherry strain. In contrast, within the $CD45^-$ population, corresponding to stromal and tumor cells, only 0.076% showed positivity for mCherry expression (compared to 0.067% background staining). These data demonstrate that the flagella and its downstream signaling impact on SPI-1, are necessary to enable epithelial cell infectivity, and that the lack thereof restricts uptake of the bacteria to only the phagocytic immune cell compartment of the tumor microenvironment (*i.e.*, tumor-resident immune/myeloid cells).

Deletion of the flagella confers multiple benefits to the immunostimulatory *S. typhimurium* strain, including eliminating TLR5-induced inflammatory cytokines that suppress adaptive immunity, reducing macrophage pyroptosis, as well as maintaining (or enhancing) tumor-specific enrichment upon systemic administration, where uptake is confined to tumor-resident phagocytic cells.

## Example 5

### *Salmonella pagP* Gene Knockout Strain Engineering and Characterization

In this example, the YS1646Δ*asd*/ΔFLG strain was further modified to delete *pagP*. The *pagP* gene is induced during the infectious life cycle of *S. typhimurium*, and encodes an enzyme (lipid A palmitoyltransferase) that modifies lipid A with palmitate. In wild-type *S. typhimurium*, expression of *pagP* results in a lipid A molecule that is hepta-acylated. In an $msbB^-$ mutant, in which the terminal acyl chain of lipid A cannot be added, the expression of *pagP* results in a hexa-acylated lipid A molecule. LPS with hexa-acylated lipid A has been shown to be highly pro-inflammatory, and to have a high affinity for TLR4 (hepta-acylated lipid A, found in wild-type, has the highest affinity for TLR4). In this example, a strain deleted of *pagP* and *msbB* can produce only penta-acylated lipid A, allowing for lower pro-inflammatory cytokines due to low affinity for TLR4, enhanced tolerability, and increased adaptive immunity when the bacteria are engineered to deliver plasmids encoding immunomodulatory proteins.

**Δ*pagP* Strain Construction**

The *pagP* gene was deleted from the YS1646Δ*asd*/ΔFLG strain using modifications of the methods described in the preceding examples. Synthetic *pagP* gene homology arm sequences that contain 203 and 279 bases of the left hand and right hand sequence, respectively, flanking the *pagP* gene, were synthesized and cloned into a plasmid called pSL0191 (SEQ ID NO:331). A kanamycin gene cassette flanked by cre/loxP sites then was cloned into pSL0191, and the *pagP* gene knockout cassette was PCR amplified with primers pagp-1 (SEQ ID NO:315) and pagp-2 (SEQ ID NO:316) (see, Table 1), gel purified, and introduced into strain YS1646Δ*asd*/ΔFLG, carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination, as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *pagP* gene knockout sequences were amplified by PCR using primers pagp-3 (SEQ ID NO:317) and pagp-4 (SEQ ID NO:318), and verified by DNA sequencing. The resulting mutant derivative of YS1646 was designated YS1646Δ*asd*/ΔFLG/Δ*pagP*.

***pagP* Deletion Mutants have LPS with Penta-Acylated Lipid A, and Induce Reduced Inflammatory Cytokines**

The *pagP* gene also was deleted from the YS1646Δ*asd* strain using the lambda-derived Red recombination system, as described in Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)) and above, to generate the strain YS1646Δ*asd*/Δ*pagP*. This strain was then electroporated with a plasmid containing a functional *asd* gene, to complement the deleted *asd* gene and to ensure plasmid maintenance *in vivo*. The lipid A was then extracted from this strain and evaluated by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI MS) and compared to lipid A from the wild-type *S. typhimurium* strain ATCC 14028, the YS1646 strain (which is deleted for *msbB* and *purI*), and the YS1646Δ*asd* strain. Wild-type *Salmonella* had a minor lipid A peak with a mass of 2034, and a major peak with a mass of 1796, corresponding to the hepta-acylated and hexa-acylated species, respectively, due to the presence of a functional *msbB* gene. The *msbB*-deleted strains, YS1646 and YS1646Δ*asd*, had major peaks at 1828 and 1585, corresponding to a mixture of hexa-acylated and penta-acylated lipid A. The *msbB*

and *pagP* deleted strain, YS1646Δ*asd*/Δ*pagP*, had only a single peak with a mass of 1585, corresponding to penta-acylated lipid A. These data demonstrate that deletion of *pagP* prevents palmitoylation of the lipid A, thereby restricting it to a single penta-acylated species.

To determine whether the LPS with penta-acylated lipid A from the Δ*pagP* mutant strains reduced TLR4 signaling, 4 μg of purified LPS from the wild-type strain, the YS1646 strain, or the YS1646Δ*asd*/Δ*pagP* strain, was added to THP-1 human monocytic cells (ATCC Catalog # TIB-202), and the supernatants were evaluated 24 hours later for the presence of inflammatory cytokines using a Cytometric Bead Array (CBA) kit (BD Biosciences). The results showed that LPS from the YS1646Δ*asd*/Δ*pagP* strain induced 25% of the amount of TNFα, compared to wild-type LPS, and induced 7-fold less IL-6 than wild-type LPS. The LPS from the YS1646Δ*asd*/Δ*pagP* strain induced 22-fold less IL-6 than strain YS1646, demonstrating that the penta-acylated LPS species from a Δ*pagP* mutant is significantly less inflammatory in human cells, and indicating that the Δ*pagP* mutant would be better tolerated in humans.

**Deletion of *pagP* Induces Significantly Less IL-6 in Primary Human M2 Macrophages**

To demonstrate that the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain also elicits less inflammatory and dose-limiting IL-6 from primary human M2 macrophages, the strain was evaluated, and compared with the YS1646Δ*asd*/ΔFLG and the parental YS1646 strains. The M2 macrophages derived from human donors are representative of the immunosuppressive phenotypes that are highly enriched in T-cell excluded solid tumors. Frozen human PBMCs, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 1X non-essential amino acids + 5% human AB serum), and washed by centrifugation for 10 minutes at 800 RPM at room temperature. PBMCs were resuspended in PBS + 2% FBS, and monocytes were negatively isolated using a CD16 depletion kit (StemCell Technologies). Isolated untouched monocytes were then washed by centrifugation in PBS + 2% FBS and resuspended in complete medium containing 100 ng/mL human macrophage colony-stimulating factor (M-CSF) and 10 ng/mL human IL-4. Isolated monocytes (3e5 per well) were then seeded in a 24-well plate with a final volume of 750 μL. Two days

after seeding, the cell culture media was entirely aspirated and replaced with fresh complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4. Two days later (on day 4), 500 µL of complete medium, containing cytokines100 ng/mL human M-CSF and 10 ng/mL human IL-4, was added per well for 48 hours. On day 6, the cell culture media was entirely aspirated and replaced with fresh complete medium without cytokines, alone, or with media containing the log-phase cultures of the *S. typhimurium* strains at an MOI of 20. Cells were infected for 1 hour, then washed with PBS, and the media was replaced with fresh media containing 50 µg/mL gentamicin to kill extracellular bacteria. The wells were then washed and replaced with fresh media and allowed to incubate at 37 °C and 5% $CO_2$. After 48 hours, supernatants were harvested and assayed for cytokines using a human IL-6 cytometric bead array (CBA) kit (BD Biosciences), according to the manufacturer's instructions.

The results demonstrated that secreted IL-6 levels from human primary M2 macrophages, infected with parental strain YS1646, yielded an average of 14839 ± 926 pg/mL, while the IL-6 levels from the YS1646Δ*asd*/ΔFLG strain were significantly lower, at 2075 ± 723 pg/mL ($p = 0.004$). This further affirms the impact that the deletion of flagella, and elimination of TLR5 signaling, has on the induction of IL-6. The YS1646Δ*asd*/ΔFLG/Δ*pagP* strain elicited the lowest IL-6 levels, at 332 ± 100 pg/mL, demonstrating the reduced ability of this modified LPS coating to stimulate TLR4, and the resulting dramatically reduced inflammatory IL-6 production.

**The Combined Flagella and *pagP* Deletions Significantly Enhance Tolerability in Mice**

To determine whether the modified strains described above are more attenuated than parental strain YS1646, a median lethal dose ($LD_{50}$) study was conducted. 6-8 week-old BALB/c mice (5 mice per group) were injected intravenously with a dose range of 3e5 to 3e7 CFUs of strain YS1646, or the derivative strains YS1646Δ*asd*/ΔFLG, YS1646Δ*asd*/Δ*pagP*, and YS1646Δ*asd*/ΔFLG/Δ*pagP*. Unlike strain YS1646, the derivative strains also carried a plasmid encoding murine IL-2, an FDA-approved cytokine that has demonstrated significant toxicity when systemically administered.

The $LD_{50}$ for strain YS1646 was found to be 4.4 x $10^6$ CFUs (average of two studies), in line with previously published $LD_{50}$ reports of YS1646, and a >1000-fold improvement compared to wild-type *S. typhimurium* (see, *e.g.*, Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002). The $LD_{50}$ for the YS1646Δ*asd*/ΔFLG strain was determined to be 2.07 x $10^7$ CFUs, demonstrating a greater than 4.5-fold reduction in virulence compared to strain YS1646. The $LD_{50}$ for the YS1646Δ*asd*/Δ*pagP* strain was determined to be 1.39 x $10^6$ CFUs, demonstrating at least a 3.2-fold reduction in virulence compared to strain YS1646, which is expected, given that the strain still has highly inflammatory flagella. The $LD_{50}$ for the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain could not be established, as no mice died at the highest dose given, but was >6.2 x $10^7$ CFUs. The YS1646Δ*asd*/ΔFLG/Δ*pagP* strain therefore demonstrates a >14-fold reduction in virulence compared to parental YS1646 strain. These data demonstrate that the genetic modifications described above reduce the virulence of the clinical *S. typhimurium* strain, YS1646 (also known as VNP20009), and therefore, lead to increased tolerability in humans.

In the Phase I clinical trial of VNP20009 (see, *e.g.*, Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152), the presence of the bacteria in patients' tumors only partially was observed at the two highest doses tested, 3 x $10^8$ CFU/$m^2$ (33% presence), and 1 x $10^9$ CFU/$m^2$ (50% presence), indicating that the tolerable dose of VNP20009 was too low to achieve tumor colonization. By improving the tolerability of the strains through the modifications described above, >14-fold higher doses can be administered, if necessary, improving the percentage of patients whose tumors will be colonized, and increasing the level of therapeutic colonization per tumor, thereby solving the observed problems with VNP20009.

**The Combined Flagella and *pagP* Deletions Significantly Limit the Generation of Anti-*S. typhimurium* Antibodies in Mice**

The surviving mice from the 3 x $10^6$ CFU dosing group described above (N=5, except for N=4 in the YS1646 dosing group) were kept for 40 days post IV dosing, at which time they were bled for serum, and assessed for antibody titers to *S. typhimurium*, by a modified flow-based antibody titering system. Overnight cultures of the YS1646Δ*asd*/ΔFLG-mCherry strain were washed and fixed with flow cytometry fixation buffer. Sera from the previously-treated mice, and from naïve

control mice, were seeded in a 96-well plate, and serial dilutions were performed in PBS. Next, 25 µL of the YS1646Δ*asd*/ΔFLG-mCherry cultures, containing 1 x $10^6$ CFUs, were added to the sera and incubated for 25 minutes at room temperature (RT). The bacteria were then washed twice with PBS by spinning them at 4000 RPM for 5 minutes. After the last wash, the bacteria were resuspended in PBS containing a secondary Goat anti-Mouse Fc AF488 antibody (1/400 dilution from stock), and incubated for 25 minutes at room temperature and protected from light. The bacteria were then washed three times with PBS by spinning them at 4000 RPM for 5 minutes. After the last wash, the bacteria were resuspended in PBS and data were acquired using the NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the MFI FlowJo™ software (Tree Star, Inc.).

To evaluate the results by flow cytometry, the highest dilution with signal in all groups was chosen (the 1250X serum dilution), and the corresponding mean fluorescence intensity (MFI) values were plotted. The limit of detection (LOD) was chosen at an MFI of 1000, as that is the MFI obtained without staining, as well as with background staining with Goat anti-Mouse Fc AF488 antibody only. Therefore, an MFI greater than 1000 was considered a positive signal, and everything equal to or under this value was considered a negative result, despite having an MFI value.

The results of this assay revealed a high MFI titer of anti-*S. typhimurium* serum antibodies from mice treated with 3 x $10^6$ CFUs of the YS1646 strain (MFI of 29196.3 ± 20730), in line with previously published data that YS1646 is able to generate serum antibodies (that are non-neutralizing). Fewer antibodies were detected in the mice treated with the YS1646Δ*asd*/ΔFLG strain (MFI of 11257 ± 9290), which can be due to the lack of adjuvant activity from the flagella. In the mice treated with the YS1646Δ*asd*/Δ*pagP* strain, significantly fewer antibodies were generated (MFI of 4494 ± 3861), as compared to strain YS1646 ($p = 0.033$), which can be due to the altered LPS surface coating. The most significant reduction in serum antibodies was demonstrated in the YS1646Δ*asd*/ΔFLG/Δ*pagP* treatment group (MFI of 1930 ± 2445), where several of the mice had MFI titers under 1000, and were thus considered negative for serum antibodies ($p = 0.021$, *vs.* strain YS1646). Thus, the combined deletions of the flagella and the *pagP* gene enable both improved safety, as well as

significantly reduced immunogenicity, which will enable repeat dosing of high CFUs in humans.

***pagP* and Flagella Deleted Strains, and their Combination, Demonstrate Significantly Higher Viability in Human Serum Compared to Strain YS1646**

Strain YS1646 exhibits limited tumor colonization in humans after systemic administration. It is shown herein that strain YS1646 is inactivated by complement factors in human blood. To demonstrate this, strains YS1646 and *E. coli* D10B were compared to exemplary immunostimulatory bacteria provided herein, that contain additional mutations that alter the surface of the bacteria. These exemplary modified strains were YS1646Δ*asd*/Δ*pagP*, YS1646Δ*asd*/ΔFLG, and YS1646Δ*asd*/ΔFLG/Δ*pagP*. These three strains, in addition to YS1646 and *E. coli* D10B cultures, were incubated with serum, or with heat-inactivated (HI) serum, from either pooled mouse blood, or pooled healthy human donors (n=3), for 3 hours at 37 °C. After incubation with serum, bacteria were serially diluted and plated on LB agar plates, and the colony forming units (CFUs) were determined.

In mouse serum, all strains remained 100% viable and were completely resistant to complement inactivation. In human serum, all strains were 100% viable in the heat-inactivated serum. The *E. coli* D10B strain was completely eliminated after 3 hours in whole human serum. In whole human serum, the YS1646 strain exhibited only 6.37% of live colonies, demonstrating that tumor colonization of the YS1646 clinical strain was limited due to complement inactivation in human blood. For the YS1646Δ*asd*/ΔFLG strain, 31.47% of live colonies remained, and for the YS1646Δ*asd*/Δ*pagP* strain, 72.9% of live colonies remained, after incubation with human serum for 3 hours. The combined YS1646Δ*asd*/ΔFLG/Δ*pagP* strain was completely resistant to complement in human serum.

These data explain why strain YS1646 (VNP20009) has very low tumor colonization when systemically administered. It is shown herein that strain YS1646 is highly sensitive to complement inactivation in human serum, but not in mouse serum. These data explain why limited tumor colonization was observed in humans, while mouse tumors were colonized at a high level. The *fljB*/*fliC* or *pagP* deletions, or the combination of these mutations, partially or completely rescues this phenotype. Thus, the enhanced stability observed in human serum with the YS1646Δ*asd*/Δ*pagP*,

YS1646Δ*asd*/ΔFLG, and YS1646Δ*asd*/ΔFLG/Δ*pagP* strains provides for increased human tumor colonization.

**Example 6**

***Salmonella ansB* Gene Knockout Strain Engineering and Characterization**

In this example, the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain was further modified to delete *ansB*, the gene encoding bacterial L-asparaginase II. Secretion of L-asparaginase II by *S. typhimurium* in the presence of T-cells has been shown to directly impair T-cell function, by reducing T-cell receptor (TCR) expression, and impairing cytolytic cytokine production. As a result, bacterially-derived asparaginases have been successfully used to treat acute lymphoblastic leukemia (ALL) for decades. Deletion of *ansB* from the bacterial genome eliminates the ability of the *S. typhimurium* strain to produce L-asparaginase II, thereby enhancing the function of T-cells in the bacterially-colonized tumor microenvironment.

**Δ*ansB* Strain Construction**

The *ansB* gene was deleted from the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain using modifications of the methods described in the preceding examples. Synthetic *ansB* gene homology arm sequences that contained 236 and 251 bases of the left hand and right hand sequence, respectively, flanking the *ansB* gene, were synthesized and cloned into a plasmid called pSL0230 (SEQ ID NO:332). A kanamycin gene cassette flanked by cre/loxP sites then was cloned into plasmid pSL0230, and the *ansB* gene knockout cassette was PCR amplified with primers ansb-1 (SEQ ID NO:319) and ansb-2 (SEQ ID NO:320), gel purified, and introduced into strain YS1646Δ*asd*/ΔFLG/Δ*pagP*, carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination, as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *ansB* gene knockout sequences were amplified by PCR using primers ansb-3 (SEQ ID NO:321) and ansb-4 (SEQ ID NO:322) (see, Table 1), and verified by DNA sequencing. The resulting mutant derivative of YS1646 was designated YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*.

**Deletion of *ansB* Eliminates Asparaginase Activity *In Vitro***

In order to determine whether the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain produced less L-asparaginase II, cultures of this strain, along with the *ansB*-intact YS1646Δ*asd*/ΔFLG/Δ*pagP* strain, were grown in LB and allowed to reach stationary phase. At this time, 50 µL of conditioned media from the cultures was analyzed for asparaginase activity, using a colorimetric Asparaginase assay kit (Sigma-Aldrich), per the manufacturer's instructions. After a 40 minute incubation, the absorbance units were read on a SpectraMax® M3 Spectrophotometer (Molecular Devices) at an absorbance wavelength of 570 nm.

Compared to the recombinant L-asparaginase II positive control, which gave an absorbance of 1.95, the absorbance of the *ansB*-intact YS1646Δ*asd*/ΔFLG/Δ*pagP* strain was 0.82. Deletion of *ansB*, in the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain, however, resulted in background levels of asparaginase activity, detected at an absorbance of 0.109. These data confirm that the Δ*ansB* mutation completely eliminates asparaginase activity.

**Deletion of *ansB* Restores T-Cell Function in an *In Vitro* Co-Culture Assay**

In order to functionally characterize the *ansB*-deleted strain for the impact of reduced L-asparaginase II activity on T-cells, a co-culture assay was established using strain-infected murine primary bone marrow-derived macrophages (BMMs) in culture with splenic purified T-cells. For this assay, spleens from healthy BALB/c mice were isolated and dissociated, and splenic $CD4^+$ and $CD8^+$ T-cells were isolated using a mouse T-cell isolation kit (StemCell Technologies), per the manufacturer's instructions. From the isolated T-cells, 2e5 cells were added per well to a Flat-bottom 96-well plate that had been previously coated with 5 µg/ml of an anti-mouse CD3ε antibody (clone 145-2C11, Thermo Fisher Scientific). Conditioned LB media from the YS1646Δ*asd*/ΔFLG/Δ*pagP* and YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* cultures, grown to stationary phase, were filtered through a 0.45 µM nylon mesh and added to the T-cells, with or without the addition of 10 µg/ml of an agonistic anti-CD28 antibody for co-stimulation. Control groups containing recombinant asparaginase at 20 U/mL, and normal culture media, were used as controls in the assay. The plate was incubated at 37 °C in a 5% $CO_2$ incubator. At 24 hours post-incubation, 100 µL of the co-culture supernatants were harvested from the wells, and a murine Th1-specific

cytokine bead array (CBA, BioLegend) was performed. Concurrently, T-cells were harvested and analyzed for surface T-cell receptor β (TCRβ) expression on $CD4^+$ and $CD8^+$ T-cells by flow cytometry, as well as for intracellular staining of IFNγ, TNFα, and IL-2.

The results confirmed that the *ansB*-intact strain (YS1646Δ*asd*/ΔFLG/Δ*pagP*) used to infect the macrophages, and subsequently co-cultured with T-cells, induces profound T-cell immunosuppression. This was exhibited by marked downregulation of TCRβ surface expression in both $CD4^+$ and $CD8^+$ T-cells (see table below), compared to media control, and to the positive control of recombinant asparaginase at a concentration of 20 U/mL. Deletion of *ansB* in the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain significantly restored TCRβ surface expression in both $CD4^+$ ($p = 0.004$) and $CD8^+$ T-cells ($p = 0.002$), as compared to the parental YS1646Δ*asd*/ΔFLG/Δ*pagP* strain.

| Treatment | TCRβ Expression (MFI Mean ± SD) | |
|---|---|---|
| | **$CD4^+$ T-Cells** | **$CD8^+$ T-Cells** |
| Media control | 8141.0 ± 405.9 | 12655.0 ± 534.6 |
| Strain YS1646Δ*asd*/ΔFLG/Δ*pagP* | 3817.0 ± 200.8 | 6492.0 ± 260.2 |
| Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* | 7047.5 ± 204.4 | 13350.0 ± 339.4 |
| Recombinant asparaginase II (20 U/mL) | 4253.5 ± 576.3 | 6305.0 ± 687.3 |

SD = Standard deviation

T-cell secretion of cytokines, 24 hours after co-culture, was measured as a marker of T-cell cytolytic function. As shown in the table below, T-cell production of the cytokines IFNγ, TNFα, and IL-2 was markedly lower after treatment with the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain, as compared to the media control, and was significantly restored by *ansB* deletion in the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain (IFNγ ($p = 0.05$), TNFα ($p = 0.012$), and IL-2 ($p = 0.006$)). These data indicate that deletion of *ansB* in the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain significantly restores T-cell cytolytic function, as compared to the parental YS1646Δ*asd*/ΔFLG/Δ*pagP* strain.

| Treatment | T-Cell Cytokine Expression (pg/mL, Mean ± SD) | | |
|---|---|---|---|
| | **IFNγ** | **TNFα** | **IL-2** |
| Media control | 4176.1 ± 20.8 | 136.0 ± 11.1 | 3118.2 ± 154.3 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP* | 238.1 ± 0.6 | 35.7 ± 7.1 | 134.9 ± 12.7 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* | 1788.9 ± 515.5 | 120.5 ± 11.2 | 1947.6 + 190.5 |

| | | | |
|---|---|---|---|
| Recombinant asparaginase II (20 U/mL) | 166.2 ± 19.7 | 33.2 ± 3.4 | 114.5 ± 4.8 |

SD = Standard deviation

**Deletion of *ansB* Restores Tumor-Resident T-Cell TCRβ Expression *In Vivo***

In *in vitro* co-culture assays, expression of *ansB* demonstrated immunosuppressive effects on T-cell function, including downregulation of TCRβ on T-cells by flow cytometry. In order to assess whether this would similarly occur *in vivo*, the MC38 mouse model of colorectal cancer was utilized.

For this experiment, 6-8 week-old female C57BL/6 mice (4 mice per group) were inoculated SC in the right flank with MC38 cells ($5x10^5$ cells in 100 µL PBS). Mice bearing large established flank tumors were IV injected on day 17 with $1x10^7$ CFUs of the YS1646Δ*asd*/ΔFLG-mCherry strain. Tumors were resected 7 days post IV dosing, and cut into 2-3 mm pieces into gentleMACS™ C tubes (Miltenyi Biotec) filled with 2.5 mL enzyme mix (RPMI-1640 containing 10% FBS with 1 mg/mL Collagenase IV and 20 µg/mL DNase I). The tumor pieces were dissociated using OctoMACS™ (Miltenyi Biotec) specific dissociation program (mouse implanted tumors), and the whole cell preparation was incubated with agitation for 45 minutes at 37 °C. After 45 minutes of incubation, a second round of dissociation was performed using the OctoMACS™ (mouse implanted tumor) program, and the resulting single cell suspensions were filtered through a 70 µM nylon mesh into a 50 mL tube. The nylon mesh was washed once with 5 mL of RPMI-1640 + 10% FBS, and the cells were filtered a second time using a new 70 µM nylon mesh, into a new 50 mL tube. The nylon mesh was washed with 5 mL of RPMI-1640 + 10% FBS, and the filtered cells were then centrifuged at 1000 RPM for 7 minutes. The resulting dissociated cells were resuspended in PBS and kept on ice before the staining process.

For the flow-cytometry staining, 100 µL of the single cell suspensions were seeded in wells of a V-bottom 96-well plate. PBS containing a dead/live stain (Zombie Aqua™, BioLegend) and Fc Blocking reagents (BD Biosciences) were added at 100 µL per well, and the cells were incubated on ice for 30 minutes in the dark. After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. Cells were then resuspended in PBS + 2% FBS containing fluorochrome-conjugated antibodies (CD45 BV570 clone 30-F11; TCRβ PE clone H57-597; and CD4 FITC clone RM4-5; all from BioLegend) and

DAPI (BioLegend), and incubated on ice for 30 minutes in the dark. After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes, and resuspended in flow cytometry fixation buffer (Thermo Fisher Scientific). Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

As shown in the table below, the average mean fluorescence intensity (MFI) for the surface expression of TCRβ on tumor-infiltrating $CD4^+$ T-cells, following their interaction within tumors with the colonized parental YS1646Δ*asd*/ΔFLG/Δ*pagP* strain, was significantly lower than with the *ansB*-deleted YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain ($p = 0.042$), which was higher than even the PBS control-treated mice.

| | **MFI for TCRβ Expression on Tumor-Infiltrating $CD4^+$ T-Cells** | | |
|---|---|---|---|
| | **PBS** | **YS1646Δ*asd*/ΔFLG/Δ*pagP*** | **YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*** |
| | 13980 | 13933 | 14412 |
| | 14543 | 13480 | 14957 |
| | 14177 | 12087 | 14844 |
| | 13931 | 14010 | 14233 |
| **AVG** | **14157.8** | **13377.5** | **14611.5** |
| **SD** | **278.0** | **891.5** | **344.7** |

MFI = mean fluorescence intensity; AVG = average; SD = standard deviation

Taken together, these data confirm the necessity of deleting the *ansB* gene in order to restore T-cell function, due to the bacterial production of immunosuppressive L-asparaginase II, and demonstrate the enhanced T-cell function observed with the *ansB* deletion in the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain.

## Example 7

### *Salmonella csgD* Gene Knockout Strain Engineering and Characterization

The YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain was further modified to delete *csgD*, a master gene that controls *S. typhimurium* curli fimbriae formation, cellulose production, and c-di-GMP production. The *csgD* gene deletion eliminates the possibility of cellulose-mediated biofilm formation, reduces pro-inflammatory signaling, and enhances uptake by host phagocytic cells. This increase in intracellular

localization would thereby enhance the effectiveness of plasmid delivery and immunomodulatory protein production.

**Δ*csgD* Strain Construction**

The *csgD* gene was deleted from the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain, using modifications of the methods described in the preceding examples. Synthetic *csgD* gene homology arm sequences that contained 207 and 209 bases of the left hand and right hand sequence, respectively, flanking the *csgD* gene, were synthesized and cloned into a plasmid called pSL0196 (SEQ ID NO:333). A kanamycin gene cassette flanked by cre/loxP sites then was cloned into plasmid pSL0196, and the *csgD* gene knockout cassette was PCR amplified with primers csgd-1 (SEQ ID NO:323) and csgd-2 (SEQ ID NO:324), gel purified, and introduced into strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*, carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination, as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *csgD* gene knockout sequences were amplified by PCR, using primers csgd-3 (SEQ ID NO:325) and csgd-4 (SEQ ID NO:326), and verified by DNA sequencing. The resulting mutant derivative of parental strain YS1646 was designated YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*.

***csgD*-Deleted Strains Cannot Form RDAR Colonies on Congo Red Plates**

The ability to form Rough Dry And Red (RDAR) colonies after growth on Congo Red plates is a well-validated assay for bacterial biofilm formation. The Rough and Dry texture occurs through cellulose production, and the red is due to the accumulation of pigment by the curli fimbriae surface structures. For this assay, the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain was compared to the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain for the ability to form the RDAR phenotype after incubation on Congo Red agar plates.

Congo Red agar plates were prepared with soytone (10 g/L) and yeast extract (5 g/L) (modified LB without NaCl), and complemented with Congo red (40 mg/L) and Coomassie brilliant blue G-250 (20 mg/L). Five microliters of a stationary phase bacterial culture was spotted onto Congo Red plates, and incubated at 37 °C for 16 hours, then transferred to 30 °C and incubated for an additional 120 hours. Visual

analysis of colony morphology and color was performed and recorded daily to confirm presence or absence of the RDAR colony morphotype.

Comparing the colony morphotypes between the two strains, the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain had a smooth phenotype, and the colonies lacked pigment. In comparison, the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain, still containing the *csgD* gene, exhibited the classic rough and dry appearance, and clear evidence of pigment uptake. Thus, the functional assay confirms that the Δ*csgD* strain is unable to form biofilms, as it lacks curli fimbriae and cellulose production.

**_csgD_-Deleted Strains Demonstrate Superior Anti-Tumor Efficacy in a Highly Refractory Mouse Model of Triple Negative Breast Cancer**

The impact of the *csgD* deletion in models where the immunostimulatory bacterial therapy colonizes tumors, but has shown limited efficacy, was assessed. This can indicate the presence of bacterially-produced cellulose that can limit uptake into tumor-resident myeloid cells, thereby limiting therapeutic benefit (see, *e.g.*, Crull *et al.* (2011) *Cellular Microbiology* 13(8):1223–1233). The difficult-to-treat EMT6 model was utilized, which is a representative model of human triple negative breast cancer (see, *e.g.*, Yu *et al.* (2018) *PLoS ONE* 13(11):e0206223). When EMT6 tumor cells are administered orthotopically into the mammary fat pad, as opposed to subcutaneously in the flank, the model is T-cell excluded, highly metastatic, and highly refractory to immunotherapy, including to all approved checkpoint antibodies (see, *e.g.*, Mariathasan *et al.* (2018) *Nature* 554:544–548).

For this experiment, 6-8 week-old female BALB/c mice (5 mice per group) were inoculated in the left mammary fat pad with EMT6 tumor cells (ATCC # CRL-2755) ($2x10^5$ cells in 100 µL PBS). Mice bearing 13 day-old established mammary tumors (~55 $mm^3$ in volumne) were IV injected with a single dose of 1 $x10^7$ CFUs of the *csgD*-deleted YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain, or the parental YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* strain, and compared to PBS control. The bacterial strains contained a plasmid encoding a constitutively active murine STING (EF-1α muSTING R283G).

The tumors in the PBS-treated mice grew evenly, reaching a max tumor volume at day 35 (1199.0 ± 298.1 $mm^3$). Mice treated with the *csgD*-intact strain,

YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*, did not demonstrate evidence of anti-tumor efficacy in this model, also reaching max tumor volume at day 35 (1689.1 ± 537.0). *Ex vivo* LB plating of these tumors revealed all tumors to be colonized. However, the *csgD*-deleted strain, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, resulted in 3 out of 5 mice being completely cured of both their primary and any metastatic disease (day 60+). Overall tumor growth inhibition (TGI) was 45.7%, with one of the other two remaining tumors partially responding before eventually growing out. The two bacterial strains contained the same plasmid payload, yet only one demonstrated significant anti-tumor efficacy. Thus, in one of the most intractable and highly metastatic syngeneic tumor models, orthotopic EMT6, a strain with a *csgD* deletion was able to induce systemic anti-tumor efficacy, and result in 60% complete responses.

***csgD*-Deleted Strains Demonstrate Enhanced Intracellular Uptake *In Vivo***

In order to determine whether the *csgD*-deleted strain demonstrated improved efficacy because of greater bacterial uptake into tumor-resident myeloid cells, an *ex vivo* gentamicin protection assay was performed (see, *e.g.*, Crull *et al.* (2011) *Cellular Microbiology* 13(8):1223–1233). For this experiment, 6-8 week-old female C57BL/6 mice (4 mice per group) were inoculated SC in the right flank with MC38 cells ($5x10^5$ cells in 100 µL PBS). Mice bearing large established flank tumors were IV injected on day 17 with $1x10^7$ CFUs of the *csgD*-deleted YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain (N=12), or the parental YS1646 strain (N=4). Tumors were resected 7 days post IV dosing, weighed, and minced in RPMI supplemented with 1 mg/mL collagenase IV and 20 mg/mL DNase I, and incubated with shaking at 37 °C for 30 minutes, to generate a single cell suspension. After 30 minutes, the suspension was passed through a 70 mm filter, and the recovered volume was divided into two separate, identical samples. Gentamicin (Thermo Fisher Scientific) was added at a concentration of 200 mg/mL to one of each of the paired samples to kill extracellular bacteria, and the samples were incubated with shaking at 37 °C for 90 minutes. Cell suspension samples were then washed and lysed with 0.05% Triton X, and plated on LB agar plates to enumerate for CFUs.

The results demonstrate that, compared to the CFUs from YS1646-treated tumors without gentamicin treatment (11925 ± 19859 CFUs), gentamicin treatment

resulted in very few CFUs detected from the tumors (51 ± 45 CFUs). This indicates that the bacteria reside largely extracellularly in these tumors, and are thus sensitive to gentamicin elimination. In the *csgD*-deleted YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* treatment group, the non-gentamicin treated tumors yielded high CFUs, as expected from well-colonized tumors, and treatment with gentamicin yielded less CFUs (1276 ± 2410 CFUs), and much more than in the parental YS1646 strain-treated tumors. This is due to more of the *csgD*-deleted bacteria residing intracellularly, and thus, being protected from gentamicin. These data demonstrate that the *csgD* deletion improves intracellular uptake of the bacteria, which can enhance plasmid delivery of immunomodulatory proteins *in vivo*.

**Example 8**

**pATI-1.75 and pATI-1.76 Vector Construction**

A plasmid was designed and synthesized that contains the following features: a pBR322 origin of replication, the *asd* gene, a kanamycin resistance gene flanked by HindIII sites for curing, and a multiple cloning site for expression cassette insertion. The expression cassette is composed of multiple elements, including eukaryotic promoters, open reading frames (ORFs), post-transcriptional regulatory elements, and polyadenylation signals, that are assembled in various configurations.

Exemplary promoters include the human cytomegalovirus (CMV) immediate early core promoter encoded directly downstream of the CMV immediate early enhancer sequence, and the core promoter for human elongation factor-1 alpha (EF-1α). Open reading frames (ORFs) can include one or more sequences that each are translated into a protein, and can be separated into distinct polypeptides by insertion of a 2A sequence, whereby eukaryotic ribosomes fail to insert a peptide bond between Gly and Pro residues within the 2A sequence. Examples of 2A sequences are the T2A peptide (see, *e.g.*, SEQ ID NO:327) from the Thosea asigna virus (TaV) capsid protein, and the P2A peptide (see, *e.g.*, SEQ ID NO:328) from porcine teschovirus (PTV). Upstream furin cleavage sites (RRKR), and other enhancer elements, are placed upstream to facilitate cleavage to separate the expressed proteins.

Examples of post-transcriptional regulatory elements (PREs) include the Woodchuck Hepatitis virus PRE (WPRE; SEQ ID NO:346) and the Hepatitis B virus PRE (HPRE; SEQ ID NO:347), which increase accumulation of the cytoplasmic

mRNA of a gene by promoting mRNA nuclear export to the cytoplasm, enhancing 3' end processing and stability. Examples of polyadenylation signal sequences include the SV40 polyadenylation (SV40polyA, or SV40pA) signal, and the bovine growth hormone polyadenylation (bGHpolyA, or bGHpA) signal, both of which are 3' regulatory elements that serve to promote transcriptional termination, and contain the sequence motif recognized by the RNA cleavage complex.

Figures 1A-C depict the pATI-1.75 and pATI-1.76 (Fig. 1A and 1B, respectively) plasmid inserts; Figure 1C shows that the Shine-Dalgarno sequence is replaced with a Kozak sequence for translation in eukaryotic cells, such as myeloid cells. Plasmids with such sequences and constructions can be used in embodiments in which the bacteria are designed to deliver RNA, which cannot be translated by the bacterial host, but can be translated *in vivo* in a eukaryote.

## Example 9

## Designed Heterologous Protein Expression Plasmids Induce Functional Protein Production from Human Cells

### Optimal Expression of Cytokines Established in Human Cells

In order to exemplify that immunostimulatory cytokines can be expressed from designed plasmids in human cells, a panel of cytokines were cloned into the pATI-1.75 plasmid under the control of the EF-1α promoter. The cytokines include, but are not limited to, murine IL-2 (muIL-2), muIL-12p70, muIL-23, and human IL-2 (huIL-2). For the muIL-15 Receptor-α fused to an IL-15 single chain (muIL-15Rα-IL-15sc), an EF-1α and CMV promoter were tested. HEK293T STING Null cells (InvivoGen) were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 200 ng of each cytokine plasmid DNA was diluted in serum-free media, and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios, with untransfected wells as negative controls (in duplicates). Cell culture supernatants from each sample were collected at 24 hours post-transfection, and assessed for protein expression by ELISAs specific for each cytokine.

The muIL-2 construct was evaluated in a murine IL-2 ELISA (R&D Systems), according to the manufacturer's instructions, and an additional version of muIL-2 with codon optimization (muIL-2 CO) also was evaluated. Concentrations of neat

supernatant were tested, and yielded an average of 1680 pg/mL of muIL-2 for the muIL-2 construct, and 1812 pg/mL of muIL-2 for the muIL-2 CO construct. These data confirmed the functionality of the constructs, and demonstrated that yield could be improved with codon optimization. The muIL-12p70 construct was evaluated in a murine IL-12 ELISA (R&D Systems), according to the manufacturer's protocol. When supernatants were added neat, a mean of 400 pg/mL of secreted muIL-12p70 was measured, although this was outside the linear range. When the supernatants were diluted 5-fold, an average of 105 pg/mL of secreted muIL-12p70 was detected. For the muIL-23 plasmid, detection of protein was achieved using the murine IL-23 ELISA (BioLegend), per kit instructions. With the supernatant added neat, a mean of 966 pg/mL of muIL-23 was detected. For the human IL-2 plasmid, detection of protein was achieved using the human IL-2 ELISA (Invitrogen), per kit instructions. With the supernatant added neat, an average of 1422 pg/mL of huIL-2 was detected.

For the muIL-15Rα-IL-15sc construct, expressed using either the EF-1α or CMV promoters, the murine IL-15 ELISA (eBioscience, Inc.) was used, per kit instructions. When added neat, the muIL-15Rα-IL-15sc plasmid with the EF-1α promoter resulted in an average of 131 pg/mL, while the muIL-15Rα-IL-15sc plasmid with the CMV promoter resulted in an average of 289 pg/mL.

These data validate the plasmid expression constructs encoding immunomodulatory cytokines, both mouse and human, in human cells. Further, they indicate that codon optimization, and the use of promoters such as CMV, can enhance protein expression.

**Post-Transcriptional Regulatory Elements Enhance Cytokine Expression**

In order to determine whether post-transcriptional regulatory elements (PREs), added at the 3' end of the ORF, enhance expression of immunostimulatory cytokines in human cells, expression of huIL-2, under the control of the EF-1α promoter, was tested with or without the addition of a Woodchuck Hepatitis virus post-transcriptional regulatory element (WPRE) in the pATI-1.75 plasmid. HEK293T STING Null cells (InvivoGen) were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 200 ng of each cytokine plasmid DNA was diluted in serum-free media and added to FuGENE® transfection reagent (Promega),

at the proper reagent:DNA ratios, with untransfected wells as negative controls (in duplicates). Cell culture supernatants from each sample were collected at 24 hours post-transfection and assessed for activity by a human IL-2 ELISA (Invitrogen), according to the manufacturer's instructions. Supernatants were added neat, or were diluted 5-fold.

The results demonstrated that, when supernatant was added neat, compared to the huIL-2 construct without a WPRE, which secreted an average of 1540 pg/mL, the huIL-2 construct with the WPRE secreted 5511 pg/mL, a 3.6-fold increase. In the 5-fold diluted supernatants, the non-WPRE huIL-2 construct secreted 315 pg/mL of huIL-2, while the huIL-2 construct with the WPRE secreted 1441 pg/mL, a 4.6-fold increase. Thus, addition of 3' post-transcriptional regulatory elements, exemplified by, but not limited to, WPRE, can significantly improve protein expression in human cells.

**Promoter Optimization and Post-Transcriptional Regulatory Elements Enhance Cytokine Production in Primary M2 Macrophages**

While expression of cytokines, such as muIL-15Rα-IL-15sc, was enhanced in human HEK293T cells by use of the CMV promoter, it was determined whether expression of cytokines could similarly be enhanced in donor-derived primary human M2 macrophages, the predominant macrophage phenotype in T-cell excluded, solid human tumors. Additionally, it was determined whether post-transcriptional regulatory elements, such as WPRE, could enhance expression in these cells.

In order to determine if protein expression could be improved, the promoters EF-1α and CMV were tested for controlling expression of muIL-2, and the WPRE post-transcriptional regulatory element was tested for expression of huIL-2. Frozen human PBMCs, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 1X non-essential amino acids + 5% Human AB serum), and washed by centrifugation for 10 minutes at 800 RPM at room temperature. PBMCs were resuspended in PBS + 2% FBS, and monocytes were negatively isolated using a CD16 depletion kit (StemCell Technologies). The isolated monocytes were cultured for 6 days in RPMI media containing M-CSF and IL-4, to generate M2 macrophages. For this, isolated untouched monocytes were washed by centrifugation in PBS + 2% FBS, and resuspended in complete medium containing 100 ng/mL human M-CSF and

10 ng/mL human IL-4. Isolated monocytes (3e5 per well) were then seeded in a 24-well plate with a final volume of 750 microliters. Two days after the seeding, the cell culture media was entirely aspirated and replaced with fresh complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4. Two days later (on day 4), 500 µL of complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4 was added per well, and incubated for 48 hours. On day 6, the cell culture media was entirely aspirated, and replaced with fresh complete medium without cytokines, for transfection with the Viromer® RED mRNA and plasmid transfection reagent (Lipocalyx).

Transfection with Viromer® RED was performed according to the manufacturer's instructions. Briefly, 500 ng of plasmid DNA, containing the EF-1α-muIL-2 construct, the CMV-muIL-2 construct, the EF-1α-huIL-2 construct, or the EF-1α-huIL-2 + WPRE construct, as well as untransfected control, were diluted in the provided buffer, and mixed with 0.2 µL of Viromer® RED, and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 24-well plate (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator for 24 hours. Supernatants were harvested at 24 hours, and assayed for cytokines using either a murine IL-2 ELISA (R&D Systems), or a human IL-2 ELISA (Invitrogen), per kit instructions.

The results demonstrated that expression of muIL-2 from neat supernatants harvested from primary human M2 macrophages, transfected with the muIL-2 construct under control of the EF-1α promoter, resulted in the secretion of an average of 59.7 pg/mL of muIL-2. The muIL-2 construct with the CMV promoter yielded an average of 275 pg/mL muIL-2, an almost 5-fold increase. For the human IL-2 ELISA, neat supernatants from the cells transfected with the plasmid lacking the WPRE yielded an average of 170 pg/mL huIL-2. The huIL-2 construct containing the WPRE yielded an average of 219 pg/mL huIL-2. These data confirm that promoters such as CMV, and post-transcriptional regulatory elements such as WPRE, can significantly improve cytokine expression in multiple cells types, including primary human M2 macrophages.

**Co-Stimulatory Receptor Ligand 4-1BBL Expressed from Human Cells**

Co-stimulatory molecules, such as 4-1BBL, when expressed on antigen-presenting cells (APCs), can engage 4-1BB expressed on T-cells to promote optimal T-cell function. 4-1BBL is negatively regulated by its cytoplasmic signaling domain. In the late-phase of 4-1BBL ligation of macrophages to T-cells, reverse signaling of the 4-1BBL cytoplasmic domain induces surface translocation of 4-1BBL to bind and form a signaling complex with TLR4. This induces high levels of TNF-α, comparable to LPS activation of TLR4, that leads to immunosuppression of the adaptive immune response (see, *e.g.*, Ma *et al.* (2013) *Sci. Signal.* 6(295):ra87).

In this example, the sequence encoding murine 4-1BBL was cloned into the pATI-1.75 vector. In order to maximally engage T-cells, the reverse signaling of the 4-1BBL cytoplasmic domain was eliminated by deleting the cytoplasmic domain (corresponding to amino acid residues 1-82 of SEQ ID NO:344), generating mu4-1BBLΔcyt. To determine whether mu4-1BBLΔcyt could be functionally expressed on the surface of human cells, HEK-293T cells were utilized. HEK293T STING Null cells (InvivoGen) were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, overnight at 37 °C in a $CO_2$ incubator, to achieve 80% confluency. The following day, 200 ng of plasmid DNA, encoding mu4-1BBLΔcyt, was diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratio, with untransfected wells as a negative control (in duplicates). After 48 hours, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were then resuspended in PBS + 2% FBS, and stained with a PE-conjugated murine anti-4-1BBL antibody (clone TKS-1, BioLegend) and DAPI (dead/live stain). After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes, and resuspended in PBS + 2% FBS. Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

As a percentage of live cells, the untransfected control cells showed a percent positive staining for murine 4-1BBL of 14.6. In comparison, 93.4% of the cells that were transfected with the plasmid encoding mu4-1BBLΔcyt, were positive for surface

expression of 4-1BBL. These data demonstrate that the pATI-1.75 plasmid can effectively be used to express 4-1BBL at high levels on the surface of human cells.

**Soluble TGFβ Receptor II Expressed from Human Cells**

Soluble mouse TGFβ receptor II variants were designed by removing the cytoplasmic and transmembrane portions of the full TGFβ receptor II. Additionally, either a FLAG or Fc tag was added for detection. These variants were cloned into the pATI-1.75 vector under the control of a CMV promoter and a 3' WPRE. The sequences were confirmed by Sanger sequencing. 1.5 x $10^6$ HEK293T cells were plated one day prior on 6-well plates coated with poly-L-lysine, to achieve 80% confluency. On the day of transfection, 3 µg of DNA was diluted in serum-free media and added to FuGENE® transfection reagent (Promega) at the proper reagent:DNA ratios. Cell culture supernatants from each sample were collected after 48 hours of incubation. Some supernatant was concentrated in a 10 kDa spin column (Millipore). Direct ELISAs with mouse TGF-β1 (R&D systems) were performed on the supernatant of transfected HEK293T cells. ELISA data, with absorbance at 450 nm, is provided in the table below.

| Construct | Absorbance at 450 nm |
|---|---|
| Concentrated soluble mouse TGFβ receptor II Fc | 1.522 ± 0.025 |
| Soluble mouse TGFβ receptor II Fc | 1.508 ± 0.018 |
| Media (control) | 0.041 ± 0.002 |

The functionality of these constructs was tested in a T-cell assay. Mouse T-cells were harvested from the spleen using a magnetic isolation kit (StemCell Technologies). T-cells were incubated with anti-mouse CD3ε antibody, with or without the soluble receptor, at various concentrations of mouse TGF-beta. T-cell activation was quantified using the mouse TH1 CBA kit (BioLegend), and flow cytometry labeling of CD4, CD8, 4-1BB, and CD69.

These data demonstrate the ability to express heterologous molecules, such as extracellular receptors fused to an Fc domain, from the plasmid engineered for delivery by the immunostimulatory bacteria to eukaryotic, such as human, cells.

**Expression of a CD3xCD19 Bispecific T-cell Engager from Human Cells**

A CD3xCD19 bispecific T-cell engager (BiTE®), containing a FLAG tag and a His tag, was cloned into the pATI-1.75 vector under the control of a CMV promoter and with a 3' WPRE. The sequences were confirmed by Sanger sequencing. 1.5 x $10^6$ HEK293T cells were plated one day prior on 6-well plates coated with poly-L-lysine, to achieve 80% confluency. On the day of transfection, 3 µg of DNA was diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios. Cell culture supernatants from each sample were collected after 48 hours of incubation. Some supernatant was concentrated in a 10 kDa spin column (Millipore).

The functionality of this construct was tested by binding of the CD3xCD19 BiTE® to Raji and Jurkat-Lucia™ NFAT cells (InvivoGen). The BiTE® was detected using an anti-FLAG-APC (BioLegend), using flow cytometry. One well of 50,000 cells was run for each condition. The mean fluorescence intensity (MFI) of the APC positive events, and the number of cells gated as APC positive, are provided in the table below.

| Sample | Number of $APC^+$ Cells | Mean Fluorescence Intensity (MFI) of $APC^+$ Cells |
|---|---|---|
| Raji cells with anti-FLAG-APC only | 41 | 1439 |
| Raji cells with CD3xCD19 BiTE® | 10307 | 7408 |
| Raji cells with concentrated CD3xCD19 BiTE® | 11248 | 16045 |
| Jurkat cells with anti-FLAG-APC only | 100 | 1519 |
| Jurkat cells with CD3xCD19 BiTE® | 18482 | 4584 |
| Jurkat cells with concentrated CD3xCD19 BiTE® | 17563 | 14089 |

Additionally, this construct was tested in a co-culture of Raji and Jurkat-Lucia™ NFAT cells. Cells were incubated with or without the CD3xCD19 BiTE®, and the luminescence (corresponding to Lucia™ luciferase activity resulting from NFAT activation) was detected at 6 hours and 24 hours post-addition of the BiTE®. The luminescence readings of this assay are provided in the table below.

| Experimental Conditions | Concentrated CD3xCD19 BiTE® (Luminescence) | CD3xCD19 BiTE® (Luminescence) | Medium (Luminescence) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| 2 Raji: 1 Jurkat, 6 hour time point | 12110.5 ± 837.9 | 3445.0 ± 717.0 | 44.5 ± 10.6 |
| 1 Raji: 1 Jurkat, 6 hour time point | 4337.0 ± 219.2 | 2057.5 ± 20.5 | 26.0 ± 15.6 |
| 2 Raji: 1 Jurkat, 24 hour time point | 47159.5 ± 1038.7 | 11274 ± 408.7 | 114.5 ± 57.3 |
| 1 Raji: 1 Jurkat, 24 hour time point | 18614 ± 1540.1 | 6017 ± 31.1 | 122 ± 15.6 |

These data demonstrate the ability to express heterologous molecules, such as scFvs, alternative antibody constructs, and bispecific T-cell engagers, in eukaryotic, such as human, cells, from the engineered plasmid that can be delivered by the immunostimulatory bacteria herein.

**Example 10**

**Immunostimulatory Bacterial Strains Efficiently Deliver Plasmids and Express Cytokines in Human Cells**

**Flagella-Deleted Strains Containing Plasmids Encoding Murine IL-2 Induce Functional IL-2 Protein Expression Following Infection in Human Monocytes**

As described above, the flagellin genes *fljB* and *fliC* were deleted from the YS1646 strain of *S. typhimurium* with the *asd* gene deleted, generating the strain YS1646Δ*asd*/ΔFLG. This strain was electroporated with a plasmid containing an expression cassette with the EF-1α promoter and the murine cytokine IL-2 (muIL-2). In addition, the YS1646Δ*asd*/ΔFLG strain was electroporated with an expression plasmid encoding murine IL-15δ, as a control for a non-cognate cytokine. Additional constructs were created using the CMV promoter.

To determine whether these strains containing expression plasmids can infect human monocytes and induce the production of murine IL-2, THP-1 human monocytic cells were plated at 50,000 cells/well in RPMI 1640 (Gibco™) + 10% Nu-Serum™ (Corning®), one day prior to infection. The cells were infected with the various strains at an MOI of 50 for one hour in RPMI, then washed 3 times with PBS, and resuspended in RPMI + 100 µg/mL gentamicin (Sigma). Supernatants were collected 48 hours later from a 96-well plate, and assessed for the concentration of murine IL-2 by ELISA (R&D Systems).

The concentration of muIL-2 detected in the YS1646Δ*asd*/ΔFLG-IL-15δ control wells was very low (6.52 pg/mL), as expected, and likely reflective of some cross-reactivity to the endogenous human IL-2 receptor. In contrast, the YS1646Δ*asd*/ΔFLG-muIL-2 strain induced an average of 35.1 pg/mL of muIL-2. These data demonstrate the feasibility of expressing and secreting functional heterologous proteins, such as IL-2, from the *S. typhimurium* immunomodulatory platform strains, in human monocytes.

**Flagella-Deleted and *pagP*-Deleted Strains, Containing Plasmids Encoding Murine IL-2, Demonstrate Enhanced IL-2 Expression, Compared to Transfected muIL-2 DNA, in Primary Human M2 Macrophages**

The relative efficiencies of transfection (*i.e.*, direct transfer of plasmid DNA) vs. bactofection (*i.e.*, transfer of plasmid DNA by the immunostimulatory bacterial strains herein), in primary human M2 macrophages, for expression of muIL-2, were compared. Frozen human PBMCs, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 1X non-essential amino acids + 5% Human AB serum), and washed by centrifugation for 10 minutes at 800 RPM at room temperature. PBMCs were resuspended in PBS + 2% FBS, and monocytes were negatively isolated using a CD16 depletion kit (StemCell Technologies). Isolated untouched monocytes were then washed by centrifugation in PBS + 2% FBS, and resuspended in complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4. Isolated monocytes (3e5 per well) were then seeded in a 24-well plate, with a final volume of 750 microliters. Two days after the seeding, the cell culture media was entirely aspirated and replaced with fresh complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4. Two days later (on day 4), 500 µL of complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4 was added per well, and incubated for 48 hours. On day 6, the cell culture media was entirely aspirated, and replaced with fresh complete medium without cytokines, for transfection with Viromer® RED mRNA and plasmid transfection reagent (Lipocalyx).

Transfection with Viromer® RED was performed according to the kit instructions. Briefly, 500 ng of plasmid DNA from the constructs encoding muIL-2 under control of the EF-1α promoter (EF-1α-muIL-2), or under control of the CMV

promoter (CMV-muIL-2), or untransfected control, were diluted in the provided buffer, mixed with 0.2 µL of Viromer® RED transfection reagent, and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 24-well plate containing the monocytes (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator for 24 hours. Additional wells of cells were infected at an MOI of 450 with the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain containing the EF-1α-muIL-2 construct, or the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain containing the CMV-muIL-2 construct, for one hour in RPMI, then washed 3 times with PBS, and resuspended in RPMI + 100 µg/mL gentamicin (Sigma).

After 24 hours, the cells were lysed with 350 µL Buffer RLT with β-mercaptoethanol (β-ME) (Qiagen), and RNA extraction was performed using the Qiagen RNeasy® Mini Kit with the following modifications. A genomic DNA elimination step, using an RNase-Free DNase kit (Qiagen) was included in the kit to remove genomic DNA from the total RNA. Total RNA concentration was measured using a NanoDrop™ One$^C$ UV-Vis Spectrophotometer (Thermo Fisher Scientific). The purity of each sample also was assessed from the $A_{260}/A_{230}$ absorption ratio. RNA was stored at -80 °C without freeze-thawing until reverse-transcription was performed. cDNA synthesis was performed using 0.4-1 µg of template RNA using a C1000 Touch Thermal Cycler (Bio-Rad) and SuperScript™ VILO™ Master Mix (Invitrogen) in a 30 µL reaction, according to the manufacturer's instructions.

qPCR (quantitative polymerase chain reaction) was performed with a CFX96™ Real-Time PCR Detection System (Bio-Rad). SYBR® primers for murine IL-2 (Assay ID: qMmuCED0060978) were purchased from Bio-Rad. The qPCR reaction (20 µL) was conducted per protocol, using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad). The standard thermocycling program on the Bio-Rad CFX96™ Real-Time System consisted of a 95 °C denaturation for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. Reactions with template-free control were included for each set of primers on each plate. All samples were run in duplicate, and the mean $C_q$ values were calculated. Quantification of the target mRNA was normalized using Gapdh (glyceraldehyde-3-phosphate dehydrogenase) reference mRNA (Bio-Rad, Assay ID: qMmuCED0027497). $\Delta C_q$

was calculated as the difference between target (mu-IL2) and reference (Gapdh) gene. $\Delta\Delta C_q$ was obtained by normalizing the $\Delta C_q$ values of the treatments, to the $\Delta C_q$ values of the non-treatment controls. Fold increase was calculated as $2^{-\Delta\Delta C_q}$. The fold increases relative to untransfected/uninfected control are shown in the table below.

| Treatment Group | Fold Increase in muIL-2 |
|---|---|
| YS1646 | <1 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*, EF-1α-muIL-2 (infection) | 74 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*, CMV-muIL-2 (infection) | 668.2 |
| Transfection, EF-1α-muIL-2 | 249.4 |
| Transfection, CMV-muIL-2 | 1527 |

The results show that, with either transfection or bactofection, the CMV promoter demonstrated superior expression of muIL-2, compared to the EF-1α promoter, in primary human M2 macrophages. While transfection using the most efficient reagent currently available gave the highest levels of muIL-2 expression, bactofection also elicited high expression levels of muIL-2, demonstrating the high efficacy of heterologous gene transfer with the bacterial platforms provided herein.

**Example 11**

**Bacterial Strains Efficiently Deliver Immunomodulatory Plasmids *In Vivo* and Demonstrate Potent Anti-tumor Activity**

**Flagella-Deleted Strains Containing Plasmids Encoding Murine IL-2 Induce Potent Tumor Inhibition without Toxicity in a Mouse Model of Colorectal Carcinoma**

In order to demonstrate that *S. typhimurium* strains containing the muIL-2 expression plasmids can induce anti-tumor efficacy without additional toxicity, the YS1646Δ*asd*/ΔFLG strain, containing the muIL-2 plasmid, was compared to PBS control, for safety and efficacy in the subcutaneous flank MC38 colorectal adenocarcinoma model. For this study, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 11 with $5x10^5$ CFUs of the YS1646Δ*asd*/ΔFLG-muIL-2 strain, or with PBS vehicle control. Tumor measurements and body weights were recorded twice weekly.

The results revealed that the YS1646Δ*asd*/ΔFLG-muIL-2 strain demonstrated significant tumor growth inhibition (TGI) compared to PBS (76.7% TGI, $P = 0.005$,

day 21), with tumors being well controlled out to day 40 post-implantation, when the PBS mice were euthanized. The therapy was well tolerated, even without further strain attenuation, and the weight loss early on was transient and resulted in only a 3.4% reduction in body weight, compared to PBS control at day 40. Thus, the immunostimulatory strain expressing muIL-2 potently inhibits tumor growth inhibition, in a safe and non-toxic manner, in a model of colorectal carcinoma.

**Flagella-Deleted Strains Containing Plasmids Encoding Murine IL-2 Induce Tumor-Specific Production of IL-2 *In Vivo***

The level of tumor muIL-2 expression, relative to spleen, was determined in order to confirm the tumor-specific nature of delivery. 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 10 with $5x10^5$ CFUs of the YS1646Δ*asd*/ΔFLG-muIL-2 strain, or with PBS vehicle control. On day 31 post tumor implantation, tumors and spleens were excised and processed for tumor extracts using the GentleMACS™ Octo Dissociator and the M tubes (Miltenyi Biotec) molecule setting in 2 mL of PBS. The homogenates were spun down at 1300 RPM for 10 minutes, and the supernatant was collected and assayed using the muIL-2 CBA kit (BD Biosciences), according to the manufacturer's instructions. Results were quantified as pg/mL of muIL-2, and standardized to per gram of tissue.

The PBS control tumors exhibited background levels of muIL-2 in the tumor, with a mean of 134 pg/mL per gram of tumor tissue. The YS1646Δ*asd*/ΔFLG-muIL-2 treated tumors yielded a much higher mean of 389.9 pg/mL of muIL-2 per gram of tumor tissue, demonstrating the ability to detect elevated muIL-2 levels due to plasmid delivery in the tumor-resident myeloid cells. The level of muIL-2 in the spleen, from mice injected with the YS1646Δ*asd*/ΔFLG-muIL-2 strain, was an average of 6.6 pg/mL per gram of tissue, which was lower than in the PBS controls. This specificity for the tumor enables delivery of immunomodulatory levels of IL-2, in a much safer manner than conventional cytokine therapies, which are not tumor-targeted.

**Attenuated Bacterial Strains Containing Plasmids Encoding Murine Co-stimulatory Receptor Ligand 4-1BBL Demonstrate Curative Effects *In Vivo***

In order to determine whether tumor-specific delivery of a co-stimulatory molecule, such as 4-1BBL, enhances anti-tumor efficacy, a bacterial strain containing a plasmid encoding 4-1BBL(Δcyt) (described above), under control of the CMV promoter and containing a 3' WPRE, was assessed in the MC38 murine model of colorectal adenocarcinoma. For this study, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 10 with $1x10^7$ CFUs of strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB* containing the CMV-4-1BBL(Δcyt)-WPRE plasmid, or with PBS vehicle control.

The therapy was very well tolerated, with only an initial weight loss of 2.2% that had fully recovered 3 days later. Compared to PBS, the 4-1BBL(Δcyt) therapy was highly effective and curative (90.7% TGI, 60% complete response (CR), day 30). These data demonstrate the potency and safety of delivering immunostimulatory bacteria containing plasmids encoding co-stimulatory molecules in a tumor-specific manner.

**Example 12**

**Identification of Gain-of-Function Mutations in Genes That Promote Constitutive Type I Interferon Production**

Instances of subjects presenting with severe auto-inflammatory conditions and vasculopathies of unknown etiology occur, and, often derive from mutations. The cause for these conditions has, and can be, identified. Steps to identify a mutational basis for such a pathology are as follows. In step one, intact genomic DNA is obtained from patients experiencing symptoms, and from healthy individuals. Whole exome sequencing is performed, then introns and exons are analyzed. Analysis of genes, and identification of mutations in products in the pathways associated with the expression of type I interferon (IFN), is performed. From this analysis, mutations are discovered in genes known to lead to constitutive functional activation of the encoded proteins, and subsequent persistent expression of type I IFN.

After identification of mutations, cDNAs encoding the full-length gene, with and without the identified mutation(s), are transfected into a reporter cell line that measures expression of type I IFN. For example, a reporter cell line can be generated

where the expression of luciferase is placed under control of the promoter for IFN-β. A gain-of-function (GOF) mutant that is constitutively active will promote the expression of IFN-β, whereas the unstimulated wild-type (WT) protein will not. In the case of known STING SAVI (STING-associated vasculopathy with onset in infancy) mutants, the WT STING stimulation of IFN-β requires the addition of increasing exogenous levels of cGAMP to directly activate WT STING. Constitutively active mutations stimulate the expression of IFN-β in a cGAMP-independent manner. Exemplary gain-of-function mutations in each of STING, RIG-I, MDA5, IRF3, and IRF7 are set forth below in Example 15, and discussed elsewhere herein. Other such genes, in which gain-of-function mutations can be identified in subjects, or produced by *in vitro* mutation and screening, include, but are not limited to, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200.

**Expression of Functional Constitutive Type I IFN Mutants in Human Cells**

Human STING (allele R232) and IRF3 gain-of-function (GOF) mutants (see, table below) were cloned into the pATI-1.75 vector, and the sequences were confirmed by PCR. To determine whether the STING and IRF3 GOF expression plasmids could induce functional type I IFN in human cells, the plasmids were assessed using HEK293T STING Null Reporter cells (InvivoGen), which do not contain endogenous STING. These cells express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous IFN-stimulated response element (ISRE) promoter, where the coding sequence of ISRE has been replaced by the SEAP ORF using knock-in technology. Type I interferon activity can be assessed by monitoring type I IFN-stimulated SEAP production in the cell culture supernatants.

To test the relative production of type I IFN by each of the GOF mutants, $1x10^5$ 293T-Dual™ Null cells (InvivoGen) were plated one day prior on plates coated with poly-L-lysine, to achieve 80% confluency, in a 24-well plate. On the day of transfection, 200 ng of plasmids encoding a panel of STING and IRF3 GOF mutants, including a STING wild-type (WT) and IRF3 WT control, and a negative control STING mutant that has been reported in the literature to be non-functional in human cells (STING V155R negative control (NC)), were diluted in serum-free media and

added to FuGENE® transfection reagent (Promega) at the proper reagent:DNA ratios. Cell culture supernatants from each sample were collected after overnight incubation, and 10 µL of the cell culture supernatants was added to 50 µL QUANTI-Blue™ reagent (InvivoGen), which is used for measuring SEAP. Type I interferon activation was determined by measuring ISRE-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), at an absorbance wavelength of 650 nm.

As shown in the table below, all GOF mutants were able to induce type I IFN activity in a STING ligand-independent manner in human cells, compared to the wild-type and negative controls, which did not induce type I IFN activity. The highest levels of type I IFN induction were observed with the human STING R284G variant, and the human IRF3 S396D phosphomimetic variant. These data support the ability of the plasmids encoding GOF mutants to produce functional, constitutively active STING and constitutively active phosphomimetic IRF3, that can induce type I IFN in a cGAMP-independent manner.

| GOF Mutant | Mean Absorbance (650 nm) | Standard Deviation |
|---|---|---|
| Plasmid control | 0.049 | ± 0.002 |
| huSTING WT | 0.144 | ± 0.004 |
| huSTING V147L | 1.399 | ± 0.015 |
| huSTING N154S | 1.382 | ± 0.008 |
| huSTING V155M | 1.360 | ± 0.048 |
| huSTING C206Y | 1.566 | ± 0.121 |
| huSTING R281Q | 1.546 | ± 0.132 |
| huSTING R284G | 1.831 | ± 0.039 |
| huSTING V155R (NC) | 0.181 | ± 0.014 |
| huIRF3 WT | 0.781 | ± 0.073 |
| huIRF3 S396D | 1.922 | ± 0.131 |

**Infection of Flagella-Deleted Strains, Containing Plasmids Encoding Constitutively Active Type I IFN Mutants, Converts Human M2 Macrophages to Type I IFN-Producing M1 Macrophages**

It was determined if primary human M2 macrophages, infected with flagella-deleted strains containing plasmids encoding constitutively active type I IFN GOF variants, could be converted to producers of type I IFN and downstream chemokines, such as CXCL10 (also known as IP-10).

Frozen human PBMCs, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 1X non-essential amino acids + 5% Human AB

serum), and washed by centrifugation for 10 minutes at 800 RPM at room temperature. PBMCs were resuspended in PBS + 2% FBS, and monocytes were negatively isolated using a CD16 depletion kit (StemCell Technologies). To generate primary human M2 macrophages, isolated untouched monocytes were washed by centrifugation in PBS + 2% FBS, and resuspended in complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4. Isolated monocytes (3e5 per well) were then seeded in a 24-well plate with a final volume of 750 microliters. Two days after the seeding, the cell culture media was entirely aspirated and replaced with fresh complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4. Two days later (on day 4), 500 µL of complete medium containing 100 ng/mL human M-CSF and 10 ng/mL human IL-4 was added per well, and incubated for 48 hours. On day 6, the cell culture media was entirely aspirated and replaced with fresh complete medium without cytokines. Duplicate wells were infected at an MOI of 450, for one hour in RPMI, with the following strains: YS1646Δ*asd*/ΔFLG containing a plasmid encoding wild-type (WT) human (hu) STING; YS1646Δ*asd*/ΔFLG containing a plasmid encoding the huSTING R284G variant; YS1646Δ*asd*/ΔFLG containing a plasmid encoding WT huIRF3; YS1646Δ*asd*/ΔFLG containing a plasmid encoding the huIRF3 S396D variant; or a strain containing a plasmid control. The cells were then washed 3 times with PBS, and resuspended in RPMI + 100 µg/mL gentamicin (Sigma). As a control, the STING agonist 3'5' RpRp c-di-AMP (InvivoGen), an analog of the clinical compound ADU-S100, was added to the cells at a concentration of 10 µg/mL.

After 24 hours, the cells were lysed with 350 µL Buffer RLT with β-ME (Qiagen), and RNA extraction was performed using the Qiagen RNeasy® Mini Kit with the following modification. A genomic DNA elimination step, using an RNase-Free DNase kit (Qiagen), was included to remove genomic DNA from the total RNA. Total RNA concentration was measured using a NanoDrop™ One$^{C}$ UV-Vis Spectrophotometer (Thermo Scientific). The purity of each sample also was assessed from the $A_{260}/A_{230}$ absorption ratio. RNA was stored at -80 °C without freeze-thawing until reverse-transcription was performed. Synthesis of cDNA was performed from 0.4-1 µg of template RNA using a C1000 Touch Thermal Cycler (Bio-Rad) and

SuperScript™ VILO™ Master Mix (Invitrogen) in a 30 µL reaction, according to the manufacturer's instructions.

qPCR was performed with a CFX96™ Real-Time System (Bio-Rad). SYBR® primers for huCXCL10 (qHsaCED0046619), huIRF3 (qHsaCID0013122), huSTING (qHsaCID0010565), and huIFNβ1 (qHsaCED0046851) were purchased from Bio-Rad. The qPCR reaction (20 µL) was conducted per protocol, using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad). The standard thermocycling program on the BioRad CFX96™ Real-Time System consisted of a 95 °C denaturation for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. Reactions with template free control were included for each set of primers on each plate. All samples were run in duplicate, and the mean $C_q$ values were calculated. Quantification of the target mRNA was normalized using Gapdh reference mRNA (Bio-Rad, qMmuCED0027497). $\Delta C_q$ was calculated as the difference between the target and reference gene. $\Delta\Delta C_q$ was obtained by normalizing the $\Delta C_q$ values of the treatments to the $\Delta C_q$ values of the non-treatment control. Fold increase was calculated as $2^{-\Delta\Delta C_q}$. The values are shown in the table below, as the average of the duplicate wells.

As shown in the table below, compared to the infection of the plasmid control, strains of YS1646Δ*asd*/ΔFLG, containing plasmids encoding WT huSTING and huSTING R284G, induced high levels of STING expression, which were significantly higher compared to the plasmid control, or the small molecule STING agonist. Similarly, the strains containing plasmids encoding WT huIRF3 and huIRF3-S396D induced high levels of IRF3 expression, which were significantly higher than the plasmid control, or the small molecule STING agonist. The bacterial strain containing a plasmid encoding the huSTING R284G variant induced much higher expression of IFNβ and CXCL10, as compared to the strain containing a plasmid encoding WT huSTING. This demonstrates the ability of the strain, containing a plasmid encoding a constitutively active STING GOF variant, to convert a human primary, immunosuppressive M2 macrophage, into an M1, type I IFN producing, cell. While the strains containing plasmids encoding WT huIRF3 and huIRF3-S396D both

induced more, or similar levels of IFNβ, they induced less CXCL10 than the huSTING-R284G variant.

| | Fold Expression Over Untransfected Control | | | |
|---|---|---|---|---|
| **GOF Mutant** | **STING** | **IRF3** | **IFNβ** | **CXCL10** |
| Plasmid Control | 22.3 | 0 | ND | ND |
| WT huSTING | 24017.1 | ND | 3.4 | 3934.5 |
| huSTING R284G | 36542.7 | ND | 20 | 23484.5 |
| WT huIRF3 | 22.7 | 478.9 | 17.5 | 10766.2 |
| huIRF3-S396D | 30.8 | 346.4 | 26.3 | 15696.1 |
| 3'5' RpRp c-di-AMP | 244.8 | 1.11 | 1.77 | 594.1 |

ND = No Data

These data demonstrate the expression of constitutively active GOF type I IFN variants in human primary M2 macrophages, and converting these cells to M1-like, type I IFN producing, cells.

## Example 13

## Immunostimulatory Bacteria, Containing Plasmids Encoding Constitutive Type I IFN Variants, Demonstrate Potent Anti-tumor Immunity in a Murine Model of Colorectal Cancer

### Human GOF STING Mutants Show Anti-Tumor Activity in Mouse Models

To demonstrate that immunostimulatory bacterial strains, containing expression plasmids encoding constitutively active STING variants, induce anti-tumor efficacy, strain YS1646Δ*asd*/ΔFLG (with a knockout of both flagellin genes *fljB* and *fliC*) was electroporated with a plasmid containing an expression cassette for human STING with the allele R232 and the GOF mutation V155M (huSTING V155M), behind the human elongation factor-1 alpha (EF-1α) promoter, and was compared to strain YS1646 alone, and to a PBS vehicle control. The gene encoding huSTING V155M was generated using DNA synthesis and cloned into the pATI-1.75 vector. In order to evaluate whether a constitutively active human STING variant could demonstrate anti-tumor activity in mice, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 8 with $5x10^5$ CFUs of strain YS1646Δ*asd*/ΔFLG-huSTING V155M, with strain YS1646, or with PBS control.

The results showed that the YS1646 parental strain was only mildly effective as an anti-tumor therapy, and was not curative (35% TGI, $p$ = NS (not significant),

day 28), in line with previously published data. The more attenuated strain, containing a plasmid encoding constitutively active human STING, YS1646Δ*asd*/ΔFLG-huSTING V155M, however, elicited significant tumor control (60% TGI, $p < 0.05$, day 28) compared to PBS, and had a cure rate of 20%. Thus, an immunostimulatory bacterial strain that delivers a constitutively active STING variant potently inhibits tumor growth, and demonstrates curative effects in a model of colorectal adenocarcinoma.

**Murine Phosphomimetic IRF3 Shows Curative Effects *In Vivo***

The murine version of the phosphomimetic human IRF3 variant was designed, designated muIRF3-S388D, and evaluated in a murine model of colorectal adenocarcinoma. Strain YS1646Δ*asd*/ΔFLG was electroporated with a plasmid containing an expression cassette for murine IRF3 with the GOF mutation S388D (muIRF3-S388D), behind the human elongation factor-1 alpha (EF-1α) promoter, and was compared to PBS vehicle control. The gene encoding muIRF3-S388D was generated using DNA synthesis and cloned into the pATI-1.75 vector. 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 10 with $5 x10^5$ CFUs of strain YS1646Δ*asd*/ΔFLG-EF-1α-muIRF3-S388D, and compared to PBS vehicle control.

The therapy was very well tolerated, with an initial weight loss nadir of only 0.3%. Compared to PBS, the bacterial strain containing the plasmid encoding the muIRF3-S388D GOF mutant was highly effective and curative (81.8% TGI, 60% CR, day 42). These data demonstrate the potency and safety of delivering constitutively active type I IFN inducing variants in a tumor-specific manner.

**Murine STING GOF Variants Show Potent and Curative Anti-Tumor Activity**

A panel of murine orthologs of the human STING variants, discovered in human patients, was designed. These orthologs differ by one codon from the human variants, and were cloned into the pATI-1.75 vector under the control of an EF-1α promoter, to yield the following set of mutants: muSTING N153S, muSTING V154M, muSTING R280Q, muSTING V146L, muSTING R283G, and muSTING C205Y, among others. The STING variants were evaluated in the MC38 model of murine adenocarcinoma for anti-tumor efficacy. For the studies, 6-8 week-old female

C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 10 with $5 x10^5$ CFUs of strain YS1646Δ*asd*/ΔFLG, containing a plasmid with EF-1α driving the expression of muSTING N153S, muSTING V154M, muSTING R280Q, muSTING V146L, or muSTING R283G, or a scrambled shRNA plasmid control, and compared to PBS vehicle control.

In this experiment, strain YS1646Δ*asd*/ΔFLG EF-1α-shSCR (scrambled plasmid control) demonstrated anti-tumor efficacy as compared to PBS control (73% TGI, day 26), which was much more potent than the YS1646 parental strain has shown historically. This can be due to inherently immunostimulatory elements on the plasmid itself, such as CpGs and RNAi stimulatory elements. However, this therapy was the least well tolerated of the group, demonstrating a weight loss nadir of 9.9% that only resolved at the very end of the study. In contrast, the constitutively active murine STING mutants resulted in a lower weight loss that was transient and that resolved within days. The relative anti-tumor efficacy of these variants revealed differences in activity, with only two variants demonstrating curative effects and enhanced efficacy over the plasmid control, muSTING N153S, and muSTING R283G.

| Murine STING GOF Mutant | TGI vs. PBS, Day 26 | Complete Response | Weight Loss Nadir and Day |
|---|---|---|---|
| Plasmid Control | 73.0% | 0/5 | 9.9%, day 19 |
| muSTING N153S | 81.7% | 1/5 | 6.2%, day 12 |
| muSTING V154M | 69.4% | 0/5 | 4.3%, day 12 |
| muSTING R280Q | 68.7% | 0/5 | 5.4%, day 12 |
| muSTING V146L | 63.4% | 0/5 | 2.8%, day 12 |
| muSTING R283G | 81.2% | 1/5 | 6.9%, day 12 |

In a follow-up study, the murine STING C205Y variant was tested along with the R283G and N153S variants, to compare their anti-tumor efficacy. 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 9 with $5 x10^5$ CFUs of strain YS1646Δ*asd*/ΔFLG containing a plasmid with EF-1α driving the expression of muSTING N153S, muSTING R283G, or muSTING C205Y, and compared to PBS

vehicle control. As before, the STING variants were well tolerated, and only a transient dip in weight loss was observed that resolved quickly. This is likely due to on-target therapy, as it is also observed with the small molecule STING agonists. The efficacy of the two constitutively active murine STING variants, muSTING N153S and muSTING R283G, was nearly identical to the previous study, although the weight loss was much less, for reasons unclear. The muSTING C205Y variant also was highly effective, although not curative.

| Murine STING GOF Mutant | TGI vs. PBS, Day 29 | Complete Response | Weight Loss Nadir and Day |
|---|---|---|---|
| muSTING C205Y | 79.4% | 0/5 | 2.6%, day 13 |
| muSTING N153S | 79.3% | 1/5 | 2.2%, day 13 |
| muSTING R283G | 85.1% | 1/5 | 1.8%, day 13 |

The STING-cured mice from these studies were re-challenged at day 40 post-initial tumor implantation on the opposite flank, SC with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Compared to naïve mice (N=5), in which all tumors grew out, all of the STING-cured mice rejected the tumors, demonstrating the engagement of adaptive immunity.

These data validate the safety and potency of the murine versions of the human constitutively active STING variants in a murine model of colorectal carcinoma, and reveal a small subset of variants that have enhanced potency compared to the other STING variants. These highly active variants also elicit protective immunity, demonstrating the potency of tumor-specific production of type I interferon.

**Murine STING GOF Variants Demonstrate Significant Tumor Remodeling Following IV Dosing**

It was next determined whether the bacterial strains containing plasmids encoding constitutively active STING variants demonstrate differences in their ability to remodel the tumor microenvironment (TME) following IV dosing. To test this, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected on day 8 with $5 x10^5$ CFUs of strain YS1646Δ*asd*/ΔFLG, containing a plasmid with EF-1α driving the expression of

muSTING N153S, muSTING V154M, muSTING R280Q, muSTING V146L, muSTING R283G, or plasmid control, and compared to PBS vehicle control.

At day 28 post tumor implantation, tumors were excised for analysis. Tumors were cut into 2-3 mm pieces into gentleMACS™ C tubes (Miltenyi Biotec) filled with 2.5 mL enzyme mix (RPMI-1640 + 10% FBS with 1 mg/mL Collagenase IV and 20 µg/mL DNase I). The tumor pieces were dissociated using OctoMACS™ (Miltenyi Biotec) specific dissociation program (mouse implanted tumors), and the whole cell preparation was incubated with agitation for 45 minutes at 37 °C. After 45 minutes of incubation, a second round of dissociation was performed using the OctoMACS™ (mouse implanted tumor) program, and the resulting single cell suspensions were filtered through a 70 µM nylon mesh into a 50 mL tube. The nylon mesh was washed once with 5 mL of RPMI-1640 + 10% FBS, and the cells were filtered a second time using a new 70 µM nylon mesh into a new 50 mL tube. The nylon mesh was washed with 5 mL of RPMI-1640 with 10% FBS, and the filtered cells were then centrifuged at 1000 RPM for 7 minutes. The resulting dissociated cells were resuspended in PBS and kept on ice before the staining process.

The percentage of live tumor-infiltrating leukocytes (TILs), including $CD4^+$ Tregs, $CD4^+$ Th1 cells, $CD8^+$ T cells, neutrophils, monocytes, dendritic cells (DCs), M1 macrophages and M2 macrophages, following the administration of strain YS1646Δ*asd*/ΔFLG, containing plasmids encoding the various GOF muSTING mutants, was determined by flow cytometry. For the flow-cytometry staining, 100 µL of the single cell suspensions were seeded in wells of a V-bottom 96-well plate. PBS containing a dead/live stain (Zombie Aqua™, BioLegend) and Fc Blocking reagents (BD Biosciences) were added at 100 µL per well, and incubated on ice for 30 minutes in the dark. After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were then resuspended in PBS + 2% FBS, containing fluorochrome-conjugated antibodies (CD4 FITC clone RM4-5; CD8a BV421 clone 53-6.7; F4/80 APC clone BM8; CD11b PE-Cy7 clone M1/70; CD45 BV570 clone 30-F11; CD3 PE clone 145-2C11; Ly6C BV785 clone HK1.4; I-A/I-E APC-Cy7 clone M5/114.15.2; Ly6G BV605 clone 1A8; and CD24 PercP-Cy5.5 clone M1/69; all from BioLegend), and incubated on ice for 30 minutes in the dark. After 30 minutes, the cells were washed twice with PBS + 2% FBS by

centrifugation at 1300 RPM for 3 minutes and resuspended in flow cytometry fixation buffer (Thermo Fisher Scientific). Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

As shown in the tables below, the YS1646Δ*asd*/ΔFLG strain with the EF-1α plasmid control demonstrated predominantly high neutrophil infiltration, despite some $CD8^+$ T-cell recruitment, likely due to immunostimulatory elements on the plasmid. In contrast, the different muSTING variants had unique tumor-infiltrating immune cell signatures, with some, such as muSTING V146L and muSTING R283G resulting in fewer immunosuppressive neutrophils than the PBS control. The most favorable immune profiles were observed in the tumors from mice that were administered the muSTING R283G and muSTING N153S mutants, with high numbers of $CD4^+$ Th1 cells and $CD8^+$ T-cells, and low numbers of neutrophils, which indicates highly favorable conditions for generating an adaptive immune response. In addition, the muSTING R283G and muSTING N153S mutants had significantly higher p15e tumor-antigen-specific $CD8^+$ T-cells in the tumor, as compared to PBS. These trends were also recapitulated in the total cell counts, as shown below. Thus, delivery of constitutively active STING variants to the tumor-resident myeloid cells leads to a complete remodeling of the immunosuppressive tumor microenvironment, towards an adaptive anti-tumor phenotype, and away from a bacterial phenotype, which is characterized by the promotion of innate immunity and the suppression of adaptive immunity.

**% of Live Tumor-Infiltrating Leukocytes (TILs)**

| | **muSTING GOF Mutants Encoded on Plasmids in IV-Administered YS1646Δ*asd*/ΔFLG Strain** | | | | | | |
|---|---|---|---|---|---|---|---|
| **% Among TILs** | **PBS** | **Plasmid Control** | **muSTING N153S** | **muSTING V154M** | **muSTING R280Q** | **muSTING V146L** | **muSTING R283G** |
| **$CD4^+$ Tregs** | 2.9 ± 1.7 | 1.8 ± 0.5 | 3.3 ± 2.6 | 1.5 ± 0.5 | 2 ± 0.9 | 1.8 ± 0.5 | 1.5 ± 0.4 |
| **$CD4^+$ Th1 Cells** | 6.6 ± 3.2 | 13 ± 4.3 | 20.7 ± 10.4 | 13.2 ± 6.1 | 22.6 ± 7.4 | 19.5 ± 4.9 | 25.6 ± 7.8 |
| **$CD8^+$ T Cells** | 16.2 ± 10.2 | 24.5 ± 11.6 | 25.8 ± 9.2 | 16.5 ± 4.4 | 20.2 ± 7.2 | 20.8 ± 3.6 | 27.8 ± 8.7 |
| **Neutrophils** | 11.3 ± 14.2 | 30.6 ± 17.4 | 13.5 ± 12.4 | 21.4 ± 12.5 | 15.9 ± 11.6 | 9.1 ± 6.5 | 6.6 ± 5.9 |
| **Monocytes** | 16.6 ± 3.5 | 12.7 ± 2.7 | 13.7 ± 3.9 | 16.4 ± 5.4 | 15.8 ± 6.5 | 15.6 ± 2.2 | 13.5 ± 1.3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **DCs** | 1.4 ± 0.4 | 0.5 ± 0.3 | 0.9 ± 0.7 | 0.4 ± 0.2 | 0.5 ± 0.3 | 0.5 ± 0.2 | 0.5 ± 0.3 |
| **M1 Macro-phages** | 10.2 ± 6.2 | 3.1 ± 2.1 | 4.6 ± 2.1 | 9.4 ± 5 | 5.6 ± 4.2 | 8.6 ± 3.7 | 5.4 ± 2 |
| **M2 Macro-phages** | 14.9 ± 10.6 | 4.6 ± 2.6 | 7.3 ± 3.1 | 11.6 ± 5.2 | 8.6 ± 6.3 | 12.8 ± 5 | 9.2 ± 4 |

**Total Cell Counts**

| | **muSTING GOF Mutants Encoded on Plasmids in IV-Administered YS1646Δ*asd*/ΔFLG Strain** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **PBS** | **Plasmid Control** | **muSTING N153S** | **muSTING V154M** | **muSTING R280Q** | **muSTING V146L** | **muSTING R283G** |
| **$CD4^+$ Tregs** | 437 ± 230 | 148 ± 102 | 530 ± 117 | 310 ± 114 | 520 ± 169 | 297 ± 207 | 438 ± 176 |
| **$CD4^+$ Th1 Cells** | 1108 ± 599 | 1059 ± 711 | 3765 ± 917 | 3349 ± 2869 | 5864 ± 1618 | 2961 ± 1800 | 7463 ± 3240 |
| **$CD8^+$ T Cells** | 2948 ± 3119 | 1571 ± 601 | 6152 ± 3820 | 3898 ± 2823 | 5446 ± 2454 | 3266 ± 1277 | 7566 ± 1782 |
| **Neutrophils** | 1531 ± 1604 | 2604 ± 1975 | 3699 ± 4400 | 4815 ± 3423 | 4301 ± 3502 | 1240 ± 1160 | 1698 ± 1485 |
| **Monocytes** | 2871 ± 1472 | 912 ± 369 | 3182 ± 1708 | 3350 ± 1183 | 4132 ± 1595 | 2524 ± 1420 | 3811 ± 996 |
| **DCs** | 233 ± 97 | 28 ± 18 | 161 ± 45 | 82 ± 31 | 130 ± 90 | 78 ± 48 | 135 ± 90 |
| **M1 Macro-phages** | 2163 ± 2025 | 227 ± 213 | 881 ± 316 | 1797 ± 750 | 1421 ± 910 | 1325 ± 856 | 1524 ± 658 |
| **M2 Macro-phages** | 3046 ± 2996 | 334 ± 275 | 1391 ± 373 | 2183 ± 608 | 2189 ± 1402 | 2043 ± 1237 | 2612 ±1330 |

## Example 14

## Expression of Multiple Immunomodulatory Proteins using Combinatorial Plasmid Expression Cassettes

### Multi-Modular Plasmid Expression Cassettes Demonstrate the Ability to Produce Multiple Immunomodulatory Proteins in Human Cells

Combinations of nucleic acids, encoding GOF type I IFN inducing variants and cytokines, were cloned into the pATI-1.75 vector, using two separate ORFs, under the control of the CMV and EF-1α promoters (dual promoter system), or using T2A sequences within the ORFs and one promoter (single promoter system). The constructs also optionally included post-transcriptional regulatory elements (PREs), such as 3' WPRE or HPRE, and/or polyadenylation signal sequences, such as the SV40 or bovine growth hormone (bGH) polyadenylation signals. The constructs prepared included those encoding the cytokines muIL-2, mu-IL-2 with codon optimization (muIL-2 CO), muIL-21, muIL-12p70, muIL-15Rα-IL-15sc, muIL-18, and muIFN-α2, and combinations thereof; the muSTING variant with the mutation

R283G; and/or the murine co-stimulatory molecule 4-1BBL with a deletion of the cytoplasmic domain (mu4-1BBLΔcyt). The sequences were confirmed by PCR.

To determine whether the combination plasmids, containing GOF type I IFN inducing variants, can induce functional type I IFN in human cells, the plasmids were assessed using HEK293T STING Null Reporter cells (InvivoGen), which do not contain endogenous STING. These cells express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous IFN-stimulated response element (ISRE) promoter, where the coding sequence of ISRE has been replaced by the SEAP ORF using knock-in technology. Type I interferon activity can be assessed by monitoring type I IFN-stimulated SEAP production in the cell culture supernatants. In addition, supernatants were collected and evaluated for relative cytokine concentrations by ELISA.

To test the relative production of type I IFN and co-expressed cytokines, $2x10^5$ 293T-Dual™ Null cells (InvivoGen) were plated one day prior on 24-well plates coated with poly-L-lysine, to achieve 80% confluency. On the day of transfection, 500 ng of plasmids encoding a panel of GOF variants, cytokines, and co-stimulatory molecules, alone, or in various combinations, were diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios. Cell culture supernatants from each sample were collected after overnight incubation, and 20 µL of the cell culture supernatants was added to 180 µL QUANTI-Blue™ reagent (InvivoGen). Type I interferon activation was determined by measuring ISRE-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices) at an absorbance wavelength of 650 nm. The muIL-2 constructs were evaluated for muIL-2 expression in a murine IL-2 ELISA (R&D Systems), according to the manufacturer's instructions. The muIL-12p70 constructs were evaluated in a murine IL-12 ELISA (R&D Systems), according to the manufacturer's recommendation. For the muIL-15Rα-IL-15sc constructs, the murine IL-15 ELISA (eBioscience) was used, per kit instructions. IFN-α2 was measured using the RAW-Lucia™ ISG reporter cell line (InvivoGen). Murine IL-18 and murine IL-21 were measured by ELISA (Invitrogen).

As shown in the table below, assays performed to detect the presence of secreted functional proteins demonstrated high expression of multiple combination

payloads. These include cytokine combinations, as well as combinations with type I IFN inducing GOF variants, and/or with co-stimulatory payloads. These data validate the ability of the platform provided herein to express multiple immunomodulatory proteins from a single expression cassette.

| Construct | Protein Expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **STING (Abs 650 nm)** | **IL-2 (Abs 650 nm)** | **IL-12 (Abs 650 nm)** | **IL-15 (pg/mL)** | **IL-15 (Abs 650 nm)** | **IFN-α2 (Luminescence)** | **IL-21 (OD at 450 nm)** | **IL-18 (pg/mL)** |
| CMV-muIL-2 | | 1.995 | | | | | | |
| CMV-muIL-2 CO-WPRE | | 2.0073 | | | | | | |
| EF-1α-muIL-21 | | | | | | | 1.138 ± 0.1853 | |
| CMV-muIL-21 | | | | | | | 0.4758 ± 0.0461 | |
| EF-1α-muIL-12p70 | | | 0.9052 | | | | | |
| CMV-muIL-15Rα-IL-15sc-WPRE | | | | 30140.8 ±535.5 | 0.8224 | | | |
| EF-1α-muIFN-α2 | | | | | | 14570 | | |
| CMV-muIFN-α2-WPRE | | | | | | 18658 | | |
| CMV-muIL-2 CO_T2A_muIFN-α2-WPRE | | 2.0998 | | | | 13066 | | |
| CMV-muIL-15Rα-sc _T2A_ muIFN-α2-WPRE | | | | 29895.9 ±630.9 | 1.0125 | 8571 | | |
| CMV-muIL-2 CO + EF-1α-muIFN-α2 | | 2.0589 | | | | 8297 | | |
| CMV-muIL-21 + EF-1α-muIFN-α2 | | | | | | 8682 | 0.3797 ± 0.0830 | |
| CMV-muIL-2 CO + EF-1α-muIL-21 | | 2.0085 | | | | | 0.7565 ± 0.1271 | |
| CMV-muIL-15Rα-IL-15sc + EF-1α-muIL-21 | | | | 12605 ± 278.6 | 0.2362 | | 0.2963 ± 0.0155 | |

| Construct | Protein Expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | STING (Abs 650 nm) | IL-2 (Abs 650 nm) | IL-12 (Abs 650 nm) | IL-15 (pg/mL) | IL-15 (Abs 650 nm) | IFN-α2 (Luminescence) | IL-21 (OD at 450 nm) | IL-18 (pg/mL) |
| CMV-muIL-12p70 + EF-1α-muIL-21 | | | 1.0893 | | | | 0.1479 ± 0.001 | |
| CMV-muIL-2 CO-HPRE-bGHpolyA | | 1.207 ± 0.121 | | | | | | |
| EF-1α-muSTING R283G | 0.4874 ± 0.0045 | | | | | | | |
| CMV-muSTING R283G-WPRE | 0.4866 ± 0.0511 | | | | | | | |
| CMV-muIL-2 CO + EF-1α-muIL-12p70 | | 0.9971 ± 0.0104 | 0.3303 ± 0.0157 | | | | | |
| CMV-muIL-15Rα-IL-15sc + EF-1α-muIL-12p70 | | | 0.3629 ± 0.0242 | | 0.1464 ± 0.0034 | | | |
| CMV-muIL-18 + EF-1α-muSTING R283G-WPRE | 0.7096 ± 0.0169 | | | | | | | 5991.4 ± 1642.0 |
| CMV-muIL-12p70_T2A_muIFN-α2 + EF-1α-IL-18-WPRE | | | 0.6538 ± 0.0205 | | | 577.3 ± 122.5 | | 12685.0 ± 4402.8 |
| CMV-muIL-12p70_T2A_muSTING R283G + EF-1α-muIL-18-WPRE | 0.454 ± 0.123 | | 0.4581 ± 0.0158 | | | | | 3805.8 ± 1369.5 |
| CMV-muIL-15Rα-IL-15sc_T2A_muSTING R283G + EF-1α-muIL-18-WPRE | 0.5867 ± 0.0333 | | | | 0.2326 ± 0.0049 | | | 3626 ± 1248 |
| CMV-muIL-15Rα-IL-15sc_T2A_muIFN-α2 + EF- | | | | | 0.3512 ± 0.0172 | 1802 ± 216.5 | | 6872 ± 1671 |

| Construct | Protein Expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | STING (Abs 650 nm) | IL-2 (Abs 650 nm) | IL-12 (Abs 650 nm) | IL-15 (pg/mL) | IL-15 (Abs 650 nm) | IFN-α2 (Luminescence) | IL-21 (OD at 450 nm) | IL-18 (pg/mL) |
| 1α-muIL-18-WPRE | | | | | | | | |
| CMV-4-1BBL(Δcyt) + EF-1α-muSTING R283G-WPRE | 0.2525 ± 0.0144 | | | | | | | |

**Constitutively Active Type I IFN Inducing Variants have Unique Cytokine and Chemokine Profiles**

Supernatants harvested from HEK293T transfection experiments with a panel of human type I IFN inducing GOF variants, tested as described above, were evaluated for downstream signaling differences using a human anti-viral CBA panel (BD Biosciences), according to the manufacturer's protocol. Each transfection was performed in duplicate, and cytokine levels were measured. The average of two measurements was calculated. Fold increase in average cytokine secretion was calculated compared to untransfected wells, in which the average cytokine secretion was set as 1.00.

As shown in the table below, low levels of human IL-12p70 were produced in the cells. However, several human type I IFN inducing GOF variants induced the production of type I IFN-α2 and/or IFN-β, including the phosphomimetic IRF3 variant (huIRF3 S396D), as well as several constitutively active STING variants. A number of these GOF variants produced high levels of secreted CXCL10, demonstrating the ability of these expressed variants to recruit T-cells into the tumor microenvironment. The highest levels of CXCL10 expression were observed following the expression of the huSTING variant with the mutation R284G.

These data validate the use of multiplexed plasmid expression constructs, containing type I IFN inducing GOF variants, for induction of functional downstream cytokines and chemokines, such as CXCL10/IP-10. These variants all have unique signatures, and STING GOF variants induced the highest levels of CXCL10 secretion, particularly huSTING R284G (corresponding to muSTING R283G).

| Constructs | Secreted Immunostimulatory Cytokines and Chemokines (Fold Relative to Untransfected) | | | |
|---|---|---|---|---|
| | IL-12p70 | IFN-α2 | IFN-β | CXCL10 |
| Untransfected control | 1.00 | 1.00 | 1.00 | 1.00 |
| EF-1α-mCherry Control | 1.50 | 1.59 | 1.50 | 2.05 |
| EF-1α-huSTING Negative Control | 1.32 | 2.10 | 1.43 | 2.16 |
| EF-1α-huSTING WT | 1.66 | 1.69 | 1.46 | 2.77 |
| EF-1α-huSTING-V147L | 1.78 | 2.35 | 21.35 | 332.14 |
| EF-1α-huSTING-N154S | 1.52 | 2.23 | 23.37 | 398.99 |
| EF-1α-huSTING-V155M | 2.00 | 2.09 | 6.02 | 121.38 |
| EF-1α-huSTING-C206Y | 1.39 | 2.02 | 16.51 | 218.38 |
| EF-1α-huSTING-R281Q | 1.35 | 1.35 | 8.17 | 301.18 |
| EF-1α-huSTING-R284G | 1.46 | 2.14 | 13.41 | 429.40 |
| EF-1α-huIRF3-WT | 1.50 | 1.49 | 1.50 | 1.11 |
| EF-1α-huIRF3-S396D | 1.93 | 9.37 | 38.06 | 65.89 |
| CMV-muIFN-α2-WPRE | 1.03 | 3.63 | 1.37 | 146.49 |
| CMV-muIL-2 CO_T2A_muIFN-α2-WPRE | 1.03 | 1.63 | 1.37 | 86.02 |
| CMV-muIL-15Rα-IL-15sc_T2A_muIFN-α2-WPRE | 1.03 | 0.80 | 1.37 | 40.85 |

**Immune Cell Co-Culture Assays Identify Optimal Combinations of Immunomodulatory Targets**

In order to determine the optimal combinations of cytokines to elicit T-cell recruitment and activation, a panel was tested in a macrophage and T-cell co-culture assay. Golden ticket (STING deficient) murine primary bone marrow-derived macrophages (BMMs) were generated as described above (see, Example 6), using a 24-well plate. Each well was transfected with the appropriate DNA constructs using FuGENE® transfection reagents (Promega). The constructs included those encoding muIL-2 CO, muIL-12p70, muSTING-R283G, muIL-2 CO + muIL-12p70, muIL-15Rα-IL-15sc + muIL-12p70, and muIL-12p70 + muSTING-R283G + muIL-18.

24 hours post-transfection, 100 µL of cell culture supernatants were harvested from the wells for a flow cytometry-based cytokine bead array (CBA). In parallel, two spleens from C57BL/6 mice were dissected, and splenic $CD4^+$ and $CD8^+$ T-cells were isolated following instructions from a mouse T-cell isolation kit (StemCell Technologies). 200,000 isolated T-cells per well were then added to the transfected cells, with or without CD3ε antibody (clone 145-2C11, BioLegend), at a final concentration of 0.5 µg/ml per well. At 24 and 48 hours post-addition of T-cells to the

transfected cells, 100 µL of the co-culture supernatants were harvested from the wells for a flow-cytometry based cytokine bead array. Supernatants from transfected bone marrow macrophages (BMMs) and bone marrow macrophage/T-cell co-cultures were analyzed for their cytokine content, using murine anti-viral and murine Th1 specific cytokine bead arrays, respectively.

As shown in the table below, only the plasmids encoding muSTING-R283G elicited CXCL10 production from the macrophages, while plasmids encoding muIL-12p70, alone or in combination with other proteins, elicited the highest levels of IFNγ from the co-cultured T-cells, which were greater than the background amount resulting from CD3ε stimulation. The combination of muIL-12p70 + muIL-18 + muSTING-R283G was able to induce both CXCL10 production from macrophages, and IFNγ production from co-cultured T-cells.

These data demonstrate the feasibility of expressing multiple immunomodulatory payloads from a single plasmid, as well as the synergistic activities of these combinations.

| Constructs | CXCL10 Production by BMMs (pg/mL) | IFNγ Production By T-Cells (pg/mL) |
|---|---|---|
| Untransfected | 3.82 | 3170.28 |
| CMV-muIL-2 CO-WPRE | 5.92 | 7633.19 |
| EF-1α-muIL-12p70 | 12.24 | 11495.65 |
| CMV-muSTING-R283G | 662.03 | 1060.82 |
| CMV-muIL-2 CO + EF-1α-muIL-12p70 | 4.59 | 8511.19 |
| CMV-muIL-15Rα-IL-15sc + EF-1α-muIL-12p70 | 5.82 | 11694.78 |
| CMV-muIL-12p70_T2A_muSTING-R283G + EF-1α-muIL-18 | 324.92 | 9331.51 |

**Combinatorial Immunotherapy Demonstrates Enhanced Anti-Tumor Activity in a Murine Model of Colorectal Adenocarcinoma**

In order to determine whether tumor-specific delivery of a combination of cytokines enhances anti-tumor efficacy, a construct encoding the combination of muIL-12p70, muIL-18, and muSTING-R283G (CMV-muIL-12p70_T2A_muIL-18 + EF-1α-muSTING-R283G-WPRE), was assessed in the MC38 murine model of colorectal adenocarcinoma. For this study, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 colorectal adenocarcinoma cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank

tumors were IV injected on day 10 with $1x10^7$ CFUs of strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*, containing a plasmid encoding the combination of muIL-12p70, muIL-18, and muSTING-R283G (CMV-muIL-12p70_T2A_muIL-18 + EF-1α-muSTING-R283G-WPRE), or with PBS vehicle control.

The combination therapy was very well tolerated, with only an initial weight loss of 3.6% that had fully recovered 3 days later. This is in marked contrast to the toxicities observed with systemic administration of these cytokines (IL-12p70 and IL-18). Compared to PBS control, the combination therapy was highly effective and curative (92.3% TGI, 60% cure rate, day 30). These data demonstrate the potency and safety of delivering combinations of cytokines in a tumor-specific manner, using the immunostimulatory bacterial strains described herein.

## Example 15

## Protein Engineering Screening to Identify Improved Gain-of-Function Mutations in STING, RIG-I, MDA5, IRF3, IRF7, and other Interferon Pathway Genes

Gain-of-function (GOF) amino acid mutants that are constitutively active and that promote interferonopathies are identified from humans. Many GOF mutations occur due to single base pair nucleotide changes that alter the amino acid codon at that particular position in the gene. For example, in STING, the V147L mutation occurs due to a mutation at c.439G→C; N154S occurs due to a mutation at c.461A→G; and V155M occurs due to a mutation at c.463G→A. The purpose of the screening was to identify constitutively active mutants that lead to high levels of type I interferon expression. Designed mutations, at sites known to promote interferonopathies when mutated in human patients, allow for a greater number of amino acid substitutions to be tested. In this example, site-directed mutagenesis with designed amino acids is performed at the positions of known mutations (see, Table below, listing mutations in genes that promote interferonopathies), to identify mutations with enhanced activity, that lead to high level type I interferon expression.

| Exemplary Normal Function Proteins in which the Mutations are Introduced | Gain-of-Function Mutations |
|---|---|
| | S102P |
| | V147L |
| | V147M |
| | N154S |

| Exemplary Normal Function Proteins in which the Mutations are Introduced | Gain-of-Function Mutations |
|---|---|
| STING/TMEM173 (SEQ ID NOs: 305-309) | V155M |
| | G166E |
| | C206Y |
| | G207E |
| | S102P/F279L |
| | F279L |
| | R281Q |
| | R284G |
| | R284S |
| | R284M |
| | R284K |
| | R284T |
| | R197A |
| | D205A |
| | R310A |
| | R293A |
| | T294A |
| | E296A |
| | R197A/D205A |
| | S272A/Q273A |
| | R310A/E316A |
| | E316A |
| | E316N |
| | E316Q |
| | S272A |
| | R375A |
| | R293A/T294A/E296A |
| | D231A |
| | R232A |
| | K236A |
| | Q273A |
| | S358A/E360A/S366A |
| | D231A/R232A/K236A/R238A |
| | S358A |
| | E360A |
| | S366A |
| | R238A |
| | S324A/S326A |
| MDA5/IFIH1 (SEQ ID NO: 310) | T331I |
| | T331R |
| | A489T |
| | R822Q |
| | G821S |
| | A946T |
| | R337G |
| | D393V |
| | G495R |
| | R720Q |

| Exemplary Normal Function Proteins in which the Mutations are Introduced | Gain-of-Function Mutations |
|---|---|
| | R779H |
| | R779C |
| | L372F |
| | A452T |
| RIG-I (SEQ ID NO: 311) | E373A |
| | C268F |

Amino acid residues R197, D205, R310, R293, T294, E296, S272, Q273, E316, D231, R232, K236, S358, E360, S366, and R238, with reference to the sequence of human STING, as set forth in SEQ ID NOs:305-309, correspond to amino acid residues R196, D204, R309, R292, T293, E295, S271, Q272, E315, D230, R231, K235, S357, E359, S365, and R237, respectively, with reference to the sequence of murine STING, as set forth in SEQ ID NO:369.

PCR primers are generated with designed substitutions flanked on the 5' and 3' ends with homologous cDNA sequences from the gene. The QuikChange® Site-Directed Mutagenesis kit (Agilent), or other comparable commercially available kit, is used to generate a PCR product incorporating the designed mutation. PCR amplified plasmids are treated with DpnI, then electroporated into competent *E. coli* cells. Individual clones are isolated, plasmid mini-preps are performed, and the sequence identity of the desired mutation is confirmed. Larger scale plasmid preparations are then performed (using a Qiagen Kit), and the DNA is transfected into HEK293T STING Reporter cells (InvivoGen), which do not contain endogenous STING. These cells express Lucia™ luciferase, a secreted luciferase, placed under the control of the endogenous IFN-β promoter; the coding sequence of IFN-β has been replaced by the Lucia™ luciferase ORF using knock-in technology. Constitutively activate mutants then are identified and ranked by measurement of IFN-β promoter induced expression of luciferase activity.

## Example 16

## Various Species of Immunostimulatory Bacteria with Inactivating Deletions Corresponding to the Deletions in *Salmonella typhimurium*

The Examples above describe exemplary modifications to the genome of *Salmonella typhimurium* to increase targeting to and accumulation of *Salmonella typhimurium* in immune-resident myeloid cells and in the tumor microenvironment, to deliver therapeutic products/payloads to tumors, and to reduce toxicity of the bacteria

by eliminating their ability to infect other cell types. These genetic modifications similarly can be introduced into other bacterial strains and species, such as by deletion of the corresponding genes in other species, as described below.

***Escherichia coli***

In-frame chromosomal deletions of the *lpxM*, *purM*, *asd*, *fliC*, *fliE*, *pagP*, *ansB* and *csgD* genes are made sequentially in *E. coli* strains using a technique based on the recombineering methods described by Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)). The genes in *E. coli* are as follows:

1) *lpxM*, encoding myristoyl-acyl carrier protein-dependent acyltransferase [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 945143];

2) *purM*, encoding phosphoribosylformylglycinamide cyclo-ligase [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 946975];

3) *asd*, encoding aspartate-semialdehyde dehydrogenase [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 947939];

4) *fliC*, encoding flagellar filament structural protein [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 949101];

5) *fliE*, encoding flagellar basal-body protein FliE [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 946446];

6) *pagP*, encoding Lipid IVA palmitoyltransferase [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 946360];

7) *ansB*, encoding L-asparaginase 2 [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 947454];

8) *csgD*, encoding DNA-binding transcriptional dual regulator CsgD [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 949119]; and

9) *rpsM*, encoding 30S ribosomal subunit protein S13 (promoter) [*E. coli* strain K-12, substrain MG1655; NCBI Gene ID: 947791].

Briefly, a specific chromosomal sequence is replaced with a selectable antibiotic resistance marker flanked by homology arms, and is subsequently removed by a cre/loxP system. The 5' and 3' flanking sequences of each target gene are identified and cloned into a plasmid vector on opposing sides of an antibiotic resistance gene. The gene deletion cassette, containing the antibiotic resistance gene and flanking 5' and 3' homology arms is PCR amplified, gel purified, and introduced

into the *E. coli* strain by electroporation. Electroporated cells are recovered, and transformants are selected for on antibiotic plates. The antibiotic marker is then cured using a cre/loxP recombination system, in which antibiotic resistant clones are transformed with a cre-expressing temperature-dependent plasmid. Colonies are selected at 30 °C and subsequently eliminated by serial passage at 42 °C, and then screened for loss of antibiotic resistance. Antibiotic sensitive clones are confirmed for gene deletion by colony PCR and sequence analysis.

**Increasing Resistance or Rendering *E. coli* Resistant to Human Complement**

Expression of the *Salmonella typhimurium rck* (resistance to complement killing) gene, the *Yersinia enterocolitica* homolog *ail* (attachment invasion locus), or the *Salmonella typhimurium pgtE* (outer membrane serine protease) gene, in the *E. coli* Δ*lpxM*/Δ*purM*/Δ*asd*/Δ*fliC*/Δ*fliE*/Δ*pagP*/Δ*ansB*/Δ*csgD* strain is achieved by encoding the *rck*, *ail*, or *pgtE* gene sequence downstream from a constitutive promoter, such as *E. coli* or *S. typhimurium* rpsM, on a plasmid (*asd* complementation system compatible), or by insertion on the bacterial chromosome (at any of the *lpxM*, *purM*, *asd*, *fliC*, *fliE*, *pagP*, *ansB*, or *csgD* loci).

***Salmonella typhi***

In-frame chromosomal deletions of the *msbB*, *purM*, *asd*, *fliC*, *flgB*, *pagP*, *ansB*, and *csgD* genes are made sequentially in *S. typhi* strains using the technique described above for *E. coli*-based strains.

Expression of the *Salmonella typhimurium rck* (resistance to complement killing) gene, the *Yersinia enterocolitica* homolog *ail*, or the *Salmonella typhimurium pgtE* gene, in the *S. typhi* Δ*msbB*/Δ*purM*/Δ*asd*/Δ*fliC*/Δ*flgB*/Δ*pagP*/Δ*ansB*/Δ*csgD* strain is achieved by encoding the *rck*, *ail*, or *pgtE* gene sequence downstream of a constitutive promoter, such as *S. typhimurium* or *S. typhi* rpsM, on a plasmid (*asd* complementation system compatible), or by insertion on the bacterial chromosome (at any of the *msbB*, *purM*, *asd*, *fliC*, *flgB*, *pagP*, *ansB*, or *csgD* loci). The genes in *S. typhi* are as follows:

1) *msbB* (STY2097), encoding lipid A acyltransferase [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1248440];

2) *purM* (STY2740), encoding phosphoribosylformylglycinamidine cyclo-ligase [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1249054];

3) *asd* (STY4271), encoding aspartate-semialdehyde dehydrogenase [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1250488];

4) *fliC* (STY2167), encoding flagellin [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1248507];

5) *flgB* (STY1213), encoding flagellar basal-body rod protein FlgB [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1247617];

6) *pagP* (STY0677), encoding antimicrobial peptide resistance and lipid A acylation protein [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1247137];

7) *ansB* (STY3259), encoding L-asparaginase [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1249541];

8) *csgD* (STY1179), encoding regulatory protein CsgD [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1247585]; and

9) *rpsM* (STY4380), encoding 30S ribosomal subunit protein S13 (promoter) [*Salmonella enterica* subspecies *enterica* serovar Typhi, strain CT18; NCBI Gene ID: 1250594].

***Listeria monocytogenes***

In-frame chromosomal deletions of the *purA*, *purQ*, *purS*, *asd*, *flaA*, *fliC*, *flgB*, and *ansB* genes in *Listeria monocytogenes* is achieved by an allelic exchange technique using a temperature-sensitive shuttle vector, such as pKSV7, that confers antibiotic resistance and allows replication at low temperatures (30 °C), but is unable to replicate at higher temperature (43 °C). The 5' and 3' flanking sequences of each target gene are identified and cloned in tandem into the pKSV7 vector, which is transformed into recipient *Listeria monocytogenes* and selected for by plating on agar plates with antibiotic. Chromosomal integration of the plasmid is induced by serial passage of antibiotic-resistant transformants at 42 °C under selection. Subsequent sequential subculturing of the strain at 30 °C results in subpopulations of cells in

which the plasmids are excised through a second crossover event, producing reversions to the original wild-type gene, or incorporation of the 5' and 3' flanking sequence homology arms to generate the targeted deletion mutant. Antibiotic sensitive clones are screened at this step by colony PCR and sequence analysis.

To increase complement resistance, expression of the *Salmonella typhimurium rck* (resistance to complement killing) gene, the *Yersinia enterocolitica* homolog *ail*, or the *Salmonella typhimurium pgtE* gene in the *Listeria monocytogenes* Δ*purA*/Δ*purQ*/Δ*purS*/Δ*asd*/Δ*flaA*/Δ*fliC*/Δ*flgB*/Δ*ansB* strain is achieved by encoding the *rck*, *ail*, or *pgtE* gene sequence downstream of a constitutive promoter, such as $P_{hyper}$ or $P_{helper}$, on a plasmid (*asd* complementation system compatible), or by insertion on the bacterial chromosome (at any of the *purA*, *purQ*, *purS*, *asd*, *flaA*, *fliC*, *flgB*, or *ansB* loci). The genes in *L. monocytogenes* are as follows:

1) *purA* (lmo0055), encoding adenylosuccinate synthetase [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 986069];

2) *purQ* (lmo1769), encoding phosphoribosylformylglycinamidine synthase II [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 985972];

3) *purS* (lmo1771), encoding phosphoribosylformylglycinamidine synthase subunit PurS [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 985970];

4) *asd* (lmo1437), encoding aspartate-semialdehyde dehydrogenase [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 986492];

5) *flaA* (lmo0690), encoding flagellin [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 987167];

6) *fliE* (lmo0712), encoding flagellar hook-basal body protein FliE [ *Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 985062];

7) *flgB* (lmo0710), encoding flagellar basal-body rod protein FlgB [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID 985059]; and

8) *ansB* (lmo1663), encoding asparagine synthetase [*Listeria monocytogenes* strain EGD-e; NCBI Gene ID: 985663].

The genes *msbB* and *pagP* are absent in *Listeria monocytogenes*, which is a Gram-positive bacterium. CsgD also is absent in *Listeria monocytogenes*. Instead, *Listeria* express *lcp*, encoding the *Listeria* cellulose binding protein that is involved in biofilm formation, which also can be deleted.

***Bifidobacterium longum***

In-frame chromosomal deletions of BL1122 (*purM*), BL0492 (*asd*) and BL1142 (encoding an L-asparaginase precursor) in *Bifidobacterium longum* is achieved by an allelic exchange technique using an incompatible plasmid vector system, in which a conditional-replication vector, such as pBS423-Δ*repA*, which lacks the plasmid replication gene, *repA*, is initially integrated into the genome, providing antibiotic resistance. A second plasmid, such as pTBR101-CM, encoding the *repA* gene, is subsequently transformed and facilitates a second crossover event that selects for excision of the initial integrant. The 5' and 3' flanking sequences of each target gene are identified and cloned in tandem into the conditional replication vector, lacking *repA*, and transformed into the *Bifidobacterium longum* ΔBL1122/ΔBL0492/ΔBL1142 strain. Crossover recombination events can occur at homology arm sequences, and successful plasmid integrants are selected for and isolated on antibiotic plates. Integrants are then transformed with an incompatible plasmid encoding a functional copy of *repA*, which facilitates a second crossover event and excision of the *repA* deficient plasmid, which is subsequently lost due to plasmid incompatibility. Genomic deletions are confirmed by colony PCR and sequence analysis, and the remaining plasmid is cured by removing selection and subsequent Rif treatment.

Expression of the *Salmonella typhimurium rck* (resistance to complement killing) gene, the *Yersinia enterocolitica* homolog *ail*, or the *Salmonella typhimurium pgtE* gene in the *Bifidobacterium longum* ΔBL1122/ΔBL0492/ΔBL1142 strain is achieved by encoding the *rck*, *ail*, or *pgtE* gene sequence downstream of a strong constitutive promoter, such as $P_{gap}$, on a plasmid (*asd* complementation system compatible), or by insertion on the bacterial chromosome (at any of the BL1122, BL0492, or BL1142 loci). The *Bifidobacterium longum* genes are as follows:

1) *purM* (BL1122), encoding phosphoribosylformylglycinamidine cyclo-ligase [*Bifidobacterium longum* strain NCC2705; NCBI Gene ID: 1022669];

2) *asd* (BL0492), encoding aspartate-semialdehyde dehydrogenase [*Bifidobacterium longum* strain NCC2705; NCBI Gene ID: 1023089];

3) BL1142, encoding an L-asparaginase precursor (Ntn_Asparaginase_2_like; L-Asparaginase type 2-like enzymes of the NTN-hydrolase superfamily) [*Bifidobacterium longum* strain NCC2705; NCBI Gene ID: 1023120]; and

4) BL1363 gap (promoter) [*Bifidobacterium longum* strain NCC2705; NCBI Gene ID: 1022828].

*Bifidobacterium longum* are non-motile and lack flagellin, and are Gram-positive and lack *msbB* and *pagP*. The *ansB* gene is present, but encodes aspartate ammonia-lyase, which catalyzes the formation of fumarate from aspartate (*aspA*/*ansB*) [BL0338, *Bifidobacterium longum* strain NCC2705; NCBI Gene ID: 1023259].

***Clostridium novyi***

In-frame chromosomal deletions of NT01CX_RS09765, NT01CX_RS07625, and NT01CX_RS04325 (*asd*); the flagellin genes NT01CX_RS04995, NT01CX_RS04990, NT01CX_RS05070, and NT01CX_RS05075; and the flagellar basal body rod protein genes NT01CX_RS05080 (*flgB*), NT01CX_RS05085 (*flgC*), and NT01CX_RS05215 (*flgG*) in *Clostridium* is achieved by an allelic exchange technique requiring a counter-selection method including toxin-antitoxin systems, which requires an inducible promoter and toxic gene, such as the *E. coli* mRNA interferase *mazF*. The 5' and 3' flanking sequences of each target gene are identified and cloned in a configuration on opposing sides of a frt-flanked antibiotic resistance cassette in an allelic exchange counter-selection-containing vector, and transformed into *Clostridium novyi*. The *mazF* gene is encoded under the control of an inducible lac promoter on the allelic exchange vector, and permits selection of a double crossover event by growth on lactose-supplemented agar plates. Genomic deletions are confirmed by colony PCR and sequence analysis. Flp-frt recombination can then be used to cure the antibiotic resistance cassette from the chromosome.

Expression of the *Salmonella typhimurium rck* (resistance to complement killing) gene, the *Yersinia enterocolitica* homolog *ail*, or the *Salmonella typhimurium pgtE* gene in the *Clostridium novyi* ΔNT01CX_RS09765/ΔNT01CX_RS07625/ΔNT01CX_RS04325/ΔNT01CX_RS04995/ΔNT01CX_RS04990/ΔNT01CX_RS05070/ΔNT01CX_RS05075/ΔNT01CX_RS05080/ΔNT01CX_RS05085/ΔNT01CX_RS05215 strain is achieved by encoding the *rck*, *ail*, or *pgtE* gene

sequence downstream from a strong constitutive promoter, such as $P_{thl}$, $P_{ptb}$, or other variants, on a plasmid (*asd* complementation system compatible), or by insertion on the bacterial chromosome (at any of the NT01CX_RS09765, NT01CX_RS07625, NT01CX_RS04325, NT01CX_RS04995, NT01CX_RS04990, NT01CX_RS05070, NT01CX_RS05075, NT01CX_RS05080, NT01CX_RS05085, or NT01CX_RS05215 loci). The *Clostridium novyi* genes are as follows:

1) NT01CX_RS09765, encoding AIR synthase [*Clostridium novyi* strain NT; NCBI Gene ID: 4541583];

2) NT01CX_RS07625, encoding phosphoribosylformylglycinamidine cyclo-ligase [*Clostridium novyi* strain NT; NCBI Gene ID: 4540669];

3) NT01CX_RS04325 (*asd*), encoding aspartate-semialdehyde dehydrogenase [*Clostridium novyi* strain NT; NCBI Gene ID: 4541762];

4) NT01CX_RS04995, encoding flagellin [*Clostridium novyi* strain NT; NCBI Gene ID: 4541703];

5) NT01CX_RS04990, encoding flagellin [*Clostridium novyi* strain NT; NCBI Gene ID: 4539984];

6) NT01CX_RS05070, encoding flagellin [ *Clostridium novyi* NT; NCBI Gene ID: 4539886];

7) NT01CX_RS05075, encoding flagellin [*Clostridium novyi* NT; NCBI Gene ID: 4539699];

8) NT01CX_RS05080 (*flgB*), encoding flagellar basal body rod protein FlgB [*Clostridium novyi* strain NT; NCBI Gene ID: 4540637];

9) NT01CX_RS05085 (*flgC*), encoding flagellar basal body rod protein FlgC [*Clostridium novyi* strain NT; NCBI Gene ID: 4540143]; and

10) NT01CX_RS05215 (*flgG*), encoding flagellar basal body rod protein FlgG [*Clostridium novyi* strain NT; NCBI Gene ID: 4540245].

*Clostridium novyi* is Gram-positive, and lacks *msbB* and *pagP*.

## Example 17

## Immunostimulatory Bacteria Modified to Express Vertebrate STING Variants that Induce Stronger Type I IFN Signaling and/or Weaker NF-κB Signaling than Human STING

STING signaling activates two signaling pathways. The first is the TANK binding kinase 1 (TBK1)/IRF3 axis, resulting in the induction of type I IFNs, and the activation of dendritic cells (DCs) and cross-presentation of tumor antigens to activate $CD8^+$ T-cell mediated anti-tumor immunity. The second is the nuclear factor kappa-light-chain-enhancer of activated B-cell (NF-κB) signaling axis, resulting in a pro-inflammatory response, but not in the activation of the DCs and $CD8^+$ T-cells that are required for anti-tumor immunity. Bacterially-based cancer immunotherapies are limited in their ability to induce type I IFN to recruit and activate the $CD8^+$ T-cells that are necessary to promote tumor antigen cross-presentation and durable anti-tumor immunity. Hence, provided are immunostimulatory bacteria herein that induce and/or increase type I IFN signaling, and that have decreased NF-κB signaling, thereby increasing the induction of $CD8^+$ T-cell mediated anti-tumor immunity, and enhancing the therapeutic efficacy of the bacteria. The immunostimulatory bacteria described above encode modified STING proteins that are gain-of-function mutants of STING that can increase induction of type I IFN compared to wild-type STING, or render the expression of type I IFN constitutive. In this example (and also described in the detailed description), the STING protein is modified to reduce or eliminate NF-κB signaling activity, and to retain the ability to induce type I IFN, and/or is modified for increased or constitutive type I IFN expression. This results in immunostimulatory bacteria that induce anti-tumor immunity, and do not induce (or induce less) NF-κB signaling that normally results from infection by bacterial pathogens.

STING proteins from different species exhibit different levels of type I IFN and NF-κB signaling activities. For example, STING signaling in human and mouse cells results in a strong type I IFN response, and a weak pro-inflammatory NF-κB response. STING signaling in ray-finned fish, such as salmon and zebrafish, in comparison, elicits robust activation of a primarily NF-κB-driven response, that is more than 100-fold higher compared with the IRF3-driven (*i.e.*, type I IFN inducing) response. In other species, such as Tasmanian devil, STING signaling results in a type I IFN response, but essentially no NF-κB response. The immunostimulatory bacteria provided herein encode STING from non-human species, such as Tasmanian devil STING, in order to exploit the ability of STING to induce a type I IFN response, but without the concomitant induction of an NF-κB response. As described herein, these

non-human STING proteins also are modified by mutation to increase the type I IFN response, or to render it constitutive. The identified mutations that have this effect in human STING are introduced into the non-human STING proteins. The corresponding residues are identified by alignment.

Also provided are chimeras in which the C-terminal tail (CTT) of STING is replaced in one species, such as human, with the CTT from a second (*e.g.*, non-human) species STING protein that exhibits little or no NF-κB signaling activity. The CTT is an unstructured stretch of approximately 40 amino acids that contains sequence motifs required for STING phosphorylation and recruitment of IRF3. It can shape downstream immunity by altering the balance between type I IFN and NF-κB signaling. This is controlled through independent modules in the CTT, including IRF3-, TBK1-, and TRAF6-binding modules. For example, human STING residue S366 (see, *e.g.*, SEQ ID NOs:305-309) is a primary TBK1 phosphorylation site that is part of an LxIS motif in the CTT, which is required for IRF3 binding, while a second PxPLR motif, including residue L374, is required for TBK1 binding. The LxIS and PxPLR motifs are highly conserved in all vertebrate STING alleles. Replacing the CTT of human STING with that of, for example, Tasmanian devil STING, produces a STING variant that induces a type I IFN response, but not an NF-κB response.

In this Example, the immunostimulatory bacteria are engineered to express a STING variant with increased type I IFN signaling, and/or reduced NF-κB signaling, compared to wild-type (WT) human STING (see, *e.g.*, SEQ ID NOs:305-309). The STING variants can be from a non-human vertebrate, such as a mammalian, bird, reptilian, amphibian, or fish species. Species from which the non-human STING proteins are derived include, but are not limited to, Tasmanian devil (*Sarcophilus harrisii*; SEQ ID NO:349), marmoset (*Callithrix jacchus*; SEQ ID NO:359), cattle (*Bos taurus*; SEQ ID NO:360), cat (*Felis catus*; SEQ ID NO:356), ostrich (*Struthio camelus australis*; SEQ ID NO:361), crested ibis (*Nipponia nippon*; SEQ ID NO:362), coelacanth (*Latimeria chalumnae;* SEQ ID NOs:363-364), boar (*Sus scrofa*; SEQ ID NO:365), bat (*Rousettus aegyptiacus*; SEQ ID NO:366), manatee (*Trichechus manatus latirostris*; SEQ ID NO:367), ghost shark (*Callorhinchus milii*; SEQ ID NO:368), and mouse (*Mus musculus*; SEQ ID NO:369). These vertebrate STING proteins readily activate immune signaling in human cells, indicating that the

molecular mechanism of STING signaling is shared in vertebrates (see, *e.g.*, de Oliveira Mann *et al.* (2019) *Cell Reports* 27:1165-1175). STING proteins from these species induce less NF-κB signal activation and/or more type I IFN signal activation, than human STING (see, *e.g.*, de Oliveira Mann *et al.* (2019), Fig. 1A). Wild-type or modified STING proteins from different non-human species can be expressed by the immunostimulatory bacteria herein, as can chimeras of human and non-human STING proteins.

The various non-human STING proteins are modified, such that the non-human STING has lower NF-κB activation, and, optionally, higher type I interferon activation, than human STING. These non-human STING proteins are modified to include a mutation or mutations so that they have increased type I IFN activity, or act constitutively, in the absence of cytosolic nucleic acid ligands (*e.g.*, CDNs). The mutations typically are amino acid mutations, such as gain-of-function mutations, that are associated with interferonopathies in humans. The corresponding mutations are introduced into the non-human species STING proteins, where corresponding amino acid residues are identified by alignment. For example, mutations include, but are not limited to, S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E, S102P/F279L, F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R197A, D205A, R310A, R293A, T294A, E296A, R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q, S272A, R293A/T294A/E296A, D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, S358A, E360A, S366A, R238A, R375A, and S324A/S326A, with reference to the sequence of human STING, as set forth in SEQ ID NOs:305-309. Corresponding mutations in STING from other species are listed in the tables below. The resulting variants of the non-human STING proteins include one or more of these mutations, and optionally, a CTT replacement, and optionally, a deletion in the TRAF6 binding site.

The STING variants include one or more replacements of the amino acid serine (S) or threonine (T) at a phosphorylation site, with aspartic acid (D), which is phosphomimetic, resulting in increased or constitutive activity. Other mutations include deletion or replacement of a phosphorylation site or sites, such as 324-326 SLS → ALA in STING, and other replacements to eliminate a phosphorylation site to

reduce NF-κB signaling in STING. Additionally, chimeras of human STING with STING from other species are provided, in which the C-terminal tail (CTT) of human STING is replaced with the CTT of STING from another species that has lower NF-κB signaling activity, and/or higher type I IFN signaling activity. The variant STING proteins can include a deletion in the TRAF6 binding site of the CTT, to reduce NF-κB signaling.

| **Human STING (SEQ ID NOs: 305-309)** | **Tasmanian devil STING (SEQ ID NO:349)** | **Marmoset STING (SEQ ID NO:359)** | **Cattle STING (SEQ ID NO:360)** | **Cat STING (SEQ ID NO:356)** | **Ostrich STING (SEQ ID NO:361)** | **Crested ibis STING (SEQ ID NO:362)** | **Coelacanth STING (SEQ ID NO:363)** |
|---|---|---|---|---|---|---|---|
| S102P | S102P | S102P | S102P | S102P | C107P | V106P | A102P |
| V147L | V147L | V145L | I147L | V146L | M152L | M151L | I147L |
| V147M | V147M | V145M | I147M | V146M | - | - | I147M |
| N154S | N154S | N152S | N154S | N153S | N159S | N158S | G154S |
| V155M | V155M | V153M | V155M | V154M | V160M | V159M | V155M |
| G166E | G166E | G164E | G166E | G165E | G171E | G170E | G166E |
| C206Y | C206Y | C204Y | C206Y | C205Y | C211Y | C210Y | C206Y |
| G207E | S207E | G205E | G207E | G206E | N212E | D211E | S207E |
| S102P/F279L | S102P/F279L | S102P/F277L | S102P/F279L | S102P/F278L | C107P/F283L | V106P/F283L | A102P/F279L |
| F279L | F279L | F277L | F279L | F278L | F283L | F283L | F279L |
| R281Q | R281Q | R279Q | R281Q | R280Q | R285Q | R285Q | K281Q |
| R284G | R284G | R282G | R284G | R283G | R288G | R288G | R284G |
| R284S | R284S | R282S | R284S | R283S | R288S | R288S | R284S |
| R284M | R284M | R282M | R284M | R283M | R288M | R288M | R284M |
| R284K | R284K | R282K | R284K | R283K | R288K | R288K | R284K |
| R284T | R284T | R282T | R284T | R283T | R288T | R288T | R284T |
| R197A | R197A | R195A | R197A | R196A | K202A | K201A | R197A |
| D205A | D205A | D203A | D205A | D204A | S210A | S209A | S205A |

| Human STING (SEQ ID NOs: 305-309) | Tasmanian devil STING (SEQ ID NO:349) | Marmoset STING (SEQ ID NO:359) | Cattle STING (SEQ ID NO:360) | Cat STING (SEQ ID NO:356) | Ostrich STING (SEQ ID NO:361) | Crested ibis STING (SEQ ID NO:362) | Coelacanth STING (SEQ ID NO:363) |
|---|---|---|---|---|---|---|---|
| R310A | R310A | R308A | R310A | R309A | R314A | R314A | R310A |
| R293A | R293A | R291A | R293A | R292A | R297A | R297A | R293A |
| T294A | T294A | T292A | T294A | I293A | T298A | T298A | T294A |
| E296A | E296A | E294A | E296A | E295A | E300A | E300A | K296A |
| R197A/D205A | R197A/D205A | R195A/D203A | R197A/D205A | R196A/D204A | K202A/S210A | K201A/S209A | R197A/S205A |
| S272A/Q273A | S272A/Q273A | S270A/Q271A | S272A/Q273A | S271A/Q272A | S276A/Q277A | S276A/Q277A | S272A/K273A |
| R310A/E316A | R310A/E316A | R308A/E314A | R310A/E316A | R309A/E315A | R314A/E320A | R314A/E320A | R310A/E318A |
| E316A | E316A | E314A | E316A | E315A | E320A | E320A | E318A |
| E316N | E316N | E314N | E316N | E315N | E320N | E320N | E318N |
| E316Q | E316Q | E314Q | E316Q | E315Q | E320Q | E320Q | E318Q |
| S272A | S272A | S270A | S272A | S271A | S276A | S276A | S272A |
| R375A | R377A | R373A | R374A | R373A | R371A | R379A | K376A |
| R293A/T294A/E296A | R293A/T294A/E296A | R291A/T292A/E294A | R293A/T294A/E296A | R292A/I293A/E295A | R297A/T298A/E300A | R297A/T298A/E300A | R293A/T294A/K296A |
| D231A | D231A | D229A | D231A | D230A | T236A | T235A | N231A |
| R232A | R232A | R230A | R232A | R231A | R237A | R236A | R232A |
| K236A | K236A | K234A | K236A | K235A | K241A | K240A | K236A |
| Q273A | Q273A | Q271A | Q273A | Q272A | Q277A | Q277A | K273A |
| S358A/E360A/S366A | S360A/E362A/S368A | S356A/E358A/S364A | S357A/E359A/S365A | S356A/E358A/S364A | S354A/D356A/S362A | S362A/E364A/S370A | S359A/E361A/S367A |

| Human STING (SEQ ID NOs: 305-309) | Tasmanian devil STING (SEQ ID NO:349) | Marmoset STING (SEQ ID NO:359) | Cattle STING (SEQ ID NO:360) | Cat STING (SEQ ID NO:356) | Ostrich STING (SEQ ID NO:361) | Crested ibis STING (SEQ ID NO:362) | Coelacanth STING (SEQ ID NO:363) |
|---|---|---|---|---|---|---|---|
| D231A/R232A/K236A/R238A | D231A/R232A/K236A/R238A | D229A/R230A/K234A/R236A | D231A/R232A/K236A/R238A | D230A/R231A/K235A/R237A | T236A/R237A/K241A/R243A | T235A/R236A/K240A/R242A | N231A/R232A/K236A/R238A |
| S358A | S360A | S356A | S357A | S356A | S354A | S362A | S359A |
| E360A | E362A | E358A | E359A | E358A | D356A | E364A | E361A |
| S366A | S368A | S364A | S365A | S364A | S362A | S370A | S367A |
| R238A | R238A | R236A | R238A | R237A | R243A | R242A | R238A |
| S324A/S326A | S326A/S328A | L322A/S324A | S324A/S326A | S323A/S325A | F328A/S330A | S328A/S330A | S327A |

| Human STING (SEQ ID NOs: 305-309) | Boar STING (SEQ ID NO:365) | Bat STING (SEQ ID NO:366) | Manatee STING (SEQ ID NO:367) | Ghost Shark STING (SEQ ID NO:368) | Mouse STING (SEQ ID NO:369) |
|---|---|---|---|---|---|
| S102P | S102P | S103P | S105P | S98P | S102P |
| V147L | I147L | V148L | I150L | I148L | V146L |
| V147M | I147M | V148M | I150M | I148M | V146M |
| N154S | N154S | N155S | N157S | N155S | N153S |
| V155M | V155M | V156M | V158M | V156S | V154M |
| G166E | G166E | G167E | G169E | G167E | G165E |
| C206Y | C206Y | C207Y | C209Y | C206Y | C205Y |
| G207E | G207E | G208E | G210E | K207E | G206E |
| S102P/F279L | S102P/F279L | S103P/F280L | S105P/F282L | S98P/F280L | S102P/F278L |
| F279L | F279L | F280L | F282L | F280L | F278L |

| Human STING (SEQ ID NOs: 305-309) | Boar STING (SEQ ID NO:365) | Bat STING (SEQ ID NO:366) | Manatee STING (SEQ ID NO:367) | Ghost Shark STING (SEQ ID NO:368) | Mouse STING (SEQ ID NO:369) |
|---|---|---|---|---|---|
| R281Q | R281Q | - | R284Q | K282Q | R280Q |
| R284G | R284G | R285G | R287G | R285G | R283G |
| R284S | R284S | R285S | R287S | R285S | R283S |
| R284M | R284M | R285M | R287M | R285M | R283M |
| R284K | R284K | R285K | R287K | R285K | R283K |
| R284T | R284T | R285T | R287T | R285T | R283T |
| R197A | R197A | R198A | R200A | K197A | R196A |
| D205A | D205A | D206A | D208A | S205A | D204A |
| R310A | R310A | R311A | R313A | R311A | R309A |
| R293A | R293A | R294A | R296A | R294A | R292A |
| T294A | T294A | T295A | T297A | T295A | T293A |
| E296A | E296A | - | E299A | K297A | E295A |
| R197A/D205A | R197A/D205A | R198A/D206A | R200A/D208A | K197A/S205A | R196A/D204A |
| S272A/Q273A | S272A/Q273A | S273A/Q274A | S275A/Q276A | T273A/N274A | S271A/Q272A |
| R310A/E316A | R310A/E316A | R311A/E317A | R313A/E319A | R311A/D317A | R309A/E315A |
| E316A | E316A | E317A | E319A | D317A | E315A |
| E316N | E316N | E317N | E319N | D317N | E315N |
| E316Q | E316Q | E317Q | E319Q | D317Q | E315Q |
| S272A | S272A | S273A | S275A | T273A | S271A |
| R375A | S374A | R376A | R378A | R374A | R374A |
| R293A/T294A/ | R293A/T294A/ | R294A/T295A/ | R296A/T297A/ | R294A/T295A/ | R292A/T293A/ |

| Human STING (SEQ ID NOs: 305-309) | Boar STING (SEQ ID NO:365) | Bat STING (SEQ ID NO:366) | Manatee STING (SEQ ID NO:367) | Ghost Shark STING (SEQ ID NO:368) | Mouse STING (SEQ ID NO:369) |
|---|---|---|---|---|---|
| E296A | E296A | | E299A | K297A | E295A |
| D231A | D231A | D232A | D234A | D231A | D230A |
| R232A | R232A | R233A | C235A | R232A | R231A |
| K236A | K236A | K237A | K239A | K236A | K235A |
| Q273A | Q273A | Q274A | Q276A | N274A | Q272A |
| S358A/E360A/S366A | S357A/E359A/S365A | H359A/E361A/S367A | S366A/E368A/S374A | S359A/E361A/S367A | S357A/E359A/S365A |
| D231A/R232A/K236A/R238A | D231A/R232A/K236A/R238A | D232A/R233A/K237A/R239A | D234A/C235A/K239A/R241A | D231A/R232A/K236A/R238A | D230A/R231A/K235A/R237A |
| S358A | S357A | H359A | S366A | S359A | S357A |
| E360A | E359A | E361A | E368A | E361A | E359A |
| S366A | S365A | S367A | S374A | S367A | S365A |
| R238A | R238A | R239A | R241A | R238A | R237A |
| S324A/S326A | S324A/S326A | S325A/S327A | S327A/S329A | G325A/S330A | S323A/S325A |

For example, modified STING variants include Tasmanian devil STING with the mutations C206Y (SEQ ID NO:350), or R284G (SEQ ID NO:351); a variant in which the CTT of human STING is replaced with the CTT of Tasmanian devil STING (SEQ ID NO:352); human STING with the mutation C206Y (SEQ ID NO:353), or R284G (SEQ ID NO:354), and where the CTT is replaced with the CTT of Tasmanian devil STING; wild-type human STING with a deletion in the TRAF6 binding domain (corresponding to residues 377-379 (DFS)) (SEQ ID NO:355); cat

STING with the mutations C205Y (SEQ ID NO:357), or R283G (SEQ ID NO:358); and other such modified STING variants.

To determine the corresponding amino acid residues for the STING mutations, the wild-type STING protein sequences from various non-human species each were aligned with the wild-type human STING protein sequence (of the allelic variants of SEQ ID NO:305 (R232 allele) or SEQ ID NO:306 (H232 allele)). The alignments were performed using the Kalign sequence alignment tool, available from ebi.ac.uk/Tools/msa/kalign/, or the EMBOSS needle sequence alignment tool, available from ebi.ac.uk/Tools/psa/emboss_needle/. Figures 2-14 depict exemplary sequence alignments for human STING compared to STING proteins from Tasmanian devil, marmoset, cattle, cat, ostrich, crested ibis, coelacanth, zebrafish, boar, bat, manatee, ghost shark, and mouse species, respectively.

**STING GOF Hybrid Variants Demonstrate Significantly Enhanced Type I Interferon to NF-κB Activity Ratios in Dendritic Cells**

In order to determine the optimal STING GOF mutant that would elicit the highest levels of the $CD8^+$ T-cell chemokine CXCL10 in mice, a panel of STING mutants were tested in murine primary bone marrow-derived dendritic cells (BMDCs). These included the Tasmanian devil STING with the constitutively active human GOF mutations C206Y (tazSTING C206Y; SEQ ID NO:350), or R284G (tazSTING R284G; SEQ ID NO:351); murine STING with the constitutive GOF mutants C205Y (muSTING C205Y; SEQ ID NO:399), or R283G (muSTING R283G; SEQ ID NO:400); cat STING with the constitutive GOF mutants C205Y (catSTING C205Y; SEQ ID NO:357), or R283G (catSTING R283G; SEQ ID NO:358); as well as variants in which the CTT of human STING (SEQ ID NO:370) was replaced with the CTT of Tasmanian devil STING (SEQ ID NO:371), and containing either wild-type human STING (huSTING tazCTT; see, *e.g.*, SEQ ID NO:352), or the human STING GOF mutations C206Y (huSTING C206Y tazCTT; see, *e.g.*, SEQ ID NO:353), or R284G (huSTING R284G tazCTT; see, *e.g.*, SEQ ID NO:354). Also included were human STING variants with the constitutive, GOF mutations C206Y (huSTING C206Y), or R284G (huSTING R284G).

To test these, murine bone marrow was isolated and flushed into 1.5 mL Eppendorf tubes, and spun at 1200 RPM for 5 minutes, to collect the bone marrow

cells. Cells were washed once in RPMI-1640 + 10% FBS, then seeded in 96-well TC-treated plates in RPMI-1640 + 10% FBS with 20 ng/ml GM-CSF. Every 2 days, 50% of the medium was replaced with fresh complete media. After six days, non-adherent cells were pipetted off the wells and re-seeded at 1e5 cells per well in RPMI-1640 + 10% FBS in a 96-well plate for transfection. Cells were transfected using Viromer® RED, according to the manufacturer's instructions. Briefly, 200 ng of plasmid DNA from a panel of STING GOF mutants, as well as untransfected control, were diluted in the provided buffer, and mixed with 0.08 µL of Viromer® RED and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 96-well plate (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator. Supernatants were harvested at 48 hours, and assayed for murine CXCL10 (IP-10) using a flow cytometry-based cytokine bead array (CBA), according to the manufacturer's protocol.

As shown in the table below, the construct that induced the highest expression of murine CXCL10 was human STING containing the GOF mutation R284G, and containing a replacement of the CTT of human STING with the CTT of Tasmanian devil STING (huSTING R284G tazCTT). The next highest expression of CXCL10 was induced by the human STING variant containing the GOF mutation C206Y (huSTING C206Y), and the Tasmanian devil STING construct containing the human STING GOF mutation R284G (tazSTING R284G). The human STING GOF mutants (huSTING C206Y and huSTING R284G) were more potent than the corresponding murine STING GOF mutants (muSTING C205Y and muSTING R283G, respectively), which were even less potent than the cat STING mutants containing the same GOF mutations (catSTING C205Y and catSTING R283G, respectively), in primary murine dendritic cells.

| **Construct** | **muCXCL10 (pg/mL)** |
|---|---|
| Untransfected | 11.48 ± 3.889 |
| huSTING C206Y | 861.0 ± 58.48 |
| huSTING R284G | 769.7 ± 95.16 |
| muSTING C205Y | 194 ± 27.15 |
| muSTING R283G | 230.1 ± 1.018 |
| huSTING C206Y tazCTT | 366.6 ± 42.61 |
| huSTING R284G tazCTT | 1326 ± 137.9 |
| tazSTING C206Y | 808.8 ± 95.78 |

| Construct | muCXCL10 (pg/mL) |
|---|---|
| tazSTING R284G | 831.3 ± 30.15 |
| catSTING C205Y | 480.7 ± 24.94 |
| catSTING R283G | 376.2 ± 6.682 |

These data demonstrate that STING proteins obtained from other species, such as Tasmanian devil, can be combined with constitutive GOF human STING mutations, to elicit potent T-cell recruiting chemokines.

**STING GOF Hybrid Variants Demonstrate Significantly Enhanced Type I Interferon to NF-κB Activity Ratios in Human Monocytes**

In order to demonstrate that the ratio of STING-induced type I interferon to NF-κB signaling can be altered using STING GOF hybrid variants from other species, a panel was tested in a human monocyte cell line. The panel included wild-type human STING (huSTING), and huSTING mutants with the constitutive human GOF mutations C206Y, or R284G; wild-type Tasmanian devil STING (tazSTING), and tazSTING mutants with the constitutive GOF mutations C206Y, or R284G; wild-type cat STING, and catSTING mutants with the constitutive GOF mutations C205, or R283G; murine STING mutants with the constitutive GOF mutations C205Y, or R283G; and the variants in which the CTT of human STING was replaced with the CTT of Tasmanian devil STING, and containing either wild-type human STING, or the human GOF STING mutations C206Y, or R284G. Also included were a wild-type human STING with a deletion in the TRAF6 binding domain (corresponding to residues 377-379, DFS, see, *e.g.*, SEQ ID NO:355), and wild-type zebrafish STING.

For this experiment, the THP1-Dual™ KO STING cells were utilized, which have been altered to lack endogenous STING, and to also express Lucia™ luciferase, a secreted luciferase, placed under the control of the endogenous IFN-β promoter. Constitutively active STING GOF mutants then were identified, and ranked by measurement of IFN-β promoter induced expression of luciferase activity. These cells also express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous NF-κB promoter, where the coding sequence of NF-κB has been replaced by the SEAP ORF using knock-in technology. NF-κB activity induced by STING GOF mutants can be assessed by monitoring SEAP production in the cell supernatants.

For this experiment, THP1-Dual™ KO STING cells were transfected using Viromer® RED, according to the manufacturer's instructions. Briefly, 200 ng of plasmid DNA from a panel of STING GOF mutants, as well as untransfected control, were diluted in the provided buffer, and mixed with 0.08 µL of Viromer® RED and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 96-well plate (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator. In addition, the wild-type STING variants were treated with or without the STING agonist 3'5' RpRp c-di-AMP (CDN, InvivoGen), an analog of the clinical compound ADU-S100, which was added to the cells after 24 hours of incubation at 10 µg/mL. Supernatants were harvested at 48 hours, and assayed for NF-κB-SEAP and IFN-Lucia reporter signals, according to the manufacturer's protocol. Briefly, 10 µL of the cell culture supernatants was added to 50 µL QUANTI-Blue™ reagent (InvivoGen) (which is used for measuring SEAP). NF-κB activation was determined by measuring NF-κB-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), at an absorbance (Abs) wavelength of 650 nm. For measuring type I interferon activity from IFN-Lucia, 10 µL of the cell culture supernatants was added to 50 µL QUANTI-Luc™, containing the coelenterazine substrate for the luciferase reaction, which produces a light signal that is quantified using a SpectraMax® M3 luminometer, and expressed as relative light units (RLUs).

As shown in the table below, the highest type I IFN responses were observed from the variant in which the CTT of human STING was replaced with the CTT of Tasmanian devil STING, and that contained the human STING GOF mutation R284G (huSTING R284G tazCTT), as well as from the wild-type zebrafish STING with the CDN STING agonist (zfSTING WT + CDN). However, unlike the wild-type zebrafish STING, which had very high NF-κB signaling, the huSTING R284G tazCTT variant had high type I IFN signaling with much lower NF-κB signaling activity. The best ratio of higher type I IFN to lower NF-κB signaling was found with the Tasmanian devil STING variant containing the human STING GOF mutation R284G (tazSTING R284G).

| STING Variant | ISRE-Lucia™ (RLUs) | ± SD | NF-κB-SEAP (Abs) | ± SD |
|---|---|---|---|---|
| Untransfected | 47.96 | 33.91 | 0.065 | 0.007 |
| huSTING WT + CDN | 170.8 | 38.15 | 0.100 | 0.014 |
| huSTING WT delTRAF6 + CDN | 164.8 | 8.48 | 0.120 | 0.014 |
| huSTING WT tazCTT + CDN | 31.47 | 10.59 | 0.060 | 0.000 |
| tazSTING WT + CDN | 143.9 | 4.24 | 0.090 | 0.014 |
| zfSTING WT + CDN | 310.2 | 23.31 | 0.690 | 0.028 |
| CMV catSTING WT WPRE + CDN | 202.3 | 6.36 | 0.125 | 0.007 |
| huSTING C206Y | 175.3 | 36.03 | 0.105 | 0.007 |
| huSTING R284G | 143.9 | 29.67 | 0.100 | 0.000 |
| huSTING C206Y tazCTT | 137.9 | 21.19 | 0.095 | 0.007 |
| huSTING R284G tazCTT | 301.2 | 61.46 | 0.250 | 0.127 |
| tazSTING C206Y | 199.3 | 2.12 | 0.120 | 0.000 |
| tazSTING R284G | 217.3 | 19.08 | 0.105 | 0.007 |
| muSTING C205Y | 43.46 | 2.12 | 0.070 | 0.000 |
| muSTING R283G | 32.97 | 4.24 | 0.070 | 0.000 |
| catSTING C205Y | 202.3 | 23.31 | 0.135 | 0.007 |
| catSTING R283G | 157.4 | 10.60 | 0.120 | 0.000 |

SD = Standard deviation

These data further demonstrate using non-human STING proteins, such as the STING protein from Tasmanian devil, and combining them with human constitutive gain-of-function STING mutations, in order to enhance the beneficial type I interferon activity, while minimizing the immunosuppressive NF-κB activity, in human monocytes.

## Example 18

### *S. typhimurium* Lipoprotein Knockout by Deletion of the *lppA* and *lppB* Genes

The live attenuated *S. typhimurium* YS1646 strain, containing the Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* gene deletions, was engineered to delete the *lppA* (SEQ ID NO:387) and *lppB* (SEQ ID NO:388) genes, in order to remove membrane surface lipoproteins. This reduces pro-inflammatory TLR2 activation, which reduces immunosuppressive cytokines and improves anti-tumor adaptive immunity. As shown below, this also enhances plasmid delivery and encoded protein expression in the tumor.

**Strain Engineering and Characterization**

**Deletion of *lppA* Gene**

The *lppA* gene was deleted from the chromosome of the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain using modifications of the method of Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)), as described in detail above. Synthetic *lppA* gene homology arm sequences that contained 231 and 200 bases of the left hand and right hand sequence, respectively, flanking the *lppA* gene, were synthesized and cloned into a plasmid called pSL0148 (SEQ ID NO:231). The sequence for the *lppA* gene is shown in SEQ ID NO:387; appropriate primers for PCR amplification were designed using the gene sequence. A kanamycin gene cassette flanked by cre/loxP sites then was cloned into plasmid pSL0148, and the *lppA* gene knockout cassette was then PCR amplified with primers lppA-1 and lppA-2, gel purified, and then introduced into the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. Electroporated cells were recovered in SOC + DAP medium, and plated onto LB agar plates supplemented with kanamycin (20 μg/mL) and diaminopimelic acid (DAP, 50 μg/mL). Colonies were selected and screened for insertion of the knockout fragment by PCR using primers lppA-3 and lppA-4. pKD46 then was cured by culturing the selected kanamycin resistant strain at 42 °C and screening for loss of ampicillin resistance. The kanamycin resistance marker then was cured by electroporation of a temperature-sensitive plasmid expressing the Cre recombinase (pJW168), and $Amp^R$ colonies were selected at 30 °C; pJW168 was subsequently eliminated by growing cultures at 42 °C. Selected *lppA* knockout clones were then tested for loss of the kanamycin marker by PCR, using primers flanking the sites of disruption (lppA-3 and lppA-4), and evaluation of the electrophoretic mobility was performed on agarose gels. This mutant derivative of strain YS1646 was designated YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppA*.

**Deletion of *lppB* Gene**

The *lppB* gene then was deleted in the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppA* strain using modifications of the methods described above. Synthetic *lppB* gene homology arm sequences that

contained 224 and 231 bases of the left hand and right hand sequence, respectively, flanking the *lppB* gene, were synthesized and cloned into a plasmid called pSL0148 (SEQ ID NO:231). The sequence for the *lppB* gene is shown in SEQ ID NO:388; appropriate primers for PCR amplification were designed using the gene sequence. A kanamycin gene cassette flanked by cre/loxP sites then was cloned into plasmid pSL0148, and the *lppB* gene knockout cassette was PCR amplified with primers lppB-5 and lppB-6, gel purified, and introduced into strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppA*, carrying the temperature-sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *lppA* and *lppB* gene knockout sequences were amplified by PCR, using primers designated lppA-3 and lppA-4, and lppB-7 and lppB-8, respectively, and verified by DNA sequencing. This mutant derivative of strain YS1646 was designated YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppAB*, or nicknamed YS1646Δ*lppAB*.

**_In vitro_ Characterization of Engineered _S. typhimurium_ Lipoprotein Knockout Strain**

The YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppAB* strain, harboring the deletions of *lppA* and *lppB*, was evaluated for growth by overnight cultures in LB. Growth was measured using a SpectraMax® M3 Spectrophotometer (Molecular Devices) at 37 °C, reading the $OD_{600}$ every 15 minutes. The results demonstrated that strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppAB* was able to replicate in LB at a growth rate comparable to the parental YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain. These data demonstrate that the elimination of lipoprotein does not decrease the fitness of *S. typhimurium in vitro*.

**Lipoprotein Deletion Enhances Plasmid Delivery to the Tumor After Systemic Administration**

As TLR2 is expressed on vascular endothelial cells, and activation of TLR2 enhances vascular permeability, the effect of the Δ*lppAB* modification, and the subsequent reduction in TLR2 agonism, on tumor colonization was assessed. The effect on payload expression also was assessed. As shown below, while colonization

of tumors was somewhat reduced, plasmid delivery and encoded gene expression was significantly increased, following systemic administration.

To demonstrate the impact of the lipoprotein knockout strains in a murine model of triple-negative breast cancer, 6-8 week-old female BALB/c mice (4 mice per group) were inoculated orthotopically in the 4$^{th}$ mammary fat pad with EMT6 cells ($5x10^5$ cells in 100 µL PBS). Mice bearing 10-day established flank tumors were IV injected with a single dose of 1 $x10^7$ CFUs of the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppAB* strain, or the parental YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain, each containing a plasmid encoding a luciferase protein (NanoLuciferase® luciferase, or NanoLuc®; Promega) that is secreted (secNanoLuc®), under the control of the CMV promoter. At day 7 post IV dosing, mice were euthanized, and tumors were homogenized and plated on LB plates, to enumerate the number of colony forming units (CFUs) per gram of tumor tissue. The parental YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain colonized tumors at a mean of 3.3 $x10^6$ CFUs per gram of tumor tissue, while the lipoprotein-deleted YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppAB* strain colonized the tumors with a 2-fold decreased mean of 1.18 x $10^6$ CFUs/g of tumor tissue.

In order to measure plasmid delivery to the tumor, and subsequent heterologous gene expression and protein secretion, the activity of the secNanoLuc® was measured. For this, homogenized tumors were assessed for luciferase activity using the NanoGlo® detection reagent (Promega), and read on a SpectraMax® M3 Spectrophotometer/Luminometer (Molecular Devices). While the parental YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain induced average luminescence relative light units (RLUs) of 4482.6, the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*lppAB* strain induced a nearly 10-fold increased average of 33,926.6 RLUs. These data demonstrate the ability of the lipoprotein-deleted strain to improve tumor colonization, and to enhance payload expression.

These data reveal that, while deletion of lipoprotein somewhat reduces tumor colonization after IV dosing, it significantly enhances plasmid delivery and payload expression in the tumor. These data demonstrate that, contrary to the expectation from the art that deletion of these genes will decrease colonization, lipoprotein deletion

enhances plasmid delivery and protein expression in the tumor microenvironment, and in tumors.

## Example 19

## *Salmonella* Full *purI* Clean Deletion Gene Knockout Strain Engineering and Characterization

The *purI* gene in strain YS1646 (VNP20009) was not deleted; it was disrupted by a transposon (Tn10) insertion that resulted in a 16.6 kbp (kilobase pair) genomic inversion event, whereby two insertion sequence (IS) elements were subsequently incorporated into the genome, one within the *purI* (*purM*) gene, and the other 16.6 kbp upstream, in the intergenic region directly flanking the 3'-end of the *acrD* gene. The region between the two IS elements is inverted and contains 18 genes, including *yffB*, DC51_2568, *upp*, uraA, *yfgE*, *yfgD*, DC51_2573, *perM*, *purC*, and others. The insertion sequence element in the intergenic region encodes a fully functional transposase (see, *e.g.*, Broadway *et al.* (2014) *J. Biotechnology* 192:177-178). The presence of such a transposase in a therapeutic strain presents a possible genetic stability concern. These elements were removed from the strain, for development of the strain as a human therapy. The presence of the complete genetic sequence of the *purI* gene, disrupted by means of a chromosomal reengagement, leaves open the possibility of reversion to a wild-type gene.

The *msbB* gene in strain YS1646 also was not fully deleted, but was disrupted by a genetically engineered 511 bp deletion (of the 972 bp gene) that resulted in an extension of the *pykA* gene (encoding pyruvate kinase), replacing the last 5 amino acid codons with 13 new codons (see, *e.g.*, Broadway *et al.* (2014) *J. Biotechnology* 192:177-178).

Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* was modified to delete the remaining portions of the *purI* gene and the two transposon-associated insertion sequence elements present in strain YS1646. It is shown below, that with a full knockout of *purI*, the resulting bacterial cells have higher viability than the cells in which the gene is inactivated by disruption, as in the parental YS1646 strain.

### A. Deletion of *purI* Gene Fragments and Transposon-Associated Insertion Sequence Elements

A first region, located between the *yffB* and *purN* genes, and containing: 1) a 1,209 bp transposon insertion sequence element, annotated as DC51_2586 in the

sequence obtained from Broadway *et. al* (2014) (see, GenBank Accession numbers CP007804 and CP008745); and 2) 740 bp of the remaining 891 bp *purI* gene fragment (referred to herein as the large *purI* gene fragment), was targeted for deletion, using modifications of the method of Datsenko and Wanner (see, *Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)). A small 151 bp portion of the 891 bp (large) *purI* gene fragment was left intact to avoid affecting the adjacent, downstream gene, *purN*. A plasmid, pSL0165, containing 284 and 262 bps of homology to the left hand and right hand regions, respectively, of the DC51_2586 insertion sequence element and the *purI* gene fragment, was transformed into DH5-alpha competent cells (Thermo Fisher Scientific). A kanamycin gene cassette flanked by loxP sites was cloned into this plasmid, and the resulting vector was designated pSL0174. The DC51_2586 insertion sequence element and the large *purI* gene fragment knockout cassette then was PCR amplified using primers purm-1 and purm-2 (SEQ ID NOs: 419 and 420, respectively; see Table 2 below), gel purified, and introduced into strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination, as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The DC51_2586 insertion sequence element and large *purI* gene fragment knockout sequences were confirmed by PCR using primers purm-3 and purm-4 (SEQ ID NOs: 421 and 422, respectively; see Table 2), and verified by DNA sequencing. The resulting mutant derivative of parental strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* was designated YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{\text{(large clean)}}$.

A second region, located between the *acrD* and DC51_2568 genes, and containing: 1) a 1,209 bp transposon insertion sequence element, annotated as DC51_2566 in the sequence obtained from Broadway *et. al* (2014) (see, GenBank Accession numbers CP007804 and CP008745); and 2) the remaining 231 bp *purI* gene fragment (referred to herein as the small *purI* gene fragment), was targeted for deletion, using modifications of the method of Datsenko and Wanner (see, *Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)). A plasmid, pSL0210, containing 241 and 265 bps of homology to the left hand and right hand regions, respectively, of the

DC51_2566 insertion sequence element and the small *purI* gene fragment, was transformed into DH5-alpha competent cells (Thermo Fisher Scientific). A kanamycin gene cassette flanked by loxP sites was cloned into this plasmid, and the resulting vector was designated pSL0212. The DC51_2566 insertion sequence element and the small *purI* gene fragment knockout cassette then was PCR amplified using primers acrd-1 and purm-5 (SEQ ID NOs: 425 and 423, respectively; see, Table 2), gel purified, and introduced into strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(\text{large clean})}$ carrying the temperature sensitive lambda red recombination plasmid pKD46, by electroporation. The kanamycin resistance gene then was cured by Cre-mediated recombination, as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The DC51_2566 insertion sequence element and small *purI* gene fragment knockout sequences were confirmed by PCR using primers purm-6 and acrd-3 (SEQ ID NOs: 424 and 426, respectively; see Table 2), and verified by DNA sequencing. The resulting mutant derivative of parental strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(\text{large clean})}$ was designated as YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(\text{full clean})}$, or alternatively, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*.

**Table 2. Primer Sequence Information**

| Primer | Primer Sequence | Primer Description | SEQ ID NO. |
|---|---|---|---|
| purm-1 | ccagatgtggcttgtttatacttcgc | Fwd primer for amplifying the DC51_2586 insertion sequence element and the large *purI* (740 bp) fragment gene KO cassette | 419 |
| purm-2 | ccttattgctgaatactgccctgag | Rev primer for amplifying the DC51_2586 insertion sequence element and the large *purI* (740 bp) gene fragment KO cassette | 420 |
| purm-3 | cgtgtcgcaattcttaatgccatag | Fwd primer for checking DC51_2586 insertion sequence element and the large *purI* (740 bp) gene fragment KO | 421 |
| purm-4 | caaacatcggactcagaatacgc | Rev primer for checking DC51_2586 insertion sequence element and the large *purI* (740 bp) gene fragment KO | 422 |
| purm-5 | caggcgataaaggctaacaaccg | Rev primer for amplifying the DC51_2566 insertion sequence element and the small *purI* (231 bp) gene fragment KO cassette | 423 |

| Primer | Primer Sequence | Primer Description | SEQ ID NO. |
|---|---|---|---|
| purm-6 | ttgacctggatcaaccaaaagcg | Rev primer for checking the DC51_2566 insertion sequence element and the small *purI* (231 bp) gene fragment KO | 424 |
| acrd-1 | cctgcgaccgatactgatgac | Fwd primer for amplifying the DC51_2566 insertion sequence element and the small *purI* (231 bp) gene fragment KO cassette | 425 |
| acrd-3 | gctactcgctacctggatgc | Fwd primer for checking the DC51_2566 insertion sequence element and the small *purI* (231 bp) gene KO | 426 |

Fwd= forward; Rev = reverse; KO = knockout

**Plasmid Information**

| Gene Sequence | Synthetic DNA Vector | Vector Name after Kanamycin Cloning | Homology Arm Length | |
|---|---|---|---|---|
| | | | Left Hand | Right Hand |
| *purI* large (740 bp) fragment | pSL0165 | pSL0174 | 284 bp | 262 bp |
| *purI* small (231 bp) fragment | pSL0210 | pSL0212 | 241 bp | 265 bp |

**B. Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* Displays Enhanced Injection Stock Cell Viability**

To evaluate the effects of the deletions of the remaining *purI* gene sequence fragments and the transposon-associated insertion sequence elements on the *in vitro* fitness of the bacteria, the cell viability of strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, with and without the full *purI* gene deletion (F-Δ*purI*), and carrying the same plasmids, was compared.

The cell viability of strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*, containing either plasmid ADN-657 (pATI1.76 CMV muIL-15Rα-IL-15sc_T2A_huSTING N154S/R284G tazCTT HPRE bGHpA), or plasmid ADN-750 (pATI2.1 CMV VCIP huIL-15Rα-IL-15sc_T2A_huSTING N154S/R284G tazCTT HPRE bGHpA), and the cell viability of strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, expressing the same plasmids (ADN-657 and ADN-750), was evaluated by directly comparing viable CFUs after culture processing for frozen injection stocks. Plasmid ADN-657 encodes murine IL-15Rα-IL-15sc and a modified human STING chimera, with a replacement of the CTT of human STING with the CTT of Tasmanian devil STING, and the GOF mutations

N154S/R284G (huSTING N154S/R284G tazCTT). The two payloads are expressed under the control of a CMV promoter, from a bicistronic construct comprising a T2A peptide. The construct also includes a Hepatitis B virus Posttranscriptional Regulatory Element (HPRE), and a bovine growth hormone polyadenylation signal sequence (bGHpA). Plasmid ADN-750 encodes human IL-15Rα-IL-15sc and the same modified human STING chimera, where expression of the two payloads is under control of a CMV promoter, and the single promoter system is achieved using an endogenous human IRES (Internal Ribosome Entry Site), known as Vascular Endothelial Growth Factor and Type 1 Collagen Inducible Protein (VCIP; SEQ ID NO:434), placed upstream of the nucleic acid encoding both payloads, as well as a T2A peptide sequence, placed between the two ORFs.

For this experiment, 100 µl of overnight stationary phase cultures, of equivalent $OD_{600nm}$ optical density (OD) values, were used to inoculate 25 ml of 4XYT media in 250 ml baffled shaker flasks with vented caps, and the cultures were incubated at 37 °C with shaking (at 225 RPM) for approximately 6 hours. Cultures were harvested at stationary phase at equivalent $OD_{600nm}$ values, washed twice, adjusted to $OD_{600nm} = 2$, aliquoted, and frozen at -80 °C. The following day, two aliquots of each strain were thawed, the $OD_{600nm}$ values were measured, and the strains were plated on agar plates to determine the titer and viability. % viability was determined from the ratio of $OD_{600nm}$ to CFUs/ml.

Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*-(ADN-657) injection stock was determined to be 77% viable, while strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-(ADN-657) injection stock was determined to be 62% viable. Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*-(ADN-750) injection stock was determined to be 72% viable, while strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-(ADN-750) injection stock was determined to be 63% viable.

These data demonstrate a similar or enhanced fitness profile for strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* compared to the parental strain, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*. Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* displayed improved viability following frozen injection stock preparation, indicating that the genomic deletions not

only diminish a potential genetic instability issue, but also provide a metabolic benefit that is manifested as increased cell viability.

**C. Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* Displays Similar Growth Characteristics in Broth Media Compared to the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* Parental Strain**

To evaluate the effects of the deletions of the remaining *purI* gene sequence fragments and the transposon-associated insertion sequence elements on the *in vitro* fitness of the bacterial strains, broth medium growth was compared between strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*, expressing either plasmid ADN-657 (pATI1.76 CMV muIL-15Rα-IL-15sc_T2A_huSTING N154S/R284G tazCTT HPRE bGHpA) or plasmid ADN-750 (pATI2.1 CMV VCIP huIL-15Rα-IL-15sc_T2A_huSTING N154S/R284G tazCTT HPRE bGHpA), and strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, expressing the same plasmids (ADN-657 and ADN-750). Frozen injection stocks were thawed at room temperature, and normalized to $1\times10^7$ CFU/mL by diluting in PBS. 10 µL of the normalized samples were used to inoculate 300 µL of LB media ($1\times10^5$ CFU/well) in a clear, flat-bottomed 96-well plate in technical quadruplicate. The plate was incubated with shaking at 37 °C, and the $OD_{600nm}$ values were monitored in 15 minute intervals over the course of 16 hours. $OD_{600nm}$ values were plotted to construct growth curves, and the slope of the log phase of growth was calculated and used to determine the doubling time for each strain.

Each of strains YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*-(ADN-657), YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-(ADN-657), YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*-(ADN-750) and YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-(ADN-750) generated comparable growth profiles, and reached similar cell densities at stationary phase. Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*-(ADN-657) had a doubling time of 77 minutes, strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-(ADN-657) had a doubling time of 62 minutes, strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*-(ADN-750) had a doubling time of 72 minutes, and strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-(ADN-750) had a doubling time of 63 minutes. These data demonstrate a similar fitness profile for strain

YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* compared to the parental strain, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*. Strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* displayed slightly increased doubling time in broth medium, but generated similar growth curve profiles and stationary phase cell densities, compared to the parental strain.

**Example 20**

**Genetically Modified Strains Reduce Inflammation in Whole Human Blood and in Primary Human Macrophages**

To evaluate the inflammatory profiles resulting from the administration of different variants of the immunostimulatory bacterial strains, strains ATCC # 14028 (wild-type (WT) *S. typhimurium*), YS1646, YS1646Δ*asd*, YS1646Δ*asd*/ΔFLG, YS1646Δ*asd*/ΔFLG/Δ*pagP*, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI*, and *E. coli* strain NEB 5-alpha, each carrying plasmids (CMV NanoLuc®) that encode the luciferase NanoLuciferase® (Promega), were incubated with human blood for 2 hours at 37 °C. The incubations were in performed in a 96-well format, with 200 µl of blood and $5x10^3$ CFUs of bacteria. At 2 hours post-infection, the blood was centrifuged at 300 relative centrifugal force (rcf) for 5 minutes, and serum was isolated for cytokine profile analysis by human antiviral cytokine bead array analysis (BioLegend), in accordance with the manufacturer's instructions.

Analysis of the levels of cytokines released from the human blood, following incubation with the various strains, showed that incubation with strain YS1646 resulted in reduced the levels of the pro-inflammatory cytokines IL-6 and TNF-α, compared to incubation with WT strain ATCC 14028. Additional genomic modifications further reduced the levels of pro-inflammatory cytokines released from the human blood.

| Strain | Cytokine Levels | | | |
|---|---|---|---|---|
| | IL-6 | | TNF-α | |
| | Mean (pg/ml) | STDEV | Mean (pg/ml) | STDEV |
| Uninfected control | 18.8 | ± 1.1 | 69.2 | ± 2.9 |
| Wild-type *S. typhimurium* strain ATCC 14028 (CMV NanoLuc®) | 155.1 | ± 32.7 | 464.3 | ± 77.9 |
| YS1646 (CMV NanoLuc®) | 48.1 | ± 20.4 | 105.0 | ± 24.4 |
| YS1646Δ*asd* (CMV NanoLuc®) | 19.3 | ± 1.2 | 68.4 | ± 6.1 |
| YS1646Δ*asd*/ΔFLG (CMV NanoLuc®) | 23.7 | ± 4.4 | 78.3 | ± 6.0 |

| | | | | |
|---|---|---|---|---|
| YS1646Δ*asd*/ΔFLG/Δ*pagP* (CMV NanoLuc®) | 17.1 | ± 5.7 | 57.8 | ± 19.9 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* (CMV NanoLuc®) | 21.1 | ± 7.6 | 74.2 | ± 33.4 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* (CMV NanoLuc®) | 21.0 | ± 1.3 | 75.1 | ± 4.9 |
| *E. coli* NEB 5-alpha (CMV NanoLuc®) | 152.9 | ± 24.1 | 519.0 | ± 236.5 |

STDEV = standard deviation

To determine the how the genomic modifications in bacteria, such as *S. typhimurium*, influence inflammation during infection of primary human myeloid cells, bactofection of human M2 macrophages was performed. Primary human monocytes were differentiated in ImmunoCult™-SF Macrophage Medium (StemCell Technologies), containing 100 ng/ml human macrophage colony-stimulating factor (M-CSF), for 5 days. On day 6, cells were supplemented with additional medium containing 150 ng/ml M-CSF and 60 ng/ml huIL-4 for 48 hours, to generate M2 macrophages. The cells then were infected with stationary phase bacterial strains (strains described above), which were grown overnight at 37 °C in 4XYT medium, at an MOI of 10. The cells were inoculated with bacteria, and centrifuged for 5 minutes at 500 rcf, followed by an incubation at 37 °C for 1 hour. The cells then were washed twice with DPBS, and then incubated in fresh ImmunoCult™-SF Macrophage Medium with 100 µg/ml gentamicin. Supernatants were harvested at 0, 2, 6, 24, and 48 hours post-infection. The cytokine measurements were performed by MESO scale analysis, according to the manufacturer's instructions.

The results, which are summarized in the table below, show that, at 24 hours post-infection, macrophages infected with strains YS1646, YS1646Δ*asd*, and YS1646Δ*asd*/ΔFLG released the highest levels of IL-6, with WT bacteria (strain ATCC 14028) inducing less IL-6. Successive genomic modifications resulting in decreased levels of IL-6 secretion, with strains YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*csgD* and YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* inducing the lowest levels of IL-6. Similar trends were observed with TNF-α.

| | **Cytokine Levels, 24 hours** | | | |
|---|---|---|---|---|
| | **IL-6** | | **TNF-α** | |
| **Strain** | **Mean (pg/ml)** | **Range (pg/ml)** | **Mean (pg/ml)** | **Range (pg/ml)** |
| Uninfected control | 1.1 | - | 1.23 | - |
| Wild-type *S. typhimurium strain* ATCC 14028 (CMV NanoLuc®) | 10450.7 | 285.0 | 2895.508 | 230.262 |
| YS1646 (CMV NanoLuc®) | 13653.2 | 332.3 | 5282.976 | 71.708 |

| | | | | |
|---|---|---|---|---|
| YS1646Δ*asd* (CMV NanoLuc®) | 14516.3 | 693.3 | 5100.7465 | 793.867 |
| YS1646Δ*asd*/ΔFLG (CMV NanoLuc®) | 13767.5 | 1540.7 | 3572.248 | 150.534 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP* (CMV NanoLuc®) | 6985.6 | 650.2 | 758.4872 | 136.6448 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*csgD* (CMV NanoLuc®) | 6746.1 | 622.0 | 1431.149 | 367.154 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* (CMV NanoLuc®) | 6584.1 | 1389.7 | 502.1081 | 44.983 |

## Example 21

## *csgD*-Deleted Strains Induce Vascular Leakage in Human Vascular Endothelial Cells

The ability to promote vascular leakage is an advantageous feature for facilitating bacterial entry into the tumor vasculature, and for promoting greater tumor colonization. It is shown in this example that Δ*csgD* strains possess this feature.

Parental strain YS1646 was compared to the derivative strains YS1646Δ*asd*/ΔFLG/Δ*pagP* and YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, for the ability to activate innate immune sensing in human umbilical vein endothelial cells (HUVECs), and for the ability to stimulate a subsequent increase in endothelial monolayer permeability. This was assessed using an *In Vitro* Vascular Permeability Assay kit (Millipore, Catalog No. ECM642). Strains of YS1646Δ*asd*/ΔFLG/Δ*pagP* and YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, each containing a plasmid encoding secreted NanoLuciferase® (EF-1α secNanoLuc®), were used. Human umbilical vein endothelial cells (HUVECs) were seeded onto semi-permeable collagen-coated membrane inserts, in a 96-well plate chamber, according to the manufacturer's instructions, at a concentration of $5x10^5$ cells/well. Confluency was monitored daily, until endothelial monolayer formation was confirmed, and determined to efficiently occlude the membrane pores (96 hours post-seeding).

The bacterial strains were grown in 3 mL of 4XYT media (TEKNOVA, Catalog No. 2Y1085), with shaking at 37 °C in vented cap 50 mL conical vials, overnight to stationary phase, and were prepared the following day by pelleting and re-suspending in PBS. Bacterial samples were normalized by $OD_{600nm}$ to a concentration of $2.5\times10^8$ CFU/mL, and were added to the HUVEC insert wells in 100 µL volume, to achieve an MOI of 50. The plate was spun at 500 rcf for 5 minutes to synchronize engagement of CFUs with HUVECs, and then the plate was incubated

for 1 hour at 37 °C. Gentamicin was then added to the insert wells to a final concentration of 200 μg/mL. At 24 hours post-bactofection, medium in the inserts was collected (and saved for cytokine analysis), and 75 μL of Fluorescein isothiocyanate–Dextran (FITC-Dextran; at a 1:40 dilution) was added, and the mixture was incubated in the dark, at room temperature, for 20 minutes. Medium from the receiver tray was collected and analyzed on a spectrophotometer at an excitation wavelength of 485 nm and an absorbance wavelength of 535 nm, and 1:40 FITC-Dextran solution was run directly as a positive control.

At 24 hours post-bactofection, and following incubation with FITC-Dextran solution, wells that were not treated with engineered, attenuated *S. typhimurium* strains (*i.e.*, medium alone) generated very low fluorescence values (35), indicating confluency and little disruption of permeability. The parental YS1646-treated wells permitted the greatest amount of FITC-Dextran to pass through the membrane (fluorescence = 155), indicating the strongest innate immune stimulation and increase in vascular permeability, while the engineered, attenuated *S. typhimurium* strains YS1646Δ*asd*/ΔFLG/Δ*pagP* and YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* permitted less FITC-Dextran to pass through (fluorescence = 84 and 91, respectively), yet still maintained the ability to promote vascular permeability.

To test whether this effect was dependent on cytokines released from the HUVECs, and in particular, IL-6, a human antiviral cytokine bead array analysis (BioLegend) was performed on HUVEC supernatants at 6 hours post-infection, in accordance with the manufacturer's instructions. HUVECs infected with the parental YS1646 strain exhibited very high IL-6 levels (1769.2 pg/mL), compared to the uninfected control (27.19 pg/mL). The YS1646Δ*asd*/ΔFLG/Δ*pagP* strain induced lower levels of IL-6 (543.3 pg/mL), and the lowest amount of IL-6 was observed with the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain (272.8 pg/mL). Therefore, despite producing lower levels of pro-inflammatory cytokines that induce vascular leakage, the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain demonstrated higher levels of leakage than the YS1646Δ*asd*/ΔFLG/Δ*pagP* strain. Thus, the ability to promote vascular leakage is an important feature for facilitating bacterial entry into the tumor vasculature, and for promoting greater tumor colonization. Strains described and provided herein possess this feature.

## Example 22

## STING Gain-Of-Function Variants Demonstrate Increased CXCL10 (IP-10) to IL-6 Ratios and Increased IFN-β to IL-6 Ratios in Primary Human M2 Macrophages

To determine the effects of various STING gain-of-function (GOF) mutants (including chimeric STING proteins with GOF mutations), on the downstream cytokine signaling in human M2 macrophages, the GOF mutants were cloned into the pATI-1.75 (also referred to as pATI1.75) vector, described in Example 8 above, which then was transfected into human M2 macrophages for expression, and the cell supernatants were assayed for secreted (expressed) cytokines.

Frozen human monocytes, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 10% FBS), and washed by centrifugation for 10 minutes at 600 x g, at room temperature. The monocytes were resuspended in ImmunoCult™-SF Macrophage Medium (StemCell Technologies) containing 100 ng/mL of human M-CSF + 20 ng/mL of human IL-4 + 20 ng/mL of human IL-10. Monocytes (8e5 to 1e6 cells per well) were then seeded in a 24-well plate, with a final volume of 750 µL. Three days later, 750 µL of ImmunoCult™-SF Macrophage Medium (StemCell Technologies), containing 100 ng/mL human M-CSF + 20 ng/mL human IL-4 + 20 ng/mL human IL-10, was added to each well, and the plate was incubated for four more days. On day seven, the cells were transfected using Viromer® RED mRNA and plasmid transfection reagent (Lipocalyx GmbH), according to the manufacturer's instructions. 500 ng of plasmid DNA from a panel of STING GOF mutants, as well as untransfected control, were diluted in the provided buffer, and mixed with the Viromer® RED transfection reagent, and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 24-well plate (in triplicate), and the plate was incubated at 37 °C in a 5% $CO_2$ incubator. Supernatants were harvested after 48 hours of incubation, and were assayed for human CXCL10 (IP-10) and IL-6 using a flow cytometry-based human anti-viral cytokine bead array (BioLegend), according to the manufacturer's protocol. The

average of three measurements was calculated, and the ratio of IP-10 to IL-6 was calculated by dividing the IP-10 concentration by the IL-6 concentration.

The results, which are summarized in the table below, show that the STING variant that resulted in the highest ratio of IP-10 to IL-6 was the huSTING N154S/R284G tazCTT variant (*i.e.*, a chimeric STING protein, containing a modified human STING with a replacement of the CTT with the CTT of Tasmanian devil STING, and the GOF mutations N154S/R284G). The STING variant, containing a replacement of the CTT of human STING with the CTT of Tasmanian devil STING (huSTING tazCTT), displayed higher ratios of IP-10 to IL-6 than the corresponding fully human STING variants with the same mutations, thus, demonstrating an improved anti-tumor response.

**Ratio of IP-10 to IL-6 Protein Expression Following Transfection of M2 Macrophages with plasmids encoding STING Variants**

| STING Variant | Mean Ratio of IP-10 to IL-6 Expression | Standard Deviation |
|---|---|---|
| Untransfected | 0.6108 | ± 0.0960 |
| huSTING N154S | 3.174 | ± 0.2006 |
| huSTING R284G | 3.938 | ± 0.6123 |
| huSTING N154S/R284G | 3.832 | ± 0.1185 |
| huSTING tazCTT N154S | 4.400 | ± 0.1957 |
| huSTING tazCTT R284G | 4.497 | ±0.1631 |
| huSTING tazCTT N154S /R284G | 5.253 | ± 0.1695 |

The ratio of IFN-β to IL-6 gene expression in human M2 macrophages, following the expression of various STING variants, was assessed. Frozen human monocytes, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 10% FBS), and washed by centrifugation for 10 minutes at 600 x g, at room temperature. The monocytes were resuspended in RPMI-1640 + 1X non-essential amino acids (NEAA) + 5% human AB serum, containing 200 ng/mL human M-CSF + 20 ng/mL human IL-4. Monocytes (8e5 to 1e6 cells per well) were then seeded in a 24-well plate, with a final volume of 750 µL. Three days later, 750 µL of RPMI with 5% human AB serum + NEAA, containing 200 ng/mL human M-CSF + 20 ng/mL human IL-4, was added to each well, and the plate was incubated for four more days. On day seven, the cells were transfected using Viromer® RED transfection reagent, according to the manufacturer's instructions. 500 ng of plasmid DNA from a panel of STING GOF mutants, including the modified chimeric STING proteins, as well as untransfected control, were diluted in the provided buffer, and

mixed with Viromer® RED transfection reagent, and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. As a positive control, the STING agonist 3'5' RpRp c-di-AMP (InvivoGen), an analog of the clinical compound ADU-S100, was added to the cells at a concentration of 10 µg/mL. The DNA/Viromer® RED complexes then slowly were added to each well of the 24-well plate (in triplicate), and the plate was incubated for 48 hours at 37 °C, in a $CO_2$ incubator.

The cells were harvested for qPCR 48 hours post-transfection, and were lysed with 350 µL Buffer RLT lysis buffer with beta-mercaptoethanol (Qiagen). RNA extraction was performed using the Qiagen RNeasy® Plus Mini Kit, with the following modifications. A genomic DNA elimination step, using an RNase-Free DNase kit (Qiagen), was included in the kit to remove genomic DNA from the total RNA. Total RNA concentration was measured using a NanoDrop™ One$^C$ UV-Vis Spectrophotometer (Thermo Fisher Scientific). The purity of each sample also was assessed from the $A_{260}/A_{230}$ absorption ratio. RNA was stored at -80 °C without freeze-thawing, until reverse-transcription was performed.

Synthesis of cDNA was performed from 0.5-1 µg of template RNA, using a CFX96™ Real-Time System (Bio-Rad) and iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) in a 20 µL reaction, according to the manufacturer's instructions. qPCR was performed with a CFX96™ Real-Time System (Bio-Rad). Primers for human IFNβ1 (huIFNβ1; Assay ID: qHsaCEP0054112; Bio-Rad) and for huIL-6 (Assay ID: qHsaCEP0051939; Bio-Rad) were used for the qPCR. The qPCR reaction (20 µL) was conducted per protocol, using either the SsoAdvanced™ Universal SYBR® Green Supermix, or the iQ™ Multiplex Powermix (Bio-Rad). The standard thermocycling program on the Bio-Rad CFX96™ Real-Time System comprised a 95 °C denaturation step for 150 seconds, followed by 39 cycles of 95 °C for 15 seconds, and 60 °C for 55 seconds. Quantification of the target mRNA was normalized using actin reference mRNA (Bio-Rad, Assay ID: qHsaCEP0036280). $\Delta C_q$ was calculated as the difference between target (huIFNβ or huIL-6) and reference (actin) gene. $\Delta\Delta C_q$ was obtained by normalizing the $\Delta C_q$ values of the treatments (transfections), to the $\Delta C_q$ values of the

non-treatment (*i.e.*, untransfected) controls. The ratios of the IFNβ $\Delta\Delta C_q$ to the IL-6 $\Delta\Delta C_q$ are shown in the table below.

The results, which are summarized in the table below, show that, of all of the STING GOF mutants screened, the huSTING N154S/R284G tazCTT variant results in the highest ratio of immunostimulatory IFN-β to pro-inflammatory IL-6 expression. Additionally, the chimeric STING constructs, containing a replacement of the CTT of human STING with the CTT of Tasmanian devil STING (*e.g.*, huSTING tazCTT), generally induced higher ratios of IFN-β to IL-6 expression than the corresponding fully human STING constructs comprising the same GOF mutation(s).

Increased expression of IFN-β indicates increased IRF3/type I IFN signaling, which is immunostimulatory and beneficial, while decreased IL-6 expression is indicative of reduced NF-κB signaling, which is pro-inflammatory and does not contribute to an anti-tumor response. A higher ratio of IFN-β to IL-6 expression is indicative of an increased anti-tumor/anti-viral type response, and a decreased pro-inflammatory response. Thus, replacement of the CTT, as well as the gain-of-function mutations, in a STING protein, increases the anti-tumor activity of the STING protein, and, hence, the immunostimulatory bacterium.

**Ratio of IFN-β to IL-6 Gene Expression Following Transfection of M2 Macrophages with STING Variants**

| STING Variant | Mean Ratio of IFN-β to IL-6 Gene Expression | Standard Deviation |
|---|---|---|
| Untransfected control | 1.00 | 0.00 |
| STING Agonist (3'5' RpRp c-di-AMP) | 4.00 | ± 4.24 |
| NanoLuc® Plasmid Control | 16.00 | ± 2.83 |
| huSTING WT | 33.00 | ± 27.84 |
| huSTING V147L | 7.00 | ± 3.46 |
| huSTING N154S | 16.33 | ± 11.24 |
| huSTING V155M | 14.00 | ± 4.58 |
| huSTING C206Y | 19.00 | ± 2.65 |
| huSTING R281Q | 21.33 | ± 18.61 |
| huSTING R284S | 6.33 | ± 4.93 |
| huSTING R284H | 6.33 | ± 2.52 |
| huSTING R284M | 7.33 | ± 0.58 |
| huSTING R284G | 9.67 | ± 2.52 |
| huSTING V155M/C206Y | 10.67 | ± 1.53 |
| huSTING N154S/C206Y | 11.00 | ± 6.56 |
| huSTING C206Y/R284G | 11.00 | ± 8.19 |
| huSTING V155M/R284G | 10.67 | ± 4.93 |
| huSTING N154S/R284G | 48.00 | ± 10.39 |
| huSTING WT tazCTT | 16.67 | ± 10.41 |

| STING Variant | Mean Ratio of IFN-β to IL-6 Gene Expression | Standard Deviation |
|---|---|---|
| huSTING V147L tazCTT | 23.00 | ± 1.00 |
| huSTING N154S tazCTT | 19.00 | ± 6.08 |
| huSTING V155M tazCTT | 22.67 | ± 4.16 |
| huSTING C206Y tazCTT | 14.67 | ± 7.51 |
| huSTING R281Q tazCTT | 10.67 | ± 3.51 |
| huSTING R284S tazCTT | 23.00 | ± 8.89 |
| huSTING R284H tazCTT | 31.33 | ± 13.43 |
| huSTING R284M tazCTT | 43.00 | ± 14.93 |
| huSTING R284G tazCTT | 38.33 | ± 12.01 |
| huSTING V155M/C206Y tazCTT | 31.00 | ± 15.72 |
| huSTING N154S/C206Y tazCTT | 26.67 | ± 11.59 |
| huSTING C206Y/R284G tazCTT | 40.67 | ± 14.01 |
| huSTING V155M/R284G tazCTT | 31.33 | ± 10.60 |
| huSTING N154S/R284G tazCTT | 108.33 | ± 50.90 |

## Example 23

### Design of IL-15 Receptor-α and IL-15 Single-Chain Fusion Proteins

Fusion proteins, containing human proteins or mouse proteins, were prepared. The mouse proteins are for use in mouse models; the human proteins are for encoding in the immunostimulatory bacteria to be used as human therapeutics.

A human IL-15 Receptor-α (IL-15Rα) fused to a human IL-15 single-chain (sc) was designed, as follows. Amino acid residues 1-108 of human IL-15Rα (SEQ ID NO:401), which correspond to the leader sequence and sushi domain of IL-15-Rα (plus an additional 13 amino acid residues of IL-15Rα; see, *e.g.*, Bouchaud *et al.* (2008) *J. Mol. Biol.* 382(1):1–12) were added in-frame with a Gly-Ser linker that has four repeats of the sequence Gly-Gly-Gly-Gly-Ser (*i.e.*, $(GGGGS)_4$). The polypeptide sequence corresponding to fully mature human IL-15sc, without the leader sequence or propeptide, and corresponding to amino acid residues 48-162 of SEQ ID NO:403, was added following the linker. The sequence of the resulting human IL-15Rα–IL-15sc fusion protein (SEQ ID NO:404) is as follows:

MAPRRARGCRTLGLPALLLLLLLRPPATRGITCPPPMSVEHADIWVKSYSLYS
RERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCIRDPALVHQRPAP
PS**GGGGSGGGGSGGGGSGGGGS**ANWVNVISDLKKIEDLIQSMHIDATLYTE
SDVHPSCKVTAMKCFLLELQVISLESGDASIHDTVENLIILANNSLSSNGNVTE
SGCKECEELEEKNIKEFLQSFVHIVQMFINTS.

where the residues corresponding to residues 1-108 of human IL-15Rα are underlined; the Gly-Ser linker is in bold letters; and the residues corresponding to residues 48-162 of human IL-15 are double underlined.

Similarly, a murine IL-15Rα-IL-15sc fusion protein (SEQ ID NO:407) was prepared by fusion of a portion of the murine IL-15 Receptor-α (IL-15Rα) protein to a portion of the murine IL-15 single-chain (sc) protein. Amino acid residues 1-132 of murine IL-15Rα (SEQ ID NO:405), which include the leader sequence and sushi domain of IL-15-Rα, were added in-frame with a $(GGGGS)_4$ linker. The polypeptide sequence corresponding to fully mature murine IL-15sc, without the leader sequence or propeptide, and corresponding to amino acid residues 49-162 of SEQ ID NO:406, was added following the linker. The sequence of the resulting murine IL-15Rα–IL-15sc fusion protein (SEQ ID NO:407), which was used for mouse model experiments, is as follows:

MASPQLRGYGVQAIPVLLLLLLLLLLPLRVTPGTTCPPPVSIEHADIRVKNYSV
NSRERYVCNSGFKRKAGTSTLIECVINKNTNVAHWTTPSLKCIRDPSLAHYSP
VPTVVTPKVTSQPESPSPSAKEPEA**GGGGSGGGGSGGGGSGGGGS**NWIDVR
YDLEKIESLIQSIHIDTTLYTDSDFHPSCKVTAMNCFLLELQVILHEYSNMTLN
ETVRNVLYLANSTLSSNKNVAESGCKECEELEEKTFTEFLQSFIRIVQMFINTS,

where the residues corresponding to residues 1-132 of murine IL-15Rα are underlined; the Gly-Ser linker is in bold letters; and the residues corresponding to residues 49-162 of murine IL-15 are double underlined.

## Example 24

## IL-15Rα-IL-15sc Induces Curative Effects and Protection from Tumor Re-Challenge in a Mouse Model of Colorectal Carcinoma

This example demonstrates that immunostimulatory bacteria that encode the IL-15Rα-IL15sc fusion protein (also referred to herein as IL-15/IL-15R alpha chain complex, IL-15/IL-15Rα chain complex, and IL-15/IL-15Rα) induce anti-tumor efficacy as a monotherapy. To demonstrate this, *S. typhimurium* strains, containing plasmids encoding murine IL-15Rα-IL15sc (muIL-15Rα-IL15sc), were prepared. The YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain, containing the plasmid encoding muIL-15Rα-IL15sc (*i.e.*, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-muIL-15Rα-IL15sc; mouse IL-15Rα-IL15sc was used for the experiments performed in the mouse model), was compared to PBS control, for safety and efficacy in the subcutaneous

(SC) flank MC38 colorectal adenocarcinoma model. For this study, 6-8 week-old female C57BL/6 mice (5 mice per group) were inoculated SC in the right flank with MC38 cells ($5x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were intravenously (IV) injected on day 8 with $2x10^7$ CFUs of the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-muIL-15Rα-IL15sc strain, or with PBS vehicle control. Tumor measurements and body weights were recorded twice weekly.

The results showed that the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-muIL-15Rα-IL15sc strain demonstrated 50% cures (4/8 vs. 0/8 for PBS, $p = 0.005$, day 21), after a single IV injection. At 66 days post-tumor implantation (day 57 post-IV dosing), cured mice (N=4) were re-implanted, on the opposite flank, with $5x10^5$ MC38 cells, and tumor growth was compared to naïve, age-matched mice (N=5). By day 30 post re-implantation, all mice in the group cured with strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-muIL-15Rα-IL15sc remained tumor-free, whereas all mice in the naïve group had reached maximum tumor volume. These data demonstrate the potent and curative effects of delivery of the IL-15Rα-IL15sc fusion protein via an immunostimulatory bacterium, such as strain YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*-muIL-15Rα-IL15sc, and demonstrate the induction of durable protective immune memory in a model of colorectal carcinoma.

## Example 25

## Anti-CTLA-4 scFv-Fc Demonstrates Superior Blockade of the CD80/CTLA-4 and CD86/CTLA-4 Interactions Compared to Anti-CTLA-4 scFv

An scFv-Fc specific for human CTLA-4 (see, SEQ ID NOs:427 and 428 for the nucleic acid and protein sequences, respectively) was designed using the amino acid sequence of ipilimumab. Ipilimumab is a fully human IgG1κ monoclonal antibody that specifically binds human CTLA-4 (see, *e.g.*, the antibody designated 10D1 in U.S. Patent Publication No. 2002/0086014 and in U.S. Patent No. 6,984,720), blocking CTLA-4's immune inhibiting interaction with CD80 (also known as B7.1 or B7-1) and CD86 (also known as B7.2 or B7-2).

To generate the ipilimumab scFv antibody fragment (see, SEQ ID NO:429), the variable light chain ($V_L$) and variable heavy chain ($V_H$) of ipilimumab were linked with a 20 amino acid long glycine-serine (GS) linker ($(GGGGS)_4$). To generate the scFv-Fc antibody fragment (see, SEQ ID NO:428), the variable heavy chain of the

ipilimumab scFv was linked to a human IgG1 Fc, containing a mutation of the free cysteine in the hinge region to a serine (at position 272 in SEQ ID NO:428). The leader sequence (METPAQLLFLLLLWLPDTTG; corresponding to residues 1-20 in SEQ ID NO:428) was derived from the sequence of the human immunoglobulin kappa variable 3-20 (IGKV3-20) protein. The sequence was codon optimized using the GenScrip GenSmart™ Codon Optimization tool.

The neutralizing ability of the anti-CTLA-4 scFv-Fc was compared to that of the anti-CTLA-4 scFv (lacking the human IgG1 Fc portion), using competitive ELISAs to measure the ability of each of the antibody fragments to block the interactions between CTLA-4 and its ligands, CD80 and CD86. HEK293T cells were transfected with 3 micrograms of DNA encoding the anti-CTLA-4 scFv-Fc or the anti-CTLA-4 scFv antibody fragment constructs, using the FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios. Forty-eight hours post-transfection, HEK293T cell-free culture supernatants were harvested, filtered, and used in a competitive ELISA to assess the blockade activity of the anti-CTLA-4 antibody fragments.

For the competitive ELISAs, mouse CD80 or CD86 recombinant proteins (R&D Systems) were coated overnight at 4 °C on a high protein-binding 96-well plate, at a concentration of 100 ng/ml. The wells were then washed one time with PBS 0.05% Tween-20, and the wells were blocked with ELISA blocking buffer for 1 hour at room temperature. The wells were then washed one time with PBS 0.05% Tween-20. HEK293T cell culture supernatants, containing each of the anti-CTLA-4 antibody fragments, were mixed with 10 ng/ml of a recombinant murine CTLA-4-human IgG1 Fc chimera (R&D Systems), and added to the wells and incubated for 2 hours at room temperature. The wells were then washed three times with PBS 0.05% Tween-20, and horseradish peroxidase (HRP)-conjugated anti-human IgG1 antibody was added to the wells (Jackson ImmunoResearch), and incubated for one hour at room temperature. The wells were then washed three times with PBS 0.05% Tween-20, and detection reagent (3,3′,5,5′-tetramethylbenzidine (TMB), Thermo Fisher Scientific) was added to the wells. The enzymatic reaction was stopped with sulfuric acid (BioLegend), and the optical densities were read at 450 nm.

The results of the competitive ELISAs are summarized in the table below. The anti-CTLA-4 scFv-Fc blocked the binding of CTLA-4 to CD86 by 75.5%, and blocked the binding of CTLA-4 to CD80 by 40.6%, whereas the anti-CTLA-4 scFv blocked the binding of CTLA-4 to CD86 by 32.5%, and blocked the binding of CTLA-4 to CD80 by 7%. A higher degree of CD86/CTLA-4 blocking activity (compared with CD80/CTLA-4 blocking activity) was observed with both antibody fragments, and a superior neutralizing activity was observed with the anti-CTLA-4 scFv-Fc, when compared to the anti-CTLA-4 scFv.

**Competitive ELISA Results**

| Anti-CTLA-4 Antibody Fragment | % CD86 Blockade Activity | % CD80 Blockade Activity |
|---|---|---|
| Anti-CTLA-4 scFv | 32.5 | 7.0 |
| Anti-CTLA-4 scFv-Fc | 75.5 | 40.6 |

An anti-murine CTLA-4 scFv (SEQ ID NO:430) and anti-murine CTLA-4 scFv-Fc (SEQ ID NO:431), derived from the 9D9 clone, also were prepared, as discussed above for the corresponding human anti-CTLA-4 antibody fragments. The scFv contains an IgK leader mouse sequence, and the $V_L$ and $V_H$ domains from clone 9D9, linked via a $(Gly_4Ser)_3$ linker. The scFv-Fc also contains a mouse IgG2a Fc linked to the $V_H$ domain.

Included among the immunostimulatory bacteria provided herein, are those that encode, on the plasmid, anti-CTLA-4 antibodies and fragments thereof, including the anti-CTLA-4 scFv antibody fragments and the anti-CTLA-4 scFv-Fc antibody fragments, as provided herein, as well as combinations with nucleic acid molecules encoding other therapeutic products.

## Example 26

## Optimized Expression Cassettes for High-Level, Multiplexed Expression of Encoded Therapeutic Products

Elements, known as Internal Ribosomal Entry Sites (IRES), can promote protein translation by enhancing ribosomal binding and stabilization of mRNA through mimicking the 5'mRNA cap. IRES elements from viruses and mammalian cells are well-known. Of these, an endogenous human IRES, known as Vascular Endothelial Growth Factor and Type 1 Collagen Inducible Protein (VCIP), previously

was demonstrated to enhance expression from a bicistronic vector, encoding Firefly and *Renilla* luciferases, where the *Renilla* luciferase, which was the downstream gene (*i.e.*, encoded after the VCIP IRES), was expressed *in vitro* and *in vivo*, without the need for a second promoter (see, *e.g.*, Licursi *et al.* (2011) *Gene Therapy* 18(6):631-636).

Plasmids containing combinations of human or mouse IL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT, or human or mouse IL-15Rα-IL-15sc, huSTING N154S/R284G tazCTT, and an anti-CTLA-4 scFv-Fc, where the bicistronic and polycistronic constructs contain 2A peptides, were tested for expression of each encoded payload/product against single expresser controls. Additionally, combination constructs, containing the VCIP IRES, also were tested.

HEK293T STING Null Cells (293-Dual™ Null Cells; InvivoGen) were used, which do not contain endogenous STING, and express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous IFN-stimulated response element (ISRE) promoter, where the coding sequence of ISRE is replaced by the SEAP ORF using knock-in technology. STING activity can thus be assessed by monitoring type I IFN induced SEAP production. The 293-Dual™ Null cells also express Lucia™ luciferase, a secreted luciferase, placed under the control of the endogenous IFN-β promoter; the coding sequence of IFN-β has been replaced by the Lucia™ luciferase ORF using knock-in technology. This allows for the assessment of STING activity by monitoring the expression of IFN-β. Using these cells, STING activity can be assessed by monitoring ISRE-induced SEAP production and/or IFN-β-dependent expression of Lucia™ luciferase. The two reporter proteins, SEAP and Lucia™ Luciferase, can be measured in the cell supernatant using standard assays and detection reagents, such as the QUANTI-Blue™ and QUANTI-Luc™ detection reagents (InvivoGen), respectively.

The cells were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, and incubated overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 300 ng of each plasmid DNA, and 40 ng of a CMV-GFP vector (*i.e.*, a vector encoding green fluorescent protein under the control of a CMV promoter), were diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios, with untransfected

wells serving as negative controls (in duplicates). Cell culture supernatants from each sample were collected 48 hours post-transfection.

The STING activity of the huSTING N154S/R284G tazCTT variant was evaluated using the ISRE-SEAP and IFN-β-Lucia™ reporter systems. The type I interferon (IFN) activity (induced by STING) was assessed by monitoring type I IFN-stimulated SEAP production in the cell supernatants. 20 µL of cell culture supernatant was added to 180 µL of QUANTI-Blue™ reagent (InvivoGen), which is used for measuring SEAP. Type I interferon activation was determined by measuring ISRE-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), at an absorbance wavelength of 650 nm. The type I interferon (IFN) activity (induced by STING) also was assessed by monitoring the type I IFN-stimulated Lucia™ luciferase production in the cell supernatants. 20 µL of cell culture supernatant was added to 50 µL of QUANTI-Luc™ reagent (InvivoGen), which is used for measuring Lucia™ luciferase activity. Type I interferon activation was determined by measuring IFNβ-induced Lucia™ luciferase activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), on the luminescence setting.

Cell culture supernatants also were assessed for the expression of human or mouse IL-15Rα-IL-15sc (see, Example 23), and for the expression of human or mouse anti-CTLA-4 scFv-Fc (see, Example 25). For the muIL-15Rα-IL-15sc constructs, the murine IL-15Rα-IL-15sc ELISA (R&D) was used, per kit instructions. For the huIL-15Rα-IL-15sc constructs, the human IL-15Rα-IL-15sc ELISA (R&D) was used, per kit instructions. Direct ELISAs, with human and mouse CTLA-4-Fc (R&D Systems), were performed on the cell culture supernatants of cells transfected with plasmids encoding anti-human CTLA-4 scFv-Fc and anti-murine CTLA-4 scFv-Fc, to measure the expression levels of these proteins.

GFP production was detected by flow cytometry, and was used to normalize the transfections to each other. 48 hours post-transfection, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells then were resuspended in PBS + 2% FBS with DAPI (dead/live stain). Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

As shown in the table below, all constructs containing the huSTING N154S/R284G tazCTT variant displayed ISRE-SEAP reporter activity and IFNβ-Lucia™ luciferase reporter activity. The construct designated 2.1 CMV VCIP muIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT, which contains the VCIP IRES and a T2A peptide for expression of the combination of muIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT, results in the highest expression levels of muIL-15Rα-IL-15sc, when normalized by GFP co-transfection. Similarly, the construct designated 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT, which contains the VCIP IRES and a T2A peptide for expression of the combination of huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT, results in the highest expression levels of huIL-15Rα-IL-15sc, when normalized by GFP co-transfection. The construct designated 1.76 CMV mu anti-CTLA-4 scFv-Fc results in the highest level of expression of the murine anti-CTLA-4 scFv-Fc, and the construct designated 1.76 CMV hu anti-CTLA-4 scFv-Fc results in the highest level of expression of the human anti-CTLA-4 scFv-Fc, when normalized by GFP co-transfection.

**Measurement of STING Activity, IL-15Rα-IL-15c Concentration, and Anti-CTLA-4 scFv-Fc Concentration, Normalized by GFP Co-Transfection, in HEK293T STING Null Cells**

| Mouse | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Construct | ISRE-SEAP (Abs) | | IFNβ-Lucia™ (RLUs) | | muIL-15Rα-IL-15sc (Abs) | | mu anti-CTLA-4 scFv-Fc (Abs) | |
| | Mean | ±SD | Mean | ±SD | Mean | ±SD | Mean | ±SD |
| Untransfected | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.76 CMV β-Actin | -1.71E-05 | 1.04E-05 | -0.01597 | 0.6049 | 0 | 0 | 0.002325 | 0.0004565 |
| 1.76 CMV muIL-15Rα-IL-15sc | 1.45E-07 | 4.79E-06 | 0.07555 | 0.3292 | 947.4 | 164.1 | NA* | NA* |
| 1.76 CMV huSTING N154S/R284G tazCTT | 4.07E-02 | 4.29E-03 | 18.25 | 1.27 | NA | NA | NA* | NA* |
| 1.76 CMV muIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT | 3.79E-02 | 2.19E-04 | 28.47 | 1.523 | 996.8 | 364.6 | NA* | NA* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2.1 CMV VCIP muIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT | 4.73E-02 | 7.60E-03 | 7.145 | 0.8812 | 1636 | 287.9 | NA* | NA* |
| 1.76 CMV mu anti-CTLA-4 scFv-Fc | -1.54E-06 | 1.81E-05 | -0.6844 | 0.1614 | NA* | NA* | 0.03733 | 0.003862 |
| 1.76 CMV mu anti-CTLA-4 scFv-Fc T2A muIL-15Rα-IL-15sc P2A huSTING tazCTT N154S/R284G tazCTT | 3.51E-02 | 1.85E-04 | 3.225 | 0.2805 | 599.6 | 190.4 | 0.01676 | 0.00193 |
| 2.1 CMV VCIP mu anti-CTLA-4 scFv-Fc T2A muIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT | 3.62E-02 | 1.26E-03 | 4.836 | 0.5915 | 610.1 | 230.7 | 0.01461 | 0.002674 |

**Human**

| **Construct** | **ISRE-SEAP (Abs)** | | **IFN β-Lucia™ (RLUs)** | | **huIL-15Rα-IL-15sc (Abs)** | | **hu anti-CTLA-4 scFv-Fc (Abs)** | |
|---|---|---|---|---|---|---|---|---|
| | **Mean** | **±SD** | **Mean** | **±SD** | **Mean** | **±SD** | **Mean** | **±SD** |
| Untransfected | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.76 CMV β-Actin | -1.71E-05 | 1.04E-05 | -0.01597 | 0.6049 | 0 | 0 | 0.001643 | 0.00007734 |
| 1.76 CMV huIL-15Rα-IL-15sc | -1.18E-05 | 9.34E-06 | 0.03296 | 0.1661 | 2407 | 4.101 | NA* | NA* |
| 1.76 CMV huSTING N154S/R284G tazCTT | 4.07E-02 | 4.29E-03 | 18.25 | ± 1.27 | NA* | NA* | NA* | NA* |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT | 4.27E-02 | 3.76E-03 | 10.5 | 0.7221 | 1439 | 869.4 | NA* | NA* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT | 4.18E-02 | 4.63E-03 | 12.79 | 1.151 | 2758 | 235.1 | NA* | NA* |
| 1.76 CMV hu anti-CTLA-4 scFv-Fc | -8.73E-06 | 5.53E-06 | -0.333 | 0.04487 | NA* | NA* | 0.06436 | 0.006539 |
| 1.76 CMV hu anti-CTLA-4 scFv-Fc T2A huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT | 3.80E-02 | 1.32E-03 | 6.962 | 0.01366 | 2071 | 557.5 | 0.02265 | 0.001188 |
| 2.1 CMV VCIP hu anti-CTLA-4 scFv-Fc T2A huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT | 3.53E-02 | 3.25E-03 | 7.12 | 1.998 | 936.3 | 149.4 | 0.01687 | 0.0007017 |

SD = Standard deviation
*NA = Not applicable

## Example 27

## Expression Plasmids Encoding the Combination of huIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT

To identify the plasmid(s), with the highest expression of huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT, plasmids containing nucleic acid molecules encoding huIL-15Rα-IL-15sc and/or huSTING N154S/R284G tazCTT, with different 2A peptides (*e.g.*, T2A or P2A), and/or different post-transcriptional regulatory elements (*e.g.*, HPRE or WPRE), and/or different poly(A) tails (*e.g.*, bovine growth hormone poly(A) (bGHpA) or simian virus 40 poly(A) (SV40pA)), and/or containing the VCIP IRES, were cloned. Additionally, in some constructs, short peptide spacers, including RRKR and RAKR, and other spacers of different lengths, were encoded following the nucleic acid encoding the first payload (*i.e.*, huIL-15Rα-IL-15sc), and before the nucleic acid encoding the 2A peptide. RRKR and RAKR are designed furin protease cleavage sites, and were placed in that position to facilitate proper processing of the 2A peptide sequences. The plasmids were first evaluated for expression and

functionality by transfection in HEK293T STING Null Cells (ISG/KI-IFN β) cells (InvivoGen).

HEK293T STING Null Cells (293-Dual™ Null Cells (ISG-SEAP/KI-[IFN-β]Lucia; InvivoGen), which do not contain endogenous STING, and express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous IFN-stimulated response element (ISRE) promoter, where the coding sequence of ISRE is replaced by the SEAP ORF using knock-in technology, were used. As discussed above, the 293-Dual™ Null cells also express Lucia™ luciferase, a secreted luciferase, placed under the control of the endogenous IFN-β promoter, where the coding sequence of IFN-β has been replaced by the Lucia™ luciferase ORF using knock-in technology. The cells were seeded in 24-well plates coated with poly-L-lysine, at 200,000 cells per well, and incubated overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 300 ng of each plasmid DNA, and 40 ng of a CMV-GFP vector, were diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios, with untransfected wells serving as negative controls (in duplicates). Cell culture supernatants from each sample were collected 48 hours post-transfection for analysis.

The STING activity of the huSTING N154S/R284G tazCTT modified STING protein was evaluated with the HEK293T STING Null (ISG-SEAP/KI-[IFN-β]Lucia) reporter cell line (InvivoGen). Using these cells, the type I interferon (IFN) activity (that is induced by STING) is assessed by monitoring type I IFN-stimulated Lucia™ luciferase production in the cell supernatants. IFNβ induction is measured with the IFNβ-Lucia reporter. 20 µL of cell culture supernatant was added to 50 µL of QUANTI-Luc™ reagent (InvivoGen), which is used for measuring Lucia™ luciferase activity. Type I interferon activation was determined by measuring IFNβ-induced Lucia™ luciferase activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), on the luminescence setting. Cell culture supernatants also were assessed for the expression of human IL-15Rα-IL-15sc by ELISA (R&D), per kit instructions.

GFP was detected by flow cytometry, and the expression levels of GFP (as measured by fluorescence) were used to normalize the transfections to one other. 48 hours post-transfection, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells then were resuspended in PBS +

2% FBS with DAPI (dead/live stain). Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

As shown in the two tables below, the plasmid construct designated 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA resulted in the highest normalized luminescence value from the IFNβ-Lucia™ reporter (see, first table below), and also, the highest normalized concentration of expressed huIL-15Rα-IL-15sc, as determined by ELISA (see, second table below).

As shown in the first table below, the construct encoding only huIL-15Rα-IL-15sc (and no STING variant), as expected, does not exhibit STING-induced type I IFN activity. The construct encoding only the huSTING N154S/R284G tazCTT exhibits a high level of STING-induced type I IFN activity. For the constructs encoding both payloads, the combination of a P2A peptide with WPRE results in higher levels of STING activity (*i.e.*, higher levels of expression of STING), than combinations of T2A and P2A with HPRE, or T2A with WPRE. Additionally, higher levels of STING activity were observed with constructs containing a bGH poly(A) tail, than an SV40 poly(A) tail. The addition of a short peptide spacer, such as RAKR or RRKR, slightly increases the level of STING activity, compared to the same constructs not containing these spacers. In certain constructs, such as those designated 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA; 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE bGHpA; 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE bGHpA; 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE SV40pA; and 2.1 CMV VCIP huIL-15Rα-IL-15sc RAKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA, the addition of a VCIP IRES increases the levels of STING activity, compared to the same constructs not containing the VCIP IRES.

As shown in the second table below, the expression levels of huIL-15Rα-IL-15sc are higher from constructs containing a bGH poly(A) tail, than an SV40 poly(A) tail, and in general, constructs containing WPRE result in higher expression levels of huIL-15Rα-IL-15sc than the same constructs containing HPRE. The addition of an

RAKR short peptide spacer, after the nucleic acid sequence encoding huIL-15Rα-IL-15sc, in the construct encoding CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA, increased the expression levels of huIL-15Rα-IL-15sc. In all constructs, except the one designated 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE SV40pA, the addition of a VCIP IRES (in addition to a 2A peptide) upstream of the construct, results in significantly increased expression of huIL-15Rα-IL-15sc. Replacement of the 2A peptide with a VCIP IRES, for example, in the construct designated 1.76 CMV huIL-15Rα-IL-15sc VCIP huSTING N154S/R284G tazCTT HPRE bGHpA, results in similar expression levels of huIL-15Rα-IL-15sc. Replacement of the 2A peptide with a VCIP IRES, and the addition of a longer spacer between the nucleic acids encoding huIL-15Rα-IL-15sc and the VCIP IRES, for example, in the construct designated 1.76 CMV huIL-15Rα-IL-15sc Longer spacer VCIP huSTING N154S/R284G tazCTT HPRE bGHpA, results in a significantly increased expression level of huIL-15Rα-IL-15sc. The spacer sequences in the constructs are nucleic acid sequences that are placed between the stop codon of the first ORF, and the VCIP IRES upstream of the second ORF. For example the spacer, designated "New spacer" in the tables below, has the sequence ACGTCTTCTCTTTTTAAAGGACCTCGTGAAATAAAAGTGC (SEQ ID NO:408), and the spacer, designated "Longer spacer" in the tables below, has the sequence TCCGAGCCAAGTAAGGAGGTCCCTCTCTCTCTCTCCCCCCACGTCTTCTCTTTTTAAAGGACCTCGTGAAATAAAAGTGC (SEQ ID NO:409).

These results indicate that the addition of a VCIP IRES generally increases the expression levels of the first payload encoded on a bicistronic construct, and increases the expression levels of the second payload in certain constructs.

**STING-Induced Type I IFN Activity, as Measured by Luminescence Levels from the IFNβ-Lucia™ Reporter, and Normalized by GFP Co-Transfection, in Transfected HEK293T STING Null Cells**

| | IFNβ-Lucia™ Reporter Luminescence (RLUs) | |
|---|---|---|
| **Construct** | **Mean** | **SD** |
| Untransfected control | 0 | 0 |
| 1.76 CMV huIL-15Rα-IL-15sc HPRE bGHpA | -0.7073 | ± 0.05241 |

| Construct | IFNβ-Lucia™ Reporter Luminescence (RLUs) | |
|---|---|---|
| | Mean | SD |
| 1.76 CMV huSTING N154S/R284G tazCTT HPRE bGHpA | 7.455 | ± 0.7934 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 3.293 | ± 0.8576 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE bGHpA | 3.758 | ± 0.1993 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE bGHpA | 3.867 | ± 1.297 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE bGHpA | 8.716 | ± 0.4241 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE SV40pA | 0.9444 | ± 0.03282 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE SV40pA | 0.4778 | ± 0.03872 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE SV40pA | 0.4436 | ± 0.1342 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE SV40pA | 0.483 | ± 0.07984 |
| 1.76 CMV huIL-15Rα-IL-15sc RAKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 3.621 | ± 0.5816 |
| 1.76 CMV huIL-15Rα-IL-15sc RRKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 4.938 | ± 0.6406 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 9.356 | ± 2.116 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE bGHpA | 4.065 | ± 0.02317 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE bGHpA | 4.648 | ± 1.199 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE bGHpA | 4.666 | ± 0.8666 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE SV40pA | -0.2763 | ± 0.4616 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE SV40pA | 0.2751 | ± 0.2559 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE SV40pA | 0.8467 | ± 0.0387 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE SV40pA | 0.3058 | ± 0.3335 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc RAKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 7.278 | ± 0.6898 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc RRKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 2.675 | ± 0.2714 |
| 1.76 CMV huIL-15Rα-IL-15sc VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 0.5301 | ± 0.09792 |
| 1.76 CMV huIL-15Rα-IL-15sc New spacer* VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 0.869 | ± 0.04427 |

| Construct | IFNβ-Lucia™ Reporter Luminescence (RLUs) | |
|---|---|---|
| | Mean | SD |
| 1.76 CMV huIL-15Rα-IL-15sc Longer spacer** VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 1.241 | ± 0.03473 |

SD = Standard deviation
*New spacer = ACGTCTTCTCTTTTTAAAGGACCTCGTGAAATAAAAGTGC
**Longer spacer =
TCCGAGCCAAGTAAGGAGGTCCCTCTCTCTCTCTCCCCCCACGTCTTCTCTTTTTAAAGGAC
CTCGTGAAATAAAAGTGC

**Concentration of Expressed huIL-15Rα-IL-15sc, as Measured by ELISA and Normalized by GFP Co-Transfection, in Transfected HEK293T STING Null Cells**

| Construct | Fluorescence (Abs) | |
|---|---|---|
| | Mean | SD |
| Untransfected control | 0 | 0 |
| 1.76 CMV huIL-15Rα-IL-15sc HPRE bGHpA | 1619 | ± 18.53 |
| 1.76 CMV huSTING N154S/R284G tazCTT HPRE bGHpA | 12.04 | ± 35.09 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 1586 | ± 88.91 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE bGHpA | 1906 | ± 35.82 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE bGHpA | 2244 | ± 477 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE bGHpA | 2056 | ± 71.49 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE SV40pA | 40.33 | ± 9.336 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE SV40pA | 268.9 | ± 10.53 |
| 1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE SV40pA | 324.5 | ± 51.87 |
| 1.76 CMV huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE SV40pA | 397.7 | ± 42.62 |
| 1.76 CMV huIL-15Rα-IL-15sc RAKR-T2A huSTING tazCTT N154S/R284G HPRE bGHpA | 1915 | ± 16.64 |
| 1.76 CMV huIL-15Rα-IL-15sc RRKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 1093 | ± 3.221 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 2758 | ± 114.5 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE bGHpA | 2457 | ± 1.527 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE bGHpA | 3616 | ± 202.8 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING tazCTT N154S/R284G WPRE bGHpA | 2327 | ± 199 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING tazCTT N154S/R284G HPRE SV40pA | 397.5 | ± 148.8 |

| Construct | Fluorescence (Abs) | |
|---|---|---|
| | Mean | SD |
| 2.1 CMVVCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE SV40pA | 184.3 | ± 39.19 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT WPRE SV40pA | 551 | ± 15.35 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT WPRE SV40pA | 662.9 | ± 99.41 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc RAKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 2553 | ± 71.83 |
| 2.1 CMV VCIP huIL-15Rα-IL-15sc RRKR-T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 1919 | ± 332.6 |
| 1.76 CMV huIL-15Rα-IL-15sc VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 1507 | ± 97.77 |
| 1.76 CMV huIL-15Rα-IL-15sc New spacer* VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 1385 | ± 28.04 |
| 1.76 CMV huIL-15Rα-IL-15sc Longer spacer** VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 2648 | ± 178.2 |

*New spacer = ACGTCTTCTCTTTTTAAAGGACCTCGTGAAATAAAAGTGC
**Longer spacer = TCCGAGCCAAGTAAGGAGGTCCCTCTCTCTCTCTCCCCCCACGTCTTCTCTTTTTAAAGGACCTCGTGAAATAAAAGTGC

The differences in the downstream IFN-β signaling, induced in human M2 macrophages by the different huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT constructs, was determined. Nucleic acids encoding huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT were cloned into vectors, with and without a VCIP IRES, placed before the start codon of either the huIL-15Rα-IL-15sc or huSTING N154S/R284G tazCTT coding sequences.

Frozen human monocytes, isolated from healthy human donors, were thawed in complete medium (RPMI-1640 + 10% FBS), and washed by centrifugation for 10 minutes at 600xg at room temperature. The monocytes were resuspended in ImmunoCult™-SF Macrophage Medium (StemCell Technologies), containing 100 ng/mL human M-CSF. The monocytes (5e5 cells per well) then were seeded in a 24-well plate with a final volume of 500 µL. Five days later, 250 µL of ImmunoCult™-SF Macrophage Medium (StemCell Technologies), containing 300 ng/mL human M-CSF + 60 ng/mL human IL-4 + 60 ng/mL human IL-10, was added to each well, and the cells were incubated for 2 more days. On day 7, the cells were transfected using Viromer® RED mRNA and plasmid transfection reagent, according to the manufacturer's instructions. 750 ng of plasmid DNA, from a panel of different

plasmid constructs, as well as a "no DNA" control (encoding secNanoLuc®), were diluted in the provided buffer, and mixed with Viromer® RED transfection reagent, and the mixture was incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 24-well plate (in triplicate), and the plate was incubated at 37 °C in a 5% $CO_2$ incubator. Cell culture supernatants were harvested at 48 hours, and were assayed for IFN-β using a human cytokine panel U-Plex assay (Meso Scale Discovery), according to the manufacturer's protocol. The average of three measurements was calculated, and background signal from the no DNA control was subtracted, to calculate the net IFN-β expression levels.

The results, which are summarized in the table below, show that the construct encoding only huIL-15Rα-IL-15sc (and no STING), does not induce any IFN-β expression in transfected human M2 macrophages. The construct that results in the highest level of IFN-β expression in transfected human M2 macrophages is the construct designated pATI2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA. This construct (designated pATI2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA) results in a higher level of IFN-β expression than constructs with the pATI1.76 backbone, and with no VCIP IRES at all, or with a VCIP IRES between the ORFs of huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT (*i.e.*, where the VCIP IRES replaces a 2A sequence). The construct designated pATI2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA results in a higher IFN-β signal, compared to the corresponding construct containing a P2A sequence instead of a T2A sequence. These results indicate that bicistronic constructs, containing a VCIP IRES upstream of the two ORFs, and a 2A sequence, particularly T2A, between the two ORFs, result in higher expression levels of the second encoded payload (in this case, a modified STING protein).

**Net Signal of IFN-β Protein Expression Following Transfection of Human M2 Macrophages with Various Constructs**

| Construct | Net IFN-β (pg/mL) | Standard Deviation |
|---|---|---|
| pATI2.1 CMV VCIP secNanoLuc® HPRE bGHpA | 0.00 | ± 133.76 |
| pATI1.76 CMV huIL-15Rα-IL-15sc HPRE bGHpA | -411.92 | ± 229.81 |

| | | |
|---|---|---|
| pATI1.76 CMV huSTING N154S/R284G tazCTT HPRE bGHpA | 2710.37 | ± 579.12 |
| pATI1.76 CMV huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA | -788.33 | ± 139.87 |
| pATI2.1 CMV VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT HPRE bGHpA | 5505.08 | ± 397.48 |
| pATI2.1 CMV VCIP huIL-15Rα-IL-15sc P2A huSTING N154S/R284G tazCTT HPRE bGHpA | 1425.51 | ± 62.06 |
| pATI1.76 CMV huIL-15Rα-IL-15sc VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 1630.63 | ± 709.30 |
| pATI1.76 CMV huIL-15Rα-IL-15sc New spacer* VCIP huSTING tazCTT N154S/R284G HPRE bGHpA | 2014.95 | ± 97.68 |
| pATI1.76 CMV huIL-15Rα-IL-15sc Longer spacer** VCIP huSTING N154S/R284G tazCTT HPRE bGHpA | 385.71 | ± 158.29 |

*New spacer = ACGTCTTCTCTTTTTAAAGGACCTCGTGAAATAAAAGTGC
**Longer spacer =
TCCGAGCCAAGTAAGGAGGTCCCTCTCTCTCTCTCCCCCCACGTCTTCTCTTTTTAAAGGAC
CTCGTGAAATAAAAGTGC

## Example 28

### Expression of Phospholipase D as a Method to Improve Plasmid Delivery

As part of its pathogenesis, *Salmonella enterica* serovar Typhimurium is phagocytized by myeloid cells, and activates a virulence program to alter phagosomes into *Salmonella*-containing vacuoles (SCVs). While ectopic expression of genes encoded on the plasmid requires plasmid translocation from the SCV to the nucleus, the mechanism of this translocation event is unknown. This example provides an engineering approach to improve plasmid transfer from the engineered immunostimulatory bacteria.

*Rickettsia prowazekii* is a Gram-negative intracellular pathogen that must escape the phagosomal compartment in order to access the host cytosol and replicate. Previous work has shown that the secreted *R. prowazekii* virulence factor, Phospholipase D (Pld), can be cloned into *S. enterica* to facilitate escape from the SCV (see, *e.g.*, Whitworth *et al.* (2005) *Infection and Immunity* 73(10):6668-6673). The *pld* gene (SEQ ID NO:432), encoding Pld (SEQ ID NO:433), was placed under the control of the ssaG promoter, which is induced in the SCV (see, *e.g.*, Walthers *et al.* (2007) *Molecular Microbiology* 65(2):477-493), to ensure high expression after the bacteria is delivered into phagocytic cells. This bacterial expression cassette was cloned into the backbone of two NanoLuciferase® reporter plasmids, designated

CMV-NanoLuc® and EF1α-NanoLuc®, and plasmid delivery was assessed in human macrophages.

To measure plasmid delivery into infected cells, primary human monocytes were differentiated in ImmunoCult™-SF Macrophage Medium (StemCell Technologies), containing 100 ng/ml GM-CSF, for 5 days. On day 6, the cells were supplemented with additional media containing 150 ng/ml GM-CSF and 60 ng/ml huIL-4 for 48 hours. The cells were then bactofected with stationary phase strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* bacteria, comprising one of the CMV-NanoLuc®, CMV-NanoLuc® + *pld*, EF1α-NanoLuc®, or EF1α-NanoLuc® + *pld* plasmids. The bacterial strains were grown overnight at 37 °C in 4XYT medium, at an MOI (multiplicity of infection) of 100. Infected cells were centrifuged for 5 minutes at 500 rcf, and then incubated at 37 °C for 1 hour, after which the cells were washed thrice with DPBS, and incubated in fresh ImmunoCult™-SF Macrophage Medium, containing 100 µg/ml gentamicin to eliminate any extracellular bacteria. At 24 and 48 hours post-infection, the cell supernatants were harvested, and the NanoLuciferase® luciferase contents were measured using a luciferase assay (Promega). The cells also were lysed with 0.1% Triton X in PBS and plated, to enumerate the number of colony forming units (CFUs) that remained inside the cells.

The results, which are summarized in the table below, show that, when placed under the control of the CMV promoter, the levels of NanoLuciferase® expressed from the *pld*-encoding group (*i.e.*, the CMV-NanoLuc® luciferase + *pld* plasmids) were greater than the levels of NanoLuciferase® expressed from the CMV-NanoLuc® luciferase plasmid alone, at 24 hours post-infection; this difference grew in magnitude at 48 hours post-infection.

Bactofection of cells with engineered immunostimulatory bacteria encoding *pld* also resulted in higher luminescence levels than corresponding infections without *pld*, when the NanoLuc® was placed under the control of the EF-1α promoter. This effect was lower than what was observed in the groups with the CMV promoter, indicating that some *pld* expression is enhanced by leakiness from the CMV promoter.

**Comparison of Luciferase Expression Levels in Cell Supernatants of Human Macrophages Infected with Bacterial Strains Containing Plasmids Encoding Pld, vs. those not Encoding Pld**

| Plasmid Delivered by the Bacteria | Average Luminescence t=0 (RLUs) | SD t=0 (RLUs) | Average Luminescence 24 hpi (RLUs) | SD 24 hpi (RLUs) | Average Luminesence 48 hpi (RLUs) | SD 48 hpi (RLUs) |
|---|---|---|---|---|---|---|
| Uninfected | 122.9 | ± 47.7 | 177.9 | ± 25.8 | 157.6 | ± 18.2 |
| CMV-NanoLuc® | 2057.0 | ± 517.2 | 2291.2 | ± 233.9 | 3443.9 | ± 124.4 |
| CMV-NanoLuc® + *pld* | 1412.6 | ± 73.6 | 8155.0 | ± 2156.2 | 12054.8 | ± 3112.9 |
| EF1α-NanoLuc® | 154.6 | ± 20.8 | 148.6 | ± 24.3 | 132.5 | ± 5.2 |
| EF1α-NanoLuc® + *pld* | 165.6 | ± 7.5 | 223.4 | ± 18.3 | 256.0 | ± 24.7 |

hpi = hours post-infection; SD = standard deviation; RLUs = relative light units.

The results also showed that the number of CFUs of the CMV-NanoLuc® + *pld*-encoding strain, that remained inside the cells at 24 and 48 hours post-infection (hpi), decreased at a greater rate than the number of CFUs of bacteria harboring the CMV-NanoLuc® luciferase plasmid alone, indicating a different fate for each strain within the same cells.

**CFUs Remaining Inside the Cells at Different Time Points**

| Strain | CFUs, 0 Hours Post-Infection | CFUs, 24 Hours Post-Infection | CFUs, 48 Hours Post-Infection |
|---|---|---|---|
| CMV-NanoLuc® | $5.4 \times 10^7$ | $1.5 \times 10^5$ | $4.2 \times 10^3$ |
| CMV-NanoLuc® + *pld* | $8.0 \times 10^7$ | $1.6 \times 10^4$ | $6.4 \times 10^2$ |
| EF1α-NanoLuc® | $7.8 \times 10^7$ | $5.5 \times 10^4$ | $2.5 \times 10^3$ |
| EF1α-NanoLuc® + *pld* | $6.8 \times 10^7$ | $2.6 \times 10^5$ | $2.3 \times 10^3$ |

To determine if encoding Phospholipase D in the bacterial plasmid enhances the delivery of immunomodulatory payloads encoded on the same plasmid, RAW-Dual™ TLR4-KO cells (which express STING; InvivoGen) were infected with bacterial strains containing plasmids with and without nucleic acid encoding *pld*. RAW-Dual™ TLR4-KO cells encode a Lucia™ luciferase reporter under the control of an Interferon-Stimulated Response Element (ISRE). The cells were cultured in DMEM + 10% FBS, seeded into wells, and allowed to adhere overnight. Bactofections were performed as described above, using strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, containing plasmids encoding huIL-15Rα-

IL-15sc and huSTING N154S/R284G tazCTT, with or without nucleic acid encoding *pld* under control of the ssaG promoter. Uninfected cells, and uninfected cells treated with 5 μg/ml of the STING agonist 3'5' RpRp c-di-AMP, were used as negative and positive controls, respectively. At 48 hours post-infection, the cell supernatants were harvested, and the levels of expressed Lucia™ luciferase were measured using a luciferase assay (Promega).

The results, which are shown in the table below, show that, when *pld* was expressed during infection, from a plasmid also encoding human IL-15Rα-IL-15sc and the STING mutant polypeptide, the ISRE activity (as measured by Lucia™ luciferase expression) was higher than in the absence of *pld* expression. The ISRE activity is a measure of the STING-induced type I IFN activity, and thus, is indicative of the delivery of the plasmids encoding the STING variant, and of expression of the STING variant in the cells. Expression of phospholipase D (pld), thus, enhances the delivery of plasmids that also encode immunomodulatory payloads.

**Effects of *pld* Expression on the Delivery of Plasmids Encoding Immunomodulatory Payload Combinations by Immunostimulatory Bacteria**

| Plasmid Construct | Average Luminescence (RLUs) | STDEV |
|---|---|---|
| Uninfected cells | 63.6 | ± 12.1 |
| Uninfected cells + 5 μg/ml 3'5' RpRp c-di-AMP | 1052.6 | ± 32.3 |
| VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT | 80.5 | ± 20.9 |
| VCIP huIL-15Rα-IL-15sc T2A huSTING N154S/R284G tazCTT (P-ssaG-*pld*) | 108.3 | ± 4.5 |

STDEV = standard deviation

## Example 29

## The Combination of Murine IL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT, and the Combination thereof with Murine Anti-CTLA-4 scFv-Fc, Induces the Secretion of CXCL10 from Myeloid Cells and Induces the Activation of T-Cells

The impact of the expression of the immunomodulatory payloads, and their combinations, on the activation and function of T-cells and myeloid cells, was evaluated. This example describes and demonstrates the impact of the delivery of various immunomodulatory payload combinations, by the immunostimulatory bacteria provided herein, on the activation of antigen-specific T-cells (in terms of

CD25 expression and IFN-γ secretion), and on the secretion of CXCL10, a key chemokine involved in the recruitment of anti-tumor T-cells, by myeloid cells. The levels of secreted cytokines, such as IFN-γ, IFN-β, and CXCL10 (IP-10), were measured as correlates of protective anti-tumor immunity, and CD25 cell surface expression was monitored as a marker of T-cell activation. This was assessed by transfecting mouse bone-marrow derived dendritic cells (BMDCs) with plasmids encoding various combinations of payloads, co-culturing the transfected dendritic cells with autologous mouse T-cells, and then identifying the resulting cytokines.

The plasmids encoding the immunomodulatory payloads/proteins included those encoding single payloads, as well as those encoding combinations of payloads. For example, as shown in the table below, encoded payloads included murine (mu) IL-15Rα-IL-15sc (also referred to as IL-15/IL-15Rα complex, as IL-15/IL-15Rα chain complex, IL-15/IL-15R alpha chain complex, IL-15 complex, and IL-15cplex); a murine anti-CTLA-4 scFv-Fc (clone 9D9); and huSTING N154S/R284G tazCTT (a chimeric protein containing human STING with the GOF mutations N154S and R284G, and a replacement of the C-terminal tail (CTT) of human STING with the CTT of Tasmanian devil STING); and combinations thereof.

Combinations of payloads included two or three payloads, expressed on a single plasmid using T2A and/or P2A peptides, such that the proteins are encoded under the control of the same promoter. The combinations of payloads included: 1) murine IL-15Rα-IL-15sc and human STING N154S/R284G tazCTT; and 2) murine anti-CTLA-4 scFv-Fc, murine IL-15Rα-IL-15sc, and human STING N154S/R284G tazCTT.

Bone marrow-derived dendritic cells (BMDCs) were differentiated from Goldenticket mice, which are STING deficient, and the cells were transfected with plasmids encoding various combinations of the investigated payloads. Twenty-four hours post-transfection, cell supernatants were harvested, and the levels of secreted CXCL10 were measured in BMDC culture supernatants, using a U-Plex assay platform from Meso Scale Discovery, according to the manufacturer's protocol.

To measure $CD8^+$ T-cell activation, transfected BMDCs were pulsed with chicken ovalbumin (OVA) SIINFEKL ($OVA_{257-264}$) peptide, a major histocompatibility complex (MHC) class I (H-2Kb)-restricted peptide epitope

recognized by $CD8^+$ T-cells. Splenic T-cells, isolated from $Rag1^{-/-}$ OT-I mice, which express T-cell receptors (TCRs) that are specific for SIINFEKL presented by the MHC class I molecule H-2Kb, were added to the BMDCs for co-culture. After 24 hours of BMDC/T-cell co-culture, supernatants were harvested, and the levels of secreted IFN-γ were measured using a U-Plex assay platform from Meso Scale Discovery, according to the manufacturer's protocol.

The co-cultured cells also were harvested, and $CD8^+$ T-cells were stained with a phycoerythrin (PE)-conjugated murine anti-CD25 antibody (clone PC61, BioLegend), to determine the expression levels of the CD25 T-cell activation marker.

The results are summarized in the table below, which shows the levels of CXCL10 secreted by BMDCs in response to transfection with plasmids encoding various single and combination payloads, as well as the levels of IFN-γ secreted by $CD8^+$ T-cells, and the levels of T-cell activation (in terms of CD25 expression), following co-culture with the transfected BMDCs.

The results show that huSTING N154S/R284G tazCTT alone induces very high levels of CXCL10 secretion by BMDCs. Combinations of murine IL-15Rα-IL-15sc with huSTING N154S/R284G tazCTT, or of murine anti-CTLA-4 scFv-Fc + murine IL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT, also induce high levels of CXCL10 secretion by BMDCs.

The results also show exemplary combinations of encoded payloads that induce high levels of secretion of IFN-γ by human T-cells, and induce the activation (CD25 expression) of T-cells. An increased effect was observed with the combination of muIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT. The combination of muIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT also results in increased activation of $CD8^+$ T-cell responses, and the expression of CD25. These results indicate that the delivery of the combination of IL-15Rα-IL-15sc and the modified STING chimera with a gain-of-function mutation or mutations and with a CTT replacement to reduce NF-κB signaling, to the tumor microenvironment, increases advantageous anti-tumor immune responses, and reduces undesirable inflammatory responses.

| BMDC Treatments (Transfections) | CXCL10 Secreted by BMDCs (pg/ml) | | IFN-γ Secreted by $CD8^+$ T-Cells (pg/ml) | | % CD25 Expression on $CD8^+$ T-Cells | |
|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM |
| Untransfected control | 11 | 0 | 1102 | 70 | 17 | 3 |
| Beta-actin (plasmid control) | 159 | 18 | 1391 | 27 | 26 | 1 |
| muIL-15Rα-IL-15sc | 286 | 43 | 1705 | 139 | 43 | 1 |
| muAnti-CTLA-4 scFv-Fc | 627 | 9 | 2182 | 87 | 41 | 7 |
| huSTING N154S/R284G tazCTT | 3612 | 62 | 2731 | 121 | 54 | 1 |
| muIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT | 2340 | 190 | 2973 | 246 | 70 | 21 |
| muAnti-CTLA-4 scFv-Fc + muIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT | 1860 | 96 | 3098 | 165 | 51 | 2 |

SEM = Standard Error of the Mean

Delivery of this combination of encoded payloads, by the immunostimulatory bacteria provided herein, or by other delivery vehicles, such as oncolytic viruses or vectors, for expression of the payloads in tumor-resident myeloid cells and/or in the tumor microenvironment, provides for increased anti-tumor responses in the treated subject. As also shown in Examples below, the combination of a cytokine and a modified STING protein with a gain-of-function mutation, including the chimeras, results in additional advantageous anti-tumor responses in a treated subject.

## Example 30

## Human Recombinant IL-15, in Combination with a Small Molecule STING Agonist, Induces the Secretion of CXCL10 from Myeloid Cells, and Induces the Activation of T-Cells

This Example shows the impact of the expression of human immunomodulatory payloads, and their combinations, on the activation and function of T-cells and myeloid cells. The levels of secreted cytokines, such as IFN-γ and CXCL10 (IP-10), were measured as indicators of protective anti-tumor immunity. This Example describes and demonstrates the impact of the recombinant monomeric human IL-15 cytokine and 2'3'-c-di-AM(PS)2 (Rp, Rp), a small molecule STING (smSTING) agonist, on the secretion of CXCL10 by myeloid cells, a chemokine that is involved in the recruitment of anti-tumor T-cells, and on the secretion of IFN-γ by antigen-specific T-cells. The smSTING agonist is a cyclic dinucleotide (CDN) known

to induce the production of type I interferons (IFNs) following its recognition by endoplasmic reticulum-resident STING. 2'3'-c-di-AM(PS)2 (Rp, Rp) is the Rp, Rp-isomer of the 2'3'-bisphosphorothioate analog of 3'3'-cyclic adenosine monophosphate (c-di-AMP). It has a higher affinity for STING than c-di-AMP, due to the presence of a 2'-5', 3'-5' mixed linkage. This analog contains two phosphorothioate diester linkages to protect it against degradation by phosphodiesterases that are present in host cells or in the systemic circulation. 2'3'-c-di-AM(PS)2 (Rp, Rp) strongly induces type I IFN and CXCL10 production in relevant cells.

Human monocyte-derived dendritic cells (ModDCs) and autologous human T-cells were co-cultured with the small molecule (smSTING) agonist and the recombinant IL-15, and the resulting secreted cytokines were identified. The human monocyte-derived dendritic cells (ModDCs) were differentiated from negatively isolated monocytes, using the ImmunoCult™ Dendritic Cell Culture Kit (STEMCELL Technologies), for 6 days, following the manufacturer's instructions. After 6 days of differentiation, ModDCs were pulsed with a pool of 32 MHC class I-restricted viral peptides from human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza virus (Flu) (extended CEF peptide pool for human CD8 T cells, MABTECH), before the addition of autologous $CD8^+$ T-cells, and treatment with 2.5 nM of recombinant human IL-15 and/or 5 µg/ml of smSTING. Pulsing the dendritic cells with the viral peptides causes the dendritic cells to present the viral antigens on their cell surfaces, and stimulates antigen-specific T-cells to produce IFN-gamma. After 48 hours of ModDC/T-cell co-culture, the levels of secreted IFN-γ and CXCL10 were measured in the cell culture supernatants, using a U-Plex assay platform from Meso Scale Discovery, according to the manufacturer's protocol.

The results, which are summarized in the table below, show the additive effect of STING activation and human IL-15 activity on the secretion of CXCL10 by human dendritic cells upon antigen-specific stimulation. The results also show that the combination of STING activation and a cytokine, such as human IL-15, synergistically activates antigen-specific $CD8^+$ T-cells, and induces high levels of IFN-γ secretion by the activated $CD8^+$ T-cells.

| Treatments | CXCL10 Secreted by ModDCs (pg/ml) | IFN-γ Secreted by $CD8^+$ T-Cells (pg/ml) |
|---|---|---|

| | Mean | SEM | Mean | SEM |
|---|---|---|---|---|
| Media (control) | 21337 | 1015 | 685 | 40 |
| smSTING (CDN) | 78407 | 3869 | 5185 | 654 |
| Recombinant human IL-15 | 38671 | 1052 | 35311 | 29713 |
| Recombinant human IL-15 + smSTING (CDN) | 100673 | 3229 | 268328 | 17505 |

SEM = Standard Error of the Mean

## Example 31

## Combinations of Gain-of-Function STING Variants and Cytokines Enhance the Anti-Tumor Immune Response

### The Combination of Human IL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT Induces the Secretion of IFN-β from Myeloid Cells, and Induces the Activation of T-Cells

This Example demonstrates the impact of the encoded human immunomodulatory payloads, and their combinations, that are delivered for expression in the TME and/or in tumor-resident myeloid cells, on the activation and function of human T-cells and dendritic cells. The levels of cytokines secreted by the dendritic cells and the T-cells, such as IFN-γ and IFN-β, respectively, were measured as correlates of protective anti-tumor immunity. This example describes and demonstrates the impact of the delivery of various immunomodulatory payload combinations to the tumor microenvironment, such as by the immunostimulatory bacteria exemplified herein, and/or by other delivery vehicles described herein, on the activation of antigen-specific T-cells (as demonstrated by IFN-γ secretion), and on the secretion of IFN-β, a key factor involved in the anti-tumor immune response. This was achieved by transfecting human monocyte-derived dendritic cells (ModDCs) with plasmids encoding various combinations of payloads, co-culturing the transfected dendritic cells with autologous human T-cells, and identifying the cytokines secreted as a result. The plasmids encoding the immunomodulatory payloads/proteins included those encoding single payloads, and those encoding combinations of payloads. For example, as shown in the table below, the encoded payloads included huIL-15Rα-IL-15sc alone, huSTING N154S/R284G tazCTT alone, and the combination of huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT.

Human monocyte-derived dendritic cells (ModDCs) were differentiated from negatively isolated monocytes using the ImmunoCult™ Dendritic Cell Culture Kit (STEMCELL Technologies) for 6 days, following the manufacturer's instructions. After 6 days of differentiation, ModDCs were transfected with plasmids encoding the various investigated payloads and combinations thereof, using the Viromer® RED mRNA and plasmid DNA transfection reagent (Origene). Four hours post-transfection, the ModDCs were pulsed with a pool of 32 MHC class I-restricted viral peptides from human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza virus (Flu) (extended CEF peptide pool for human CD8 T cells, MABTECH), before the addition of autologous $CD8^+$ T-cells to the cell culture. The transfected dendritic cells also were pulsed with an irrelevant peptide from the Human Immunodeficiency virus 1 (HIV-1) reverse transcriptase, and used as a negative control for the peptide stimulation of T-cells. $CD8^+$ T-cells from HIV-1 negative donors were used. After 48 hours of ModDC/T-cell co-culture, the levels of IFN-γ secreted by the T-cells were measured in the cell culture supernatants, using a U-Plex assay platform from Meso Scale Discovery, according to the manufacturer's protocol.

In a separate experiment, ModDCs were transfected with plasmids encoding the various investigated payloads and combinations thereof, using the Viromer® RED mRNA and plasmid DNA transfection reagent (Origene), and cultured for forty-eight hours. Cell culture supernatants from the transfected ModDCs were harvested forty-eight hours post-transfection, and the levels of secreted IFN-β were measured using a U-Plex assay platform from Meso Scale Discovery, according to the manufacturer's protocol. In order to analyze the impact of the encoded payloads on the secretion of IFN-β by the human dendritic cells, the concentration of IFN-β measured for the plasmid control group (Beta-actin) was subtracted from all the other groups in the study, providing the net IFN-β concentrations resulting only from the activities of the encoded payloads.

The results, which are summarized in the table below, show the synergistic effect of the combination of huIL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT activities on the secretion of IFN-β by human dendritic cells. The results also show the combined effects of huIL-15Rα-IL-15sc and huSTING N154S/R284G

tazCTT, which are at least additive, on the activation of antigen-specific $CD8^+$ T-cells, and the induction of high levels of secreted IFN-γ from the activated T-cells.

| ModDC Treatments (Transfections) | Net IFN-β Secreted by ModDCs (pg/ml) | IFN-γ Secreted by $CD8^+$ T-Cells (pg/ml) | | | |
|---|---|---|---|---|---|
| | Mean | HIV Peptides | | CEF Peptides | |
| | | Mean | SEM | Mean | SEM |
| Untransfected control | 0 | 205 | 62 | 1661 | 169 |
| Beta-actin (plasmid control) | 0 | 7639 | 2422 | 18252 | 619 |
| huSTING N154S/R284G tazCTT | 647 | 11746 | 825 | 31452 | 3280 |
| huIL-15Rα-IL-15sc | 226 | 10670 | 2397 | 39245 | 6439 |
| huIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT | 1281 | 16953 | 3595 | 42942 | 8083 |

SEM = Standard Error of the Mean

It is shown in the next Example, that the combined effects of the encoded payloads lead to synergistic results, as evidenced by the cures observed in mouse models of cancer.

## Example 32

## Combinations of muIL-15Rα-IL15sc, huSTING N154S/R284G tazCTT, and Murine Anti-CTLA-4 scFv-Fc, Demonstrate Superior Anti-Tumor Efficacy in a Highly Refractory Mouse Model of Triple Negative Breast Cancer

The synergistic effects observed in the previous Example (Example 31), are further shown in this Example, which shows that the combinations of payloads act synergistically in effecting cures. Various payload combinations were evaluated for *in vivo* efficacy in an orthotopic, T-cell excluded, and metastatic model of CPI (immune checkpoint inhibitor) refractory triple-negative breast cancer. For this experiment, 6-8 week-old female BALB/c mice (8 mice per group) were inoculated in the left mammary fat pad with EMT6 tumor cells (ATCC® CRL-2755™) ($1x10^6$ cells in 100 µL PBS). Mice bearing 7 day-old established mammary tumors (~65 $mm^3$ in volume) were intravenously (IV) injected with a single dose of $1 x10^7$ CFUs of strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* containing plasmids encoding the various payloads, alone, or in combination with weekly intraperitoneal (IP) injections of 100 µg of the anti-PD-L1 antibody atezolizumab. The YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strains contained plasmids encoding muIL-

15Rα-IL15sc + huSTING N154S/R284G tazCTT, or muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT + muAnti-CTLA-4 scFv-Fc, or the control plasmid encoding β-actin, and were compared to treatment with PBS control.

The results are summarized in the table below. The tumors in the PBS-treated mice grew evenly, reaching a max tumor volume at day 31. Treatment of mice with the control β-actin plasmid did not demonstrate much evidence of anti-tumor efficacy (5% tumor growth inhibition (TGI), 2/8 cures), nor did treatment with IP anti-PD-L1 alone (17.9% TGI, 1/8 cures). Mice that were IV treated with the bacterial strain containing a plasmid encoding muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT demonstrated monotherapy efficacy (59.8% TGI, 3/8 cures), which improved with the combination of IP anti-PD-L1 (77.3% TGI, 5/8 cures). The combination of muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT + muAnti-CTLA-4 scFv-Fc also demonstrated significant monotherapy efficacy (62.4% TGI, 4/8 cures).

The therapeutic payload combinations were very well tolerated, and mice did not lose weight during the study. These data demonstrate the *in vivo* potency of IV administered immunostimulatory bacteria encoding the combinations of muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT, alone, or enhanced with IP administered anti-PD-L1, and muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT + muAnti-CTLA-4 scFv-Fc, in an orthotopic, T-cell excluded, and metastatic model of checkpoint inhibitor refractory triple-negative breast cancer (TNBC).

**Effects of Combinations of Encoded Payloads, Delivered by Immunostimulatory Bacteria, with and without addition of IP Anti-PD-L1, on the Inhibition of Tumor Growth and on the Cure Rates of Mice with Checkpoint Inhibitor Refractory Triple-Negative Breast Cancer**

| Encoded Payload(s) and Anti-PD-L1 Treatments | Tumor Growth Inhibition (TGI) | Cures |
|---|---|---|
| Beta-actin (plasmid control) | 5 % | 2/8 |
| Intraperitoneal (IP) Anti-PD-L1 alone | 17.9% | 1/8 |
| muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT | 59.8% | 3/8 |
| muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT + IP Anti-PD-L1 | 77.3% | 5/8 |
| muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT + muAnti-CTLA-4 scFv-Fc | 62.4% | 4/8 |

A follow-up study was performed to evaluate the efficacy of higher doses of the muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT combination therapy, and

for comparison with the efficacies of individually administered muIL-15Rα-IL15sc and huSTING N154S/R284G tazCTT. Strains of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*, containing plasmids encoding muIL-15Rα-IL15sc, huSTING N154S/R284G tazCTT, or the combination thereof, were administered intravenously (IV), at a dose of 3e7 CFUs, to mice bearing 7 day-old established mammary tumors, and were compared to PBS control. The tumors in the PBS-treated mice grew evenly, reaching a maximum tumor volume at day 31. As shown in the table below, mice treated with the bacterial strain containing a plasmid encoding muIL-15Rα-IL15sc or huSTING N154S/R284G tazCTT alone demonstrated 2/10 cures each, whereas the combination of muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT resulted in 7/10 cures.

| **Encoded Payload(s)** | **Cures** |
|---|---|
| PBS Control | 0/10 cures |
| muIL-15Rα-IL15sc | 2/10 cures |
| huSTING N154S/R284G tazCTT | 2/10 cures |
| muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT | 7/10 cures |

These data show the synergistic potency of combinations of a cytokine and a modified STING protein, such as the combination of muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT, for promoting complete responses. It is particularly significant, since these results were achieved in a highly refractory model of breast cancer, which highlights the general anti-tumor therapeutic potency of the combination. These results indicate the synergistic anti-tumor potency of the combination of a cytokine, such as the IL-15Rα-IL15sc fusion protein, and a STING protein, particularly a highly active STING protein, such as a gain-of-function constitutively active STING variant, or a gain-of-function constitutively active STING variant that is further modified to have reduced NF-κB signaling.

Cured mice from the muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT combination treatment groups (described above) then were evaluated for the ability to promote durable anti-tumor immunity in an orthotopic EMT6 tumor re-challenge study, and in a $CD8^+$ T-cell dependent manner. For this study, 20 cured mice were divided into two groups of 10 mice each, and each mouse received IP injections of either 100 μg of an anti-CD8β antibody (which does not deplete $CD8\alpha^+$ dendritic

cells), or 100 μg of an IgG isotype control, on days 3 and 1 prior to tumor re-challenge (day 56 and day 58 post-initial tumor implantation, respectively). The mice were bled prior to tumor re-challenge to confirm $CD8^+$ T-cell depletion, and average circulating $CD8^+$ T-cells were determined to be 5.72% for the isotype control, and 0.48% for the anti-CD8β antibody. The mice were then re-challenged with 1e6 EMT6 tumor cells, orthotopically on the opposite mammary fat pad, and compared to naïve, age-matched control mice (N=10). Mice from the naïve group all grew tumors to maximum tumor volume by day 30. Re-challenged mice from the anti-CD8β antibody-depleted group grew tumors even more aggressively than the naïve mice, whereas all 10 re-challenged mice from the IgG isotype antibody control group (*i.e.*, mice without $CD8^+$ T-cell depletion) were protected from tumor re-challenge. These data demonstrate the significant and durable anti-tumor efficacy of the combination of a cytokine and a modified gain-of-function constitutively active STING protein variant, such as when delivered in an immunostimulatory bacterium, such as a strain of YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* bacteria, comprising plasmids encoding a combination of muIL-15Rα-IL15sc + huSTING N154S/R284G tazCTT. The combination of therapeutic payloads/proteins induces a high cure rate after IV administration of the bacteria, and mice are protected from tumor re-challenge in a $CD8^+$ T-cell dependent manner.

**Example 33**

**DLL3xCD3 T-Cell Redirection Antibody (TCRA) Design**

T-cell redirection antibodies (TCRAs), or T-cell redirecting bispecific antibodies (TRBAs), are T-cell receptor (TCR) function-independent T-cell based immunotherapies, where the epsilon (ε) domain of cluster of differentiation 3 (CD3), a component of the TCR complex, is targeted by one binding domain, while a second binding domain targets a tumor cell surface antigen. TCRAs include, for example, bispecific T-cell engagers (sold under the trademark BiTEs®), which are engineered antibody-based immunotherapies, that are fusion proteins of two single-chain variable fragments (scFvs), linked together by a peptide linker.

TCRAs induce the formation of a cytolytic synapse between the T-cell and the tumor cell, in an MHC-independent and TCR function-independent manner, by simultaneously binding to a tumor cell surface antigen, and to CD3 on T-cells,

directing the cytolytic activity of T-cells selectively to the targeted tumor cells. Following the formation of the cytolytic synapse, the T-cells release cytotoxic proteins, such as perforin and granzymes (*e.g.*, granzyme B), resulting in the apoptosis of the tumor cells. The activation of the T-cells results in the release of cytokines, engaging other immune cells and inducing a broader anti-tumor immune response. This results in the conversion of a non-inflamed (or cold) tumor environment to an inflamed (or hot) one, and results in the infiltration and proliferation of T-cells, and the killing of tumor cells. The original BiTE® antibodies have a short half-life; later generation BiTE® antibodies have an extended half-life by virtue of fusion to an Fc (see, *e.g.*, Hipp *et al.* (2020) *Clin. Cancer Res.* 26:5258-5268; and Strohl, W. R. and Naso, M. (2019) *Antibodies* 8:41).

Delta-like ligand 3 (DLL3) is an inhibitory Notch pathway ligand that plays a role in Notch signaling during embryonal development. DLL3 is expressed intracellularly during embryonal development in normal tissues, with the highest expression in the fetal brain, but is absent in adult normal tissues. DLL3 is overexpressed in certain tumors, such as small cell lung cancers (SCLCs) and other high-grade neuroendocrine tumors, where, instead of being expressed intracellularly, it escapes to the cell surface, making it amenable to targeting with antibody-based therapeutics (see, *e.g.*, Hipp *et al.* (2020) *Clin. Cancer Res.* 26:5258-5268). DLL3 also is expressed in other tumor types of neuroendocrine origin, including melanoma, glioblastoma multiforme, small cell bladder cancer, metastatic castration-resistant prostate cancer, and neuroendocrine lung tumors (see, *e.g.*, Owen *et al.* (2019) *Journal of Hematology & Oncology* 12:61); hence, it is a useful target for TCRA-based therapies.

A DLL3xCD3 TCRA (see, SEQ ID NOs: 410 and 411 for the nucleotide and amino acid sequences, respectively; see, also, Hipp *et al.* (2020) *Clin. Cancer Res.* 26:5258-5268) was prepared from the light chain and heavy chain variable regions ($V_L$ and $V_H$, respectively) of the anti-DLL3 clone hSC16.56 antibody (see, *e.g.*, International Application Publication No. WO 2013/126746), and the light chain and

heavy chain variable regions of the anti-CD3ε clone 145-2C11 antibody (available from BioLegend).

The construct included the mouse IgGκ leader/signal sequence from the pSecTag2 vector (corresponding to residues 1-21 of SEQ ID NO:411; see, *e.g.*, addgene.org) for secretion of the TCRA. The anti-DLL3 $V_H$ domain (corresponding to residues 22-139 of SEQ ID NO:411) was linked to the anti-DLL3 $V_L$ domain (corresponding to residues 155-261 of SEQ ID NO:411) via a 15 amino acid long $(Gly_4Ser)_3$ linker (corresponding to residues 140-154 of SEQ ID NO:411), forming an anti-DLL3 scFv. A 5 amino acid long $Gly_4Ser$ linker (corresponding to residues 262-266 of SEQ ID NO:411) was inserted between the anti-DLL3 scFv and the anti-CD3 scFv. The anti-CD3 scFv contains the anti-CD3 $V_H$ domain (corresponding to residues 267-382 of SEQ ID NO:411), linked, via a second $(Gly_4Ser)_3$ linker (corresponding to residues 383-397 of SEQ ID NO:411), to the anti-CD3 $V_L$ domain (corresponding to residues 398-504 of SEQ ID NO:411). This construct was designated DLL3HL x CD3HL, to indicate the order in which the heavy (H) and light (L) chain variable domains of the anti-DLL3 and anti-CD3 antibodies appear in the molecule. A FLAG tag (DYKDDDDK; SEQ ID NO:412) can be added to the C-terminal end of this construct for detection (for the FLAG tag-labeled DLL3xCD3 TCRA, designated DLL3HL x CD3HL-FLAG TCRA, see SEQ ID NO:413).

Another DLL3xCD3 TCRA construct, designated DLL3LH x CD3HL TCRA (see, SEQ ID NOs: 414 and 415 for the nucleotide and amino acid sequences, respectively), was constructed similarly to the DLL3HL x CD3HL construct, but, as the designation implies, the anti-DLL3 scFv contains the anti-DLL3 $V_L$ domain placed N-terminally to the anti-DLL3 $V_H$ domain. The anti-CD3 scFv portion of the TCRA, the GS linkers, and the leader sequence for secretion, all are as described above for the DLL3HL x CD3HL construct. A FLAG tag-labeled version of the DLL3LH x CD3HL TCRA construct, designated DLL3LH x CD3HL-FLAG TCRA (see, SEQ ID NOS: 416 and 417 for the nucleotide and amino acid sequences, respectively), also was constructed.

**Example 34**

**Evaluation of T-Cell Redirection Antibodies (TCRAs) For Target Binding, Conjugation, And Cytotoxicity**

Antibodies directed against CD (cluster of differentiation) antigens, such as CD19 and CD3, are anti-cancer targets. Expression of certain CD antigens is restricted to specific lineage lymphohematopoietic cells. Antibodies directed against lymphoid-specific antigens have been developed as therapeutics. CD19 is a useful target because it is expressed on B cells, it is not shed, it is uniformly expressed on all lymphoma cells, and it is absent from stem cells. CD3 is expressed on T-cells as part of the T-cell receptor (TCR) complex, which contains three chains: CD3ε, CD3δ, and CD3γ. The clustering of CD3 on T-cells, such as by immobilized anti-CD3 antibodies, results in T-cell activation that is similar to engagement of the T-cell receptor (TCR), but is independent of its clone typical specificity. A bispecific antibody, directed against each of the CD19 and the CD3 antigens, retargets T-cell cytotoxicity towards CD-19 positive cells (*e.g.*, lymphoma cells), in an MHC-independent manner, and without the need for T-cell pre-stimulation or co-stimulation. Thus, such a molecule is particularly useful in inducing durable T-cell mediated anti-tumor immunity, and as an anti-cancer therapeutic.

The FLAG-tag variant sequence of the anti-CD19 and anti-CD3 TCRA, designated MT103 (SEQ ID NO:418; see, also, expired U.S. Patent No. 7,112,324), was cloned into a vector designated pATI1.75 (described above). The MT103 TCRA construct contains a leader sequence (corresponding to residues 1-19 of SEQ ID NO:418), followed by a FLAG tag (DYKDDDDK; corresponding to residues 20-27 of SEQ ID NO:418), and an anti-CD19 scFv linked to an anti-CD3 scFv. The anti-CD19 scFv contains an anti-CD19 $V_L$ domain (corresponding to residues 28-138 of SEQ ID NO:418), linked to an anti-CD19 $V_H$ domain (corresponding to residues 154-277 of SEQ ID NO:418), via a $(GGGGS)_3$ linker (corresponding to residues 139-153 of SEQ ID NO:418). The anti-CD19 scFv is linked to the anti-CD3 scFv via a GGGGS linker (corresponding to residues 278-282 of SEQ ID NO:418). The anti-CD3 scFV contains an anti-CD3 $V_H$ domain (corresponding to residues 283-401 of SEQ ID NO:418), linked via a peptide linker (VEGGSGGSGGSGGSGGVD; corresponding to residues 402-419 of SEQ ID NO:418), to an anti-CD3 $V_L$ domain

(corresponding to residues 420-525 of SEQ ID NO:418). The construct also includes a His tag ($His_6$; corresponding to residues 526-531 of SEQ ID NO:418).

HEK293T STING Null Cells (293-Dual™ Null Cells; InvivoGen) were seeded in 6-well plates coated with poly-L-lysine at 1.5e6 cells per well, and incubated overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 3000 ng of the MT103 plasmid DNA was diluted in serum-free media, and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios, and the cells were transfected, with untransfected wells serving as negative controls (in duplicates). Cell culture supernatants from each sample were collected 48 hours post-transfection. The supernatant was either kept neat, or was concentrated using an Amicon® Ultra 4 mL centrifugal filter (Millipore Sigma).

To confirm the functionality of the expressed MT103 (CD19xCD3) TCRA, a flow cytometry experiment using Raji cells (purchased from ATCC) and Jurkat-Lucia™ NFAT cells (InvivoGen) was performed. The Raji cells are human lymphoblast cells that highly express CD19, and Jurkat cells are human T-lymphocytes that express CD3. 200,000 Raji cells, and 200,000 Jurkat cells, each were washed in PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. Then, 50 µL of either neat or concentrated cell culture supernatant, containing the expressed TCRA, was added to the cells, and incubated for 30 minutes at 37 °C in a 5% $CO_2$ incubator. The cells then were resuspended in PBS + 2% FBS, and stained with an APC-labeled anti-FLAG tag antibody (BioLegend). The APC-labeled anti-FLAG tag antibody is added to label any MT103 TCRA (which contains a FLAG tag) that binds to CD19 on the Raji cells, and/or CD3 on the Jurkat cells. After a 30 minute incubation, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes, and then were resuspended in PBS + 2% FBS with DAPI (dead/live stain). Flow cytometry was performed, to detect any binding of the CD19xCD3 MT103 TCRA to CD19 on the Raji cells, and/or binding of the TCRA to CD3 on the Jurkat cells. 50,000 events were acquired for each sample. Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.) and analyzed using the FlowJo™ software (Tree Star, Inc.).

As shown in the table below, the median fluorescence intensity (MFI) of APC detected from the Raji and the Jurkat cells that were incubated with either neat or

concentrated MT103-containing cell culture supernatant, is much higher than the MFI detected from the cells incubated with untransfected cell culture supernatant, indicating that the MT103 TCRA binds to the CD19- and CD3-expressing cells.

**Flow Cytometry Data for Cells Treated with the MT103 (CD19xCD3) TCRA**

| Sample | MFI of APC + Cells |
|---|---|
| Raji cells stained with anti-FLAG-APC antibody only | 1021 |
| Raji cells stained with untransfected supernatant | 1064 |
| Raji cells stained with neat MT103-containing supernatant | 6334 |
| Raji cells stained with concentrated MT103-containing supernatant | 14544 |
| Jurkat cells stained with anti-FLAG-APC antibody only | 1035 |
| Jurkat cells stained with untransfected supernatant | 996 |
| Jurkat cells stained with neat MT103-containing supernatant | 4062 |
| Jurkat cells stained with concentrated MT103-containing supernatant | 12538 |

Additionally, the CD19xCD3 MT103 TCRA construct was tested in a co-culture of Raji and Jurkat-Lucia™ NFAT cells. The Jurkat-Lucia™ NFAT reporter cell line contains an NFAT-inducible Lucia™ construct, where expression of Lucia™ luciferase is driven by an ISG54 minimal promoter, fused to six copies of the NFAT (nuclear factor of activated T-cells; a transcription factor) consensus transcriptional response element. The Jurkat-Lucia™ NFAT cells, thus, can be used to study NFAT activation by monitoring Lucia™ luciferase activity; the levels of Lucia™ luciferase can be measured in the cell culture supernatant with QUANTI-Luc™, a Lucia™ luciferase detection reagent. In this experiment, the binding of the MT103 TCRA to both the CD19-expressing Raji cells, and the CD3-expressing Jurkat cells, activates the Jurkat cells (T-cells), resulting in NFAT-inducible Lucia™ luciferase secretion, which can be measured to determine the level of activation of the Jurkat cells.

Jurkat cells were seeded at 5e4 cells per well in a 96-well tissue culture plate. Raji cells were seeded at either 1e5 or 5e4 cells per well, for an Effector:Target (Jurkat:Raji) ratio of 1:2 or 1:1, respectively. The co-cultured cells were incubated with and without the neat or concentrated MT103-containing cell culture supernatant. 10-fold, 100-fold, and 1000-fold dilutions of the neat or concentrated supernatant were prepared and incubated with the co-cultures. The luminescence (*i.e.*, secreted Lucia™ luciferase activity) resulting from the induced NFAT reporter was detected at 6 hours and 24 hours post-addition of the TCRA to the co-cultures.

The results are summarized in the table below, which shows that the activity of the MT103 TCRA results in increased luminescence from the Jurkat-Lucia™ NFAT reporter cell line, compared to the control samples without the addition of MT103, and to the control samples without the co-culture with Raji (target) cells. The luminescence resulting from the NFAT-inducible Lucia™ reporter construct decreases with the dilution of MT103, in a dose-dependent manner. A longer incubation time (24 hours vs. 6 hours) results in increased luminescence, as expected, which is indicative of increased T-cell activation.

**NFAT-Lucia™ Reporter Values from Co-Cultures of Jurkat and Raji Cells Treated with Different Concentrations of MT103, and with Different Effector:Target Ratios**

| **1:2 Effector:Target (Jurkat:Raji) Ratio, 6 Hour Incubation** | | | | | | |
|---|---|---|---|---|---|---|
| | **MT103 Neat** | | **MT103 Concentrated** | | **No MT103** | |
| | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| No dilution | 12110.5 | 837.9 | 3445.0 | 717.0 | 44.5 | 10.6 |
| 10-fold dilution | 4285.0 | 209.3 | 5880.0 | 581.2 | 70.0 | 15.6 |
| 100-fold dilution | 2253.5 | 507.0 | 3415.5 | 161.9 | 48.0 | 36.8 |
| 1000-fold dilution | 270.0 | 110.3 | 954.5 | 10.6 | 37.0 | 0.0 |
| No Raji (target) cells | 63.0 | 5.7 | 55.5 | 16.3 | 37.0 | 21.2 |
| **1:1 Effector:Target (Jurkat:Raji) Ratio, 6 Hour Incubation** | | | | | | |
| | **MT103 Neat** | | **MT103 concentrated** | | **No MT103** | |
| | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| No dilution | 4337.0 | 219.2 | 2057.5 | 20.5 | 26.0 | 15.6 |
| 10-fold dilution | 2113.0 | 36.8 | 2767.5 | 41.7 | 78.0 | 15.6 |
| 100-fold dilution | 965.5 | 26.2 | 1620.5 | 241.1 | 48.0 | 15.6 |
| 1000-fold dilution | 188.5 | 5.0 | 566.0 | 46.7 | 41.0 | 15.6 |
| No Raji (target) cells | 63.0 | 5.7 | 55.5 | 16.3 | 37.0 | 21.2 |
| **1:2 Effector:Target (Jurkat:Raji) Ratio, 24 Hour Incubation** | | | | | | |
| | **MT103 Neat** | | **MT103 concentrated** | | **No MT103** | |
| | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| No dilution | 47159.5 | 1038.7 | 11274.0 | 408.7 | 114.5 | 57.3 |
| 10-fold dilution | 12843.5 | 1047.2 | 13995.5 | 225.6 | 159.5 | 26.2 |
| 100-fold dilution | 5114.0 | 21.2 | 8767.5 | 204.4 | 189.0 | 36.8 |
| 1000-fold dilution | 562.5 | 94.0 | 1979.5 | 57.3 | 148.0 | 62.3 |
| No Raji (target) cells | 159.0 | 67.9 | 181.5 | 5.0 | 77.5 | 4.95 |
| **1:1 Effector:Target (Jurkat:Raji) Ratio, 24 Hour Incubation** | | | | | | |
| | **MT103 Neat** | | **MT103 concentrated** | | **No MT103** | |
| | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| No dilution | 18614.0 | 1540.1 | 6017.0 | 31.3 | 122.0 | 15.6 |
| 10-fold dilution | 7083.0 | 209.3 | 7479.0 | 717.0 | 181.5 | 26.2 |
| 100-fold dilution | 2908.5 | 41.7 | 5210.5 | 72.9 | 174.0 | 15.6 |
| 1000-fold dilution | 444.0 | 9.9 | 1213.5 | 94.0 | 163.0 | 52.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| No Raji (target) cells | 159.0 | 67.9 | 181.5 | 5.0 | 77.5 | 4.95 |

SD = standard deviation

A cytotoxicity assay was performed using a co-culture of human $CD8^+$ T-cells and Raji cells (Burkitt's lymphoma cells; *i.e.*, tumor cells). Human $CD8^+$ T-cells were isolated using the EasySep™ Human T-cell Isolation Kit (StemCell Technologies), and then were resuspended in RPMI + 2% FBS, and seeded in a 96-well plate. $CD8^+$ T-cells were seeded at 1e5 cells per well, or at 5e4 cells per well, and Raji cells were seeded at 10,000 cells per well, for Effector:Target (T-cell:Raji cell) ratios of 10:1 or 5:1, respectively. Concentrated or neat MT103-containing supernatant was diluted 10-fold, 100-fold, and 1000-fold, in RPMI + 2% FBS, and then 50 µL of each supernatant sample was added to the co-cultures. The supernatant was collected after 5 hours of co-culture incubation, and analyzed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), per the manufacturer's instructions. The % cytotoxicity was calculated according to the manufacturer's instructions

As shown in the table below, higher amounts (concentrations) of MT103 result in higher % cytotoxicity. The % cytotoxicity decreases as the MT103-containing supernatant is diluted 10-fold, 100-fold, and 1000-fold, in a dose dependent manner.

**Lysis of Raji Cells (% Cytotoxicity) in Co-Culture with $CD8^+$ T-Cells and Varying Concentrations of MT103**

| **5:1 Effector:Target ($CD8^+$ T-Cell:Raji Cell) Ratio, 5 Hour Incubation** | | | | | | |
|---|---|---|---|---|---|---|
| | **MT103 Neat** | | **MT103 Concentrated** | | **No MT103** | |
| | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| No dilution | NA | NA | NA | NA | 28.2 | 1.63 |
| 10-fold dilution | 59.0 | 9.6 | 78.8 | 7.5 | NA | NA |
| 100-fold dilution | 36.0 | 5.1 | 54.5 | 6.4 | NA | NA |
| 1000-fold dilution | 3.7 | 3.6 | 32.8 | 2.4 | NA | NA |
| **10:1 Effector:Target ($CD8^+$ T-Cell:Raji Cell) Ratio, 5 Hour Incubation** | | | | | | |
| | **MT103 Neat** | | **MT103 Concentrated** | | **No MT103** | |
| | **Mean** | **± SD** | **Mean** | **± SD** | **Mean** | **± SD** |
| No dilution | NA | NA | NA | NA | 18.0 | 1.0 |
| 10-fold dilution | 51.9 | 5.4 | 77.6 | 1.6 | NA | NA |
| 100-fold dilution | 38.5 | 2.6 | 51.9 | 2.2 | NA | NA |
| 1000-fold dilution | NA | NA | 16.8 | 2.2 | NA | NA |

SD = Standard deviation; NA = not applicable

## Example 35

## Immunostimulatory Bacterial Delivery of Tumor-Associated Antigens Encoded in Expression Plasmids

This Example describes exemplary tumor-associated antigens (TAAs) that can be delivered by the immunostimulatory bacteria. Upon treatment with the engineered immunostimulatory bacteria, the delivery of tumor-associated antigens to tumor-resident myeloid cells, such as macrophages and dendritic cells, allows for the priming and activation of antigen-specific T-cells to result in an anti-tumor response.

Among the tumor-associated antigens encoded by the plasmids in the immunostimulatory bacteria are those categorized as oncofetal or oncoviral. Some of the antigens are either overexpressed by, or they accumulate in tumors. The antigens include those that are lineage-restricted, mutated, or post-translationally altered. Non-limiting examples of tumor-associated antigens in each of these categories, and their associated cancer types (indications), are set forth in the following table.

| **Category of Tumor-Associated Antigen (TAA)** | **Name / Family Name of TAA** | **Cancer Histology** |
|---|---|---|
| ***Oncofetal*** | Carcinoembryonic antigen (CEA) | Colorectal carcinoma |
| | Immature laminin receptor | Renal cell carcinoma (RCC) |
| | Tumor-associated glycoprotein 72 (TAG-72) | Prostate carcinoma |
| ***Oncoviral*** | HPV E6, HPV E7 | Cervical carcinoma |
| ***Overexpressed/Accumulated*** | BING-4 | Melanoma |
| | Epidermal Growth Factor Receptor (EGFR) | Colorectal carcinoma (CRC) |
| | Wilms' Tumor Protein | Multi |
| | Calcium-activated chloride channel 2 | Lung carcinoma |
| | Cyclin-B1 | Multi |
| | 9D7 | RCC |
| | Epithelial cell adhesion molecule (EpCAM) | Breast carcinoma |
| | Ephrin type-A receptor 3 (EphA3) | Multi |
| | Human epidermal growth factor receptor 2 (HER2/Neu) | Multi |
| | Telomerase | Multi |
| | Mesothelin | Ductal pancreatic carcinoma |
| | Stomach-cancer–associated protein tyrosine phosphatase 1 (SAP-1) | Colorectal carcinoma (CRC) |
| | Survivin | Multi |

| | | |
|---|---|---|
| *Cancer-Testis* | BAGE (B melanoma antigen) family | Multi |
| | CAGE family | Multi |
| | GAGE family | Multi |
| | MAGE (melanoma antigen) family | Multi |
| | SAGE (sarcoma antigen) family | Multi |
| | PAGE family | Multi |
| | XAGE family | Multi |
| | CT9, CT10 | Multi |
| | New York esophageal squamous cell carcinoma 1 (NY-ESO-1), LAGE-1 | Multi |
| | Preferentially expressed antigen in melanoma (PRAME) | Multi |
| | synovial sarcoma/X breakpoint 2 (SSX-2) | Melanoma, Multi |
| *Lineage-Restricted* | Melanoma antigen recognized by T-cells 1 (Melan-A/MART-1) | Melanoma |
| | gp100/Pmel17 | Melanoma |
| | Tyrosinase | Melanoma |
| | Tyrosinase related protein (TRP)-1, TRP-2 | Melanoma |
| | P.polypeptide | Melanoma |
| | Melanocortin 1 receptor (MC1R) | Melanoma |
| | Prostate-specific antigen (PSA) | Prostate carcinoma |
| *Mutated* | β-catenin | Melanoma, Prostate carcinoma, Hepatocellular carcinoma (HCC) |
| | BRCA1, BRCA2 | Breast carcinoma, ovarian carcinoma |
| | Cyclin dependent kinase 4 (CDK4) | Multi |
| | Chronic Myelogenous Leukemia Tumor Antigen 66 (CML66) | Chronic myelogenous leukemia (CML) |
| | Fibronectin | Multi |
| | Melanoma antigen recognized by T-cells 2 (MART-2) | Melanoma |
| | p53 | Multi |
| | Ras | Multi |
| | TGF-β receptor type II (TGF-βRII) | Colorectal carcinoma |

| | | |
|---|---|---|
| ***Post-Translationally Altered*** | Mucin 1, Cell Surface Associated (MUC1) | Ductal carcinoma, RCC |
| ***Idiotypic*** | Immunoglobulin (Ig), T-cell Receptor (TCR) | B-cell leukemia, T-cell leukemia, lymphoma, myeloma |

**Example 36**

**Ectopic Protein Synthesis in Eukaryotic Cells from Bacterial-Derived RNA Delivery**

As described in the detailed description, in addition to plasmid DNA delivery, immunostimulatory bacterial can be used to directly deliver bacterial-derived RNA, such as mRNA, into infected myeloid cells. Direct delivery of RNA obviates the need for plasmid DNA delivery, which relies on the eukaryotic transcription and translation machinery, as well as proper trafficking of plasmid DNA to the host cell nucleus. Direct delivery of RNA, such as mRNA, bypasses host trafficking and transcriptional processes, to rely only on eukaryotic translation machinery for protein production. The delivery of bacterial-derived RNA requires the de-coupling of bacterial transcription from bacterial translation, in order to prevent problematic translation of foreign proteins in the bacteria. Many eukaryotic proteins are complex proteins that require proper folding conditions, an endoplasmic reticulum, proper post-translational modifications, including disulfide bonds, and host chaperones for folding. Over-expression of foreign proteins in bacteria can reduce strain fitness, decrease doubling time, and reduce strain stability.

To demonstrate that mRNA delivery occurs during treatment with the immunostimulatory bacteria, a series of plasmids were engineered with either eukaryotic or bacterial promoters driving the expression of mu4-1BBL_T2A_muIL-12p70 HPRE bGHpA, with the presence and absence of an intervening encephalo-myocarditis virus Internal Ribosome Entry Site (EMCV IRES). The EMCV IRES, which is non-functional in bacteria, reduces prokaryotic translation and enhances eukaryotic translation. There is only one known eukaryotic IRES, from the *Dicistroviridae* family of viruses, that functions in bacteria (see, *e.g.*, Colussi *et al.* (2015) *Nature* 519(7541):110-113). Thus, in general, IRES elements do not function in bacteria. The promoter-IRES (or promoter-Kozak) combinations also were cloned

into an mCherry fluorescent reporter gene (Takara Bio), so that bacterial expression from the regulatory sequences could be rapidly assessed by fluorescence.

To measure the bacterial expression of the fluorescent mCherry protein, overnight cultures of bacterial strains were washed in PBS, and normalized by $OD_{600}$ to 0.1. For every bacterial culture, the percentage of mCherry expression was analyzed by flow cytometry using the NovoCyte® Flow Cytometer (ACEA Biosciences, Inc.). The level of ectopic gene expression after bactofection, using a co-culture system of infected primary human M2 macrophages and HEK-Blue™ IL-12 reporter cells (InvivoGen), was measured. HEK-Blue™ IL-12 cells are HEK293 cells that express the IL-12 receptor, as well as genes in the IL-12 signaling pathway, and a STAT4-inducible SEAP reporter gene. The binding of IL-12 to the IL-12 receptor on the surface of HEK-Blue™ IL-12 cells triggers a signaling cascade leading to the activation of STAT-4, with the subsequent production of SEAP. Detection of SEAP in the supernatant of HEK-Blue™ IL-12 cells can be readily assessed using QUANTI-Blue™ Solution. Thus, these reporter cells are used to measure the expression of IL-12.

Primary human monocytes were differentiated in ImmunoCult™-SF Macrophage Medium, containing 100 ng/ml M-CSF, for 3 days, and then supplemented with additional medium containing 200 ng/ml M-CSF, 20 ng/ml IL-4, and 20 ng/ml IL-10, for 4 more days. The cells were then infected with stationary phase immunostimulatory bacteria, which were grown overnight at 37 °C in 4XYT medium, at an MOI of 50. The cells were inoculated with bacteria and centrifuged for 5 minutes at 500 rcf, followed by an incubation at 37 °C for 1 hour. The cells were then washed twice with DPBS, and then incubated in fresh ImmunoCult™-SF Macrophage Medium, containing 100 µg/ml gentamicin, to remove extracellular bacteria. Infected macrophages were then co-cultured with the HEK-Blue™ IL-12 reporter cells, at a ratio of 2.5:1 (macrophages to HEK-Blue™ cells). At 48 hours post-infection, 5 µl of cell culture supernatants were incubated with 180 µl of QUANTI-Blue™ solution (which detects SEAP), and incubated at 37 °C, to allow for the color to develop. Thus, the expression of IL-12 by the infected macrophages is assessed by measuring the SEAP levels in the supernatants, which is produced by the HEK-Blue™ cells upon binding of IL-12 to the IL-12 receptor on their cell surfaces.

The results, which are summarized in the Table below, show that the infected control (EF-1α-NanoLuc® luciferase) stimulates a baseline level of IL-12 expression in primary human M2 macrophages, and very little fluorescence from mCherry expression in the bacteria. The CMV-Kozak pair drives a higher level of IL-12 expression after bactofection, as well as a higher level of mCherry protein expression. This is attributable to the low levels of bacterial "leakiness" that occur from the CMV promoter in bacteria. The two bacterial promoters that were tested, rpsM and MTL, when paired with a Kozak sequence, induce levels of IL-12 expression in primary human M2 macrophages that are lower than the CMV promoter, but higher than EF-1α-NanoLuciferase control, and both promoters drive a high level of mCherry protein expression in bacteria. These results indicate that mRNA delivery is occurring, because the bacterial promoters are non-functional in eukaryotic hosts. The levels of expression of the IL-12 reporter protein are higher when the bacterial promoters are coupled with an IRES, indicating that protein expression from delivered mRNA is enhanced at the translational level by the presence of a eukaryotic IRES. The presence of an IRES also reduces the amount of mCherry expression in the bacteria, indicating that the secondary structure of the RNA inhibits expression even when coupled with a bacterial promoter.

Including an IRES in the constructs, downstream from the promoter and before a start codon, inhibits or prevents translation in bacteria, so that the bacteria serve as delivery agents for mRNA. The bacteria, such as the immunostimulatory bacteria provided in this application, are designed to infect host cells, such as tissue-resident macrophages (*e.g.*, tumor-resident myeloid cells), and to deliver the encoded RNA into the host cells. The bacteria can auxotrophic, such as *asd*$^{-}$, so that they can be grown *in vitro*, but such that they do no grow when introduced into a host, such as a human. The bacteria deliver their contents to host cells, and do not reproduce.

| **Promoter Type** | **IRES** | **Promoter-IRES or Promoter-Kozak** | **Absorbance (650 nm) IL-12-SEAP** | **IL-12 Range** | **Fluorescence (mCherry Expression in Bacteria)** |
|---|---|---|---|---|---|
| Eukaryotic | No | EF-1α-Kozak-NanoLuc® | 0.4585 | 0.1042 | 0.00403 |
| Eukaryotic | No | CMV-Kozak | 0.60545 | 0.1315 | 9.43 |

| Promoter Type | IRES | Promoter-IRES or Promoter-Kozak | Absorbance (650 nm) IL-12-SEAP | IL-12 Range | Fluorescence (mCherry Expression in Bacteria) |
|---|---|---|---|---|---|
| Prokaryotic | No | rpsM-Kozak | 0.47785 | 0.0233 | 21.1 |
| Prokaryotic | Yes | rpsM-IRES | 0.5802 | 0.106 | 0.82 |
| Prokaryotic | No | MTL-Kozak | 0.55295 | 0.0757 | 85.8 |
| Prokaryotic | Yes | MTL-IRES | 0.75665 | 0.0801 | 4.19 |

Bacteria are cultured *in vitro* to produce encoded RNA, which, because of the presence of an IRES or other such regulatory sequence that prevents or inhibits bacterial translation, results in the production of RNA, such as mRNA by the bacteria. Upon infection of eukaryotic host cells, such as human macrophages, by the bacteria, the RNA is translated, producing the encoded product, such as, for example, an antigen.

Bacteria are advantageous vessels for RNA delivery, because bacteria readily can be cultured in large amounts, and then stored and/or formulated or provided as powder, a tablet, or a liquid for injection. They can be administered by any suitable route, including intravenously, and mucosally through the nose or lungs. They are very stable, and will provide large, stable quantities of RNA.

The immunostimulatory bacteria provided herein, when used for the delivery of RNA, such as mRNA, for example, when used as vaccines, or to encode viral antigens and/or other proteins to protect against pathogens, such as Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19), can encode the full length wild-type SARS-CoV-2 spike protein (see, *e.g.*, SEQ ID NO:438), or can encode the full length receptor binding domain (RBD) of the SARS-CoV-2 spike protein (see, SEQ ID NO:440), or can encode a portion of the spike protein RBD (see, *e.g.*, SEQ ID NO:441) that is sufficient to induce or cause an immune response, and/or to immunize or vaccinate or protect a subject against SARS-CoV-2. The immunostimulatory bacteria also can deliver mRNA encoding mutants of the SARS-CoV-2 spike protein, with mutations in the spike protein or a portion thereof, such as the RBD or a portion thereof, that increase or enhance the expression

of the spike protein or RBD or portions thereof, and/or that increase or enhance the binding of the spike protein or RBD, or the portions thereof to the ACE2 receptor.

The spike protein or spike protein RBD mutants include any described herein and known in the art, including, but not limited to, those comprising the mutations V367F, D614G, G476S, V483A, H49Y, N501Y, N501F, N501W, N501V, F817P, A892P, A899P, A942P, K986P, V987P, V417K, G502D, N501T, Q498Y, W436R and D364Y, and those comprising mutations at residues corresponding to residues N439/R426, L452/R426, T470/N457, E484/P470, Q498/Y484 and N501/T487 of the spike protein or portion thereof. The immunostimulatory bacteria can be used as vaccines against SARS-CoV-2 by delivering mRNA encoding any of the antigenic sequences or modified forms of the SARS-CoV-2 spike protein or RBD, including portions thereof, as described, for example, in U.S. Patent Nos. 10,973,908 and 10,702,600. The immunostimulatory bacteria also can be used as vaccines/for mRNA delivery, to deliver the mRNA uin the Pfizer-BioNTech COVID-19 vaccine, and in the Moderna COVID-19 vaccine, as described elsewhere herein. SARS-CoV-2 proteins for use as antigens, include, for example, spike protein, nucleocapsid and M protein and antigenic portions thereof. The immunostimulatory bacteria can be used to deliver universal flu antigens, such as flu vaccines that elicit antibodies against HA proteins from a variatey of flue strains, such as influenza type A strains (H1 and H3) and influenza type B strains. Other combinations include repeating patters of HA eptiopes, such as the FlusMos-v1. See, *e.g.*, Nachbaguer *et al.* (2021) *Nature Medicine 27*:106-114, which provides a chimeric hemagglutinin-based universal influenza vaccine.

**Example 37**

**Immunomodulatory Strains Containing Human IL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT Colonize Autochthonous Murine Tumors**

It previously has been reported that the attenuated *Salmonella* strain YS1646 is able to colonize tumors in the 4T1 murine breast transplant model (average tumor size 400 $mm^3$, average CFUs/g = $10^8$), but is significantly less able to colonize the spontaneous BALB-NeuT breast tumor model (average tumor size 400 $mm^3$, average CFUs/g = $10^3$; see, *e.g.*, Drees *et al.* (2015) *J. Cancer* 6(9):843-848).

In order to show that the immunostimulatory bacteria provided herein do not have a similar deficiency in colonizing spontaneous tumors compared to transplanted tumors, tumors were collected from the orthotopically-transplanted EMT6 mouse model of breast cancer, and compared to tumors collected from the spontaneous MMTV-PyMT breast cancer model. For the EMT6 model, 6-8 week-old female BALB/c mice (5 mice per group) were inoculated orthotopically in the 4$^{th}$ mammary fat pad with EMT6 cells (1x10$^6$ cells in 100 µL PBS). Mice bearing 7-day established flank tumors (average tumor size of 56 mm$^3$) were IV injected with a single dose of 3 x10$^7$ CFUs of the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain, containing a plasmid encoding the combination of huIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT. At day 4 post-IV dosing, mice were euthanized, and tumors were homogenized and plated on LB plates to enumerate the number of colony forming units (CFUs) per gram of tumor tissue. The YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain colonized tumors at a mean of 1.7 x10$^7$ CFUs per gram of tumor tissue.

For the spontaneous model of breast cancer, MMTV-PyMT mice were IV injected with 3 x10$^7$ CFUs of the same bacterial strain when their largest tumors measured an average of 272 mm$^3$ in volume. Tumors were collected at day 6 post-IV dosing. Tumors were homogenized and plated on LB plates to enumerate the number of CFUs per gram of tumor tissue. All tumors collected were found to be well colonized despite a range of tumor weights (0.05 g to 0.36 g, N=7), with a mean of 2.89 x 10$^6$ CFUs/g. These data demonstrate that, unlike the parental YS1646 strain, the immunomodulatory YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain, containing the plasmid encoding the combination of huIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT, was able to colonize spontaneous MMTV-PyMT tumors at a comparable level to the transplanted EMT6 tumors ($P$ = 0.18, NS).

Since, unlike the transplanted tumors, the spontaneous tumors have vasculature that is more comparable to human tumors, these data indicate that the immunostimulatory bacteria will colonize human tumors at a much higher rate/level than the parental YS1646 strain was reported to in a phase I clinical trial of advanced cancer patients (see, *e.g.*, Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152).

## Example 38

## Immunomodulatory Bacterial Strains, Containing Plasmids Encoding Human IL-15Rα-IL-15sc and huSTING N154S/R284G tazCTT, or a NanoLuciferase® Luciferase Control Plasmid, are Well Tolerated in Non-Human Primates

In a phase I human clinical trial, it was reported that the maximum tolerated dose (MTD) of the parental YS1646 strain is 3 x $10^8$ CFUs/$m^2$, and that the dose-limiting toxicity (DLT) dose was 1 x $10^9$ CFUs/$m^2$ (see, *e.g.*, Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152). At these doses, the toxicities and adverse events, such as fever, hypotension, thrombocytopenia, anemia, vomiting, diarrhea, nausea, and hypophosphatemia, were attributed to very high serum levels of pro-inflammatory cytokines, measured at 4 hours post-IV dosing, including TNF-α (approximately 500,000 pg/mL), IL-6 (approximately 500,000 pg/mL), and IL-1β (approximately 200 pg/mL). In a separate study, strain YS1646 was evaluated in a non-human primate (NHP) study using cynomolgus monkeys. In this study, 1 x $10^9$ CFUs/monkey was found to be the MTD (Human Equivalent Dose (HED) = 4 x $10^9$ CFUs/$m^2$), while a dose of 1 x $10^{10}$ CFUs/monkey was considered not tolerated (HED = 4 x $10^{10}$ CFUs/$m^2$). DLTs at the top dose were attributed to liver-associated adverse events (AEs), and serum cytokines were not measured (see, *e.g.*, Lee *et al.* (2000) *International Journal of Toxicology* 19:19–25). Since the NHPs tolerated strain YS1646 much better than the humans, with an MTD value of a log higher than the human MTD, it can be inferred that the cytokine levels of the monkeys would be approximately a log lower than those measured in humans.

To determine the MTD and serum cytokine profile of immunostimulatory bacteria provided herein, such as strains that lack flagella and that are *msbB*$^-$/*pagP*$^-$, in NHPs, a tolerability study was performed. For this study, 15 previously untreated male cynomolgus monkeys, with ages ranging from 24 to 50 months, were utilized. The NHPs were IV administered the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* strain (discussed above), containing a plasmid encoding the combination of huIL-15Rα-IL-15sc + huSTING N154S/R284G tazCTT, at doses of 3 x $10^8$ CFUs/monkey, 1 x $10^9$ CFUs/monkey, or 3 x $10^9$ CFUs/monkey (3 NHPs per dosing group), or were IV administered the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* strain, containing a plasmid encoding NanoLuciferase® luciferase, at a dose of 3 x $10^8$

CFUs/monkey (3 NHPs per group), and compared to the saline vehicle control group (N=3). The NHPs were bled prior to dosing, 4 hours post-dosing, and 24 hours post-dosing, and serum cytokine levels were measured using a monkey cytokine U-Plex panel (Meso Scale Discovery), according to the manufacturer's protocol.

The results, which are summarized in the tables below, show that the bacterial strains were well-tolerated at all dose levels tested, and no clinical findings were reported that differed significantly from the saline (PBS) control group. Thus, no MTD could be established in this study. Serum cytokine levels were very low overall, particularly for the cytokines that were attributed to dose-limiting toxicities (DLTs) in the human clinical trial with strain YS1646, such as the cytokines measured 4 hours post-IV dosing in the 3 x $10^9$ CFUs/monkey dose group (HED = 1.2 x $10^{10}$ CFUs/$m^2$), including TNF-α (mean concentration of 4.6 pg/mL), IL-6 (mean concentration of 376.9 pg/mL), and IL-1β (mean concentration of 0.88 pg/mL). Only the serum cytokine levels of IP-10/CXCL10 (mean concentration of 10,549.6 pg/mL) and MCP-1/CCL2 (mean concentration of 6247.7 pg/mL) were high in the top dose level group, 4 hours post-dosing, and remained elevated at 24 hours post-dosing. These analytes are not associated with toxicity, and can indicate a more favorable immune profile. In summary, the immunostimulatory bacteria provided herein, including those containing plasmids encoding payloads, are very well-tolerated in NHPs. It can thus be inferred, from these data, that there will be a high level of tolerability of the immunostimulatory bacterial strains in humans.

**Serum Cytokines Levels Prior to Dosing**

| | | **Immunostimulatory Bacterial Strain and Dose (CFUs/NHP)** | | | | |
|---|---|---|---|---|---|---|
| | | **PBS Control** | **YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/ F-Δ*purI* + p2.1 CMV VCIP huIL-15Rα-IL15sc_T2A_STING N154S/R284G tazCTT** | | | **YS1646Δ*asd*/Δ FLG/Δ*pagP*/Δ *ansB*/Δ*csgD* + NanoLuc® Plasmid** |
| | | - | **3 x $10^8$ CFUs/NHP** | **1 x $10^9$ CFUs/NHP** | **3 x $10^9$ CFUs/NHP** | **3 x $10^8$ CFUs/NHP** |
| **IFN-γ** | Mean (pg/ml) | 3.87 | 0.24 | 0.80 | 3.78 | 0.00 |
| | ± SD | 0.00 | 0.00 | 0.78 | 0.00 | 0.00 |
| **IL-10** | Mean (pg/ml) | 0.08 | 0.03 | 0.05 | 0.09 | 0.08 |
| | ± SD | 0.06 | 0.02 | 0.03 | 0.02 | 0.02 |
| **IL-12p70** | Mean (pg/ml) | 0.56 | 0.32 | 1.43 | 1.23 | 0.30 |

| | | Immunostimulatory Bacterial Strain and Dose (CFUs/NHP) | | | | |
|---|---|---|---|---|---|---|
| | | PBS Control | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* + p2.1 CMV VCIP huIL-15Rα-IL15sc_T2A_STING N154S/R284G tazCTT | | | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* + NanoLuc® Plasmid |
| | | - | 3 x $10^8$ CFUs/NHP | 1 x $10^9$ CFUs/NHP | 3 x $10^9$ CFUs/NHP | 3 x $10^8$ CFUs/NHP |
| | ± SD | 0.27 | 0.16 | 1.87 | 1.08 | 0.21 |
| **IL-1β** | Mean (pg/ml) | 0.08 | 0.05 | 0.70 | 0.09 | 0.26 |
| | ± SD | 0.00 | 0.05 | 1.15 | 0.12 | 0.00 |
| **IL-2** | Mean (pg/ml) | 0.00 | 2.29 | 0.00 | 17.32 | 0.00 |
| | ± SD | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **IL-6** | Mean (pg/ml) | 0.38 | 0.41 | 1.01 | 2.56 | 0.79 |
| | ± SD | 0.23 | 0.17 | 0.14 | 4.10 | 0.47 |
| **IP-10** | Mean (pg/ml) | 605.50 | 639.54 | 813.35 | 766.50 | 684.53 |
| | ± SD | 154.57 | 54.59 | 248.99 | 297.89 | 46.50 |
| **MCP-1** | Mean (pg/ml) | 361.83 | 249.90 | 284.35 | 275.21 | 268.46 |
| | ± SD | 11.71 | 28.90 | 65.43 | 210.65 | 65.80 |
| **TNF-α** | Mean (pg/ml) | 0.33 | 0.41 | 0.83 | 0.37 | 0.45 |
| | ± SD | 0.15 | 0.48 | 0.55 | 0.13 | 0.00 |
| **IL-15** | Mean (pg/ml) | 11.39 | 9.05 | 8.25 | 9.54 | 13.03 |
| | ± SD | 1.52 | 2.29 | 1.31 | 2.64 | 7.02 |

SD = standard deviation

**Serum Cytokine Levels, 4 Hours After Dosing**

| | | Immunostimulatory Bacterial Strain and Dose (CFUs/NHP) | | | | |
|---|---|---|---|---|---|---|
| | | PBS Control | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* + p2.1 CMV VCIP huIL-15Rα-IL15sc_T2A_STING N154S/R284G tazCTT | | | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* + NanoLuc® Plasmid |
| | | - | 3 x $10^8$ CFUs/NHP | 1 x $10^9$ CFUs/NHP | 3 x $10^9$ CFUs/NHP | 3 x $10^8$ CFUs/NHP |
| **IFN-γ** | Mean (pg/ml) | 1.35 | 6.87 | 26.49 | 22.62 | 4.03 |
| | ± SD | 1.09 | 5.48 | 34.96 | 21.59 | 2.51 |
| **IL-10** | Mean (pg/ml) | 0.068 | 0.481 | 0.508 | 0.586 | 0.387 |
| | ± SD | 0.050 | 0.149 | 0.125 | 0.269 | 0.113 |
| **IL-12p70** | Mean (pg/ml) | 0.05 | 0.52 | 1.21 | 1.17 | 0.45 |
| | ± SD | 0.01 | 0.36 | 1.03 | 1.12 | 0.06 |
| **IL-1β** | Mean (pg/ml) | 0.08 | 0.34 | 0.55 | 0.88 | 0.33 |

| | | Immunostimulatory Bacterial Strain and Dose (CFUs/NHP) | | | | |
|---|---|---|---|---|---|---|
| | | PBS Control | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* + p2.1 CMV VCIP huIL-15Rα-IL15sc_T2A_STING N154S/R284G tazCTT | | | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* + NanoLuc® Plasmid |
| | | - | $3 \times 10^8$ CFUs/NHP | $1 \times 10^9$ CFUs/NHP | $3 \times 10^9$ CFUs/NHP | $3 \times 10^8$ CFUs/NHP |
| | ± SD | 0.04 | 0.14 | 0.16 | 0.62 | 0.13 |
| **IL-2** | Mean (pg/ml) | 0.00 | 0.52 | 0.11 | 10.33 | 0.00 |
| | SD | 0.00 | 0.00 | 0.00 | 11.24 | 0.00 |
| **IL-6** | Mean (pg/ml) | 3.0 | 95.3 | 116.7 | 376.9 | 34.8 |
| | ± SD | 1.4 | 105.3 | 70.7 | 318.1 | 26.4 |
| **IP-10** | Mean (pg/ml) | 363.0 | 9529.3 | 11838.7 | 10549.6 | 11143.2 |
| | ± SD | 19.6 | 4596.6 | 82.0 | 2362.7 | 1231.4 |
| **MCP-1** | Mean (pg/ml) | 475.5 | 5017.5 | 5642.4 | 6247.7 | 3700.4 |
| | ± SD | 143.0 | 3755.6 | 1622.2 | 2492.8 | 2209.3 |
| **TNF-α** | Mean (pg/ml) | 0.1 | 1.8 | 3.4 | 4.6 | 1.1 |
| | ± SD | 0.1 | 1.5 | 0.2 | 4.1 | 0.5 |
| **IL-15** | Mean (pg/ml) | 12.73 | 14.45 | 14.84 | 15.32 | 14.9 |
| | ± SD | 2.5 | 4.07 | 3.29 | 3.91 | 3.48 |

SD = standard deviation

**Serum Cytokine Levels, 24 Hours After Dosing**

| | | Immunostimulatory Bacterial Strain and Dose (CFUs/NHP) | | | | |
|---|---|---|---|---|---|---|
| | | PBS Control | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* + p2.1 CMV VCIP huIL-15Rα-IL15sc_T2A_STING N154S/R284G tazCTT | | | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* + NanoLuc® Plasmid |
| | | - | $3 \times 10^8$ CFUs/NHP | $1 \times 10^9$ CFUs/NHP | $3 \times 10^9$ CFUs/NHP | $3 \times 10^8$ CFUs/NHP |
| | Mean (pg/ml) | 0.71 | 0.79 | 1.65 | 3.32 | 6.63 |
| **IFN-γ** | ± SD | 0.83 | 0.51 | 2.15 | 3.13 | 0.97 |
| | Mean (pg/ml) | 0.0577 | 0.0906 | 0.0672 | 0.0988 | 0.1000 |
| **IL-10** | ± SD | 0.0362 | 0.0428 | 0.0629 | 0.0631 | 0.0617 |
| | Mean (pg/ml) | 0.25 | 0.35 | 0.98 | 2.51 | 0.31 |
| **IL-12p70** | ± SD | 0.03 | 0.22 | 1.38 | 2.50 | 0.11 |
| | Mean (pg/ml) | 0.02 | 0.02 | 0.36 | 0.15 | 0.09 |
| **IL-1β** | ± SD | 0.02 | 0.00 | 0.57 | 0.04 | 0.03 |
| **IL-2** | Mean (pg/ml) | 0.00 | 0.00 | 0.00 | 3.19 | 0.00 |

| | | Immunostimulatory Bacterial Strain and Dose (CFUs/NHP) | | | | |
|---|---|---|---|---|---|---|
| | | PBS Control | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/F-Δ*purI* + p2.1 CMV VCIP huIL-15Rα-IL15sc_T2A_STING N154S/R284G tazCTT | | | YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD* + NanoLuc® Plasmid |
| | | - | $3 \times 10^8$ CFUs/NHP | $1 \times 10^9$ CFUs/NHP | $3 \times 10^9$ CFUs/NHP | $3 \times 10^8$ CFUs/NHP |
| | ± SD | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | Mean (pg/ml) | 0.6 | 8.0 | 5.3 | 19.8 | 3.0 |
| **IL-6** | ± SD | 0.2 | 1.9 | 3.0 | 17.4 | 0.7 |
| | Mean (pg/ml) | 475.6 | 1579.7 | 2354.0 | 3638.2 | 2762.1 |
| **IP-10** | ± SD | 180.7 | 339.5 | 1475.8 | 4036.5 | 1752.8 |
| | Mean (pg/ml) | 266.5 | 340.1 | 258.2 | 525.7 | 393.1 |
| **MCP-1** | ± SD | 5.9 | 39.9 | 41.2 | 285.5 | 270.7 |
| | Mean (pg/ml) | 0.1 | 0.2 | 0.4 | 0.4 | 0.5 |
| **TNF-α** | ± SD | 0.1 | 0.0 | 0.3 | 0.2 | 0.6 |
| **IL-15** | Mean (pg/ml) | 11.09 | 16.07 | 12.17 | 15.87 | 18.63 |
| | ± SD | 2.51 | 4.26 | 1.56 | 4.83 | 2.81 |

SD = standard deviation

## Example 39

### Thymidine Auxotroph strains

The *Salmonella typhimurium* essential gene *thyA* encodes thymidylate synthase, a key enzyme in DNA synthesis. Deletion or mutation of the gene renders the strain auxotrophic for thymidine. Upon deprivation of the complementing substrate, bacterial cell death occurs and it has been demonstrated that no macromolecules are released from *thyA* mutants upon thymidine starvation (Loessner *et al.*, *FEMS Microbiol Lett.* 265(1): 81-88 (2006)), reducing potential release of PAMPs and subsequent activation of innate inflammatory responses. To generate a thymidine auxotroph that is incapable of replication in vivo and rapidly dies in the absence of supplemented thymidine, *thyA* was deleted from strain YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI.

A 690 bp region containing the *thyA* gene (SEQ ID NO: 464; DC51_3078), was targeted for deletion using modifications of the method of Datsenko and Wanner (*Proc. Natl. Acad. Sci. U.S.A.* 97:6640–6645 (2000)). To disrupt thyA gene, a thyA gene knockout cassette was constructed by overlapping PCR. This cassette contains a Kanamycin resistance ($Kan^R$) cassette flanked by two I-SceI cut site, and homologous

regions on both the 5' end (345 bp) and 3' end (332 bp) to facilitate recombination. Immediately 5' of the $Kan^R$ cassette there is 75 bp of DNA sequence that is perfectly homologous to 75 bp on the 3' of the $Kan^R$ cassette. This repeat element (thyA MHA) leaves a scarless deletion upon excision of the $Kan^R$ cassette.

Specifically, with reference to the sequences in the table below, the thyA left homology arm sequence was amplified from YS1646 using primers thya-1 and thya-2, was PCR overlapped with a $Kan^R$ cassette using primers thya-1 and scFv-thyA right homology arm sequence amplified from YS1646 using primer thya-3 and thya-4, was PCR overlapped with thyA MHA ( prepared as a synthetic gBlock gene fragment from a supplier (IDT) using primer loxp-4 and thya-4 ).

**Table 3: thyA gene KO homology arm length information**

| Gene name | Homology arm length | |
|---|---|---|
| | LHA | RHA |
| *thyA* (SEQ ID NO: 464_ | 345 bp (PCR amplified from VNP20009) | 332 bp (PCR amplified from VNP20009) |

**Table 4: thyA KO primer sequence information**

| Gene | Primer | Primer sequence | Primer description | SEQ ID NO. |
|---|---|---|---|---|
| thyA | thya-1 | ccttcccagttgtatgagttggc | Forward primer amplifying thyA LHA and KO cassette | 471 |
| | thya-2 | CTGTTATCCCTAgaagcagctccagcctacacctgtgtgccttcatccagca | Reverse primer amplifying thyA LHA | 472 |
| | thya-3 | gatgaaggcacacagtgaaattgaaggctatgatccgcac | Forward primer amplifying thyA RHA | 473 |
| | thya-4 | gttgccagccataaagtcctgtg | Reverse primer amplifying thyA RHA and KO cassette | 474 |
| | thya-5 | ccatgctctttcctggctca | fwd primer checking thyA KO | 475 |
| | thya-6 | ctgacaaagcaccatgaaccg | rev primer checking thyA KO | 476 |
| | FRT-3 | gtgtaggctggagctgcttc | Fwd primer amplifying Kan gene cassette | 477 |
| | loxp-4 | ggcttcccaaccttaccagag | Fwd primer for overlapping thyA MHA and RHA | 478 |
| | scFv-3 | cataatctgggtccttggtctgc | Reverse primer for amplifying Kan gene cassette and for overlapping Kan gene cassette with thyA LHA | 223 |

>thyA MHA gBlock fragment
Note: thyA KO middle homology arm
(ggcttcccaaccttaccagagggcgccccagctggcaattccgGGTACCggcaagTAGGGATAACAGGGTAATgttaTCTGCAgaccaaggacccagattatgcagcaacacgtttcctgaggaaccatgaaacagtatttagaactgatgcaaaaagtgctggatgaaggcacacagtgaaattgaaggctatgatccgcac; SEQ ID NO: 479) was used for the lambda red recombination step during Kan gene removal using I-SceI.

The full length thyA gene knockout cassette then was constructed by overlap PCR using primers thya-1and thya-4 (see above Table), gel purified, and introduced into strain YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI carrying the temperature sensitive lambda red recombination plasmid pSL0304 by electroporation. The kanamycin resistance gene then was cured by I-SceI/lambda red mediated recombination as previously described Yang and Yang (*Applied and Environmental Microbiology*, 80: 3826-3834 (2014)), and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *thyA* gene fragment knockout sequence was confirmed by PCR using primers thya-5 and thya-6 (Table 2), and verified by DNA sequencing. The resulting mutant derivative of parental strain, YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI, was designated YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA.

Thymidine deletion strains were grown in a DasGip fermentation system supplemented with 250$\mu$g/mL thymidine for 6 hours and culture material was subsequently processed for injection stocks. YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA was cultured in the presence of 250$\mu$g/mL thymidine (Sigma Aldrich T1895-1G) and 50$\mu$g/mL diaminopimelic acid to generate electrocompetent cells. YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA was transformed with plasmids ADN-86, ADN-870 or ADN-872 (see table below), to generate strains STST-321, STST-326 and STST-328, respectively. YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA strains, designated STST-321, STST-326 and STST-328 containing the noted plasmids (see Table below), were grown in a DasGip fermentation system at 1L scale for 6hrs in the presence of 250$\mu$g/mL thymidine in parallel with CRST-2000, YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI bearing plasmid ADN-838, in the absence of thymidine supplementation.

| | | |
|---|---|---|
| STST-321 | **ADN-865** | PurI del-CsgD-**ThyA**-pATI1.76 MTL EMCV IRES Ovalbumin-SIY Myc HPRE bGHpA-ADN-865 |
| STST-326 | **ADN-870** | PurI del-CsgD-**ThyA**-pATI1.76 MTL EMCV IRES tPA leader 4xSIINFEKYL HPRE bGHpA-ADN-870 |
| STST-328 | **ADN-872** | PurI del-CsgD-**ThyA**-pATI1.76 MTL tPA leader 4xSIINFEKYL HPRE bGHpA-ADN-872 |
| CRST-2000 | **ADN-838** | PurI del-CsgD-pATI2.1 CMV VCIP IRES huIL-15cplex T2A hSTINGtCTT N154S+R284G HPRE bGHpA-ADN-838 |

Fermentation culture was analyzed for $OD_{600}$ throughout the fermentation. The growth of the thymidine deletion strains were observed to be comparable to the CRST-2000 control.

To confirm thymidine auxotrophy, growth of strains STST-321, STST-326 and STST-328 was evaluated in medium with or without 250$\mu$g/mL thymidine supplementation in comparison to control CRST-2000. Injection stocks were thawed at room temperature and 2.5$\mu$L was used to inoculate 250$\mu$L media +/- thymidine in triplicate wells in a clear bottom 96 well plate; growth was evaluated for 12 hours, shaking at 37°C, by OD600 in a Spectramax microplate reader (Molecular Devices). Thymidine deletions strains were grown in broth with or without 250$\mu$g/mL thymidine supplementation, and $OD_{600}$ was monitored in 15 minutes intervals over the course of 12 hours.

The results show that no growth was observed for STST-321, STST-326 and STST-328 in the absence of thymidine supplement, while CRST-2000 demonstrated robust growth to stationary phase. In the presence of 250$\mu$g/mL thymidine supplementation, STST-321, STST-326 and STST-328 grew to stationary phase with a growth profile similar to CRST-2000, demonstrating precise and controlled auxotrophy for thymidine.

**Example 40**

**Optimized Expression of IRF3, IFN$\alpha$2, and IFN-$\beta$ Expression Cassettes**

Plasmids containing combinations of human IRF3 S396D or mouse IRF3 S388D, or human or mouse IFN$\alpha$2, or human or mouse IFN-$\beta$, where the bi-cistronic constructs contain T2A peptides, were tested for expression of each encoded payload/product against single expresser controls.

HEK293T STING Null Cells (293-Dual™ Null Cells; InvivoGen),which do not contain endogenous STING, and express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous IFN-stimulated response element (ISRE) promoter, where the coding sequence of ISRE is replaced by the SEAP ORF using knock-in technology, were used. STING activity, thus, is assessed by monitoring type I IFN induced SEAP production. The 293-Dual™ Null cells also express Lucia™ luciferase, a secreted luciferase, placed under the control of the endogenous IFN-$\beta$ promoter; the coding sequence of IFN-$\beta$ has been replaced by the

Lucia™ luciferase ORF using knock-in technology. This allows for the assessment of STING activity by monitoring the expression of IFN-β. Using these cells, STING activity can be assessed by monitoring ISRE-induced SEAP production and/or IFN-β-dependent expression of Lucia™ luciferase. The two reporter proteins, SEAP and Lucia™ Luciferase, can be measured in the cell supernatant using standard assays and detection reagents, such as the QUANTI-Blue™ and QUANTI-Luc™ detection reagents (InvivoGen), respectively.

The cells were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, and incubated overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 300 ng of each plasmid DNA, and 40 ng of a CMV-GFP vector (*i.e.*, a vector encoding green fluorescent protein under the control of a CMV promoter), were diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios, with untransfected wells serving as negative controls (in duplicates). Cell culture supernatants from each sample were collected 48 hours post-transfection.

The activity of the constructs were evaluated using the ISRE-SEAP and IFN-β-Lucia™ reporter systems. The type I interferon (IFN) activity was assessed by monitoring type I IFN-stimulated SEAP production in the cell supernatants. 20 µL of cell culture supernatant was added to 180 µL of QUANTI-Blue™ reagent (InvivoGen), which is used for measuring SEAP. Type I interferon activation was determined by measuring ISRE-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), at an absorbance wavelength of 650 nm. The type I interferon (IFN) activity also was assessed by monitoring the type I IFN-stimulated Lucia™ luciferase production in the cell supernatants. 20 µL of cell culture supernatant was added to 50 µL of QUANTI-Luc™ reagent (InvivoGen), which is used for measuring Lucia™ luciferase activity. Type I interferon activation was determined by measuring IFNβ-induced Lucia™ luciferase activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), on the luminescence setting.

**A. Cytokines Resulting from Transfection of Mouse IRF3, IFNα2, and IFNb in BMDCs**

Various single expressing plasmids and combination plasmids containing mouse IRF3 S388D, mouse IFNα2, mouse IFN-β were compared to the hSTING N154S/R284G tazCTT plasmid. The expression results are provided in the table below. ISRE-SEAP reporter activity is higher than hSTING N154S/R284G tazCTT in all of the human constructs, as well as mIFNα2 T2A mIRF3 S388D and mIRF3 S388D T2A mIFNα2. IFNβ-Lucia reporter activity is higher than hSTING N154S/R284G tazCTT for the phoshpmimetic hIRF3 alone, hIFNα2 T2A hIRF3 S388D and hIRF3 S388D T2A hIFNα2. These results demonstrate high levels of type I IFN production can be induced through expressing individual type I IFN signaling components, as well as combinations of constitutive type I IFN signaling components.

**Mouse and Human IRF3, IFNα2, and IFN-β Expression Normalized by Transfection Efficiency**

| | **ISRE-SEAP (Absorbance / %GFP)** | | **IFNβ-Lucia (Luminesence / %GFP)** | |
|---|---|---|---|---|
| | Mean | ± SD | Mean | ± SD |
| Untransfected | ND | ND | ND | ND |
| 1.76 CMV huSTING tazCTT DM | 0.0228583 | 0.00085119 | 11.2064602 | 0.45461337 |
| 1.76 CMV mIFNα2 | 0.03989167 | 0.00145511 | 0.32920588 | 0.28396577 |
| 1.76 CMV mIFN-β | ND | ND | ND | ND |
| 1.76 CMV mIRF3 S388D | 0.01381649 | 0.00062722 | 5.20826023 | 0.36717461 |
| 1.76 CMV mIFNα2 T2A mIFN-β | 0.01767895 | 0.00052589 | ND | ND |
| 1.76 CMV muIFNα2 T2A muIRF3 S388D | 0.02566539 | 0.00030503 | 6.64847586 | 1.66829016 |
| 1.76 CMV muIRF3 S388D T2A muIFNα2 | 0.03512478 | 0.0016182 | 6.37450819 | 1.35199207 |
| 1.76 CMV huIFNα2 | 0.05600093 | 0.0009781 | 0.8808781 | 0.67044592 |
| 1.76 CMV huIRF3 S396D | 0.03945822 | 0.00031064 | 42.716536 | 4.91527029 |
| 1.76 CMV huIFN-β | 0.06714092 | 0.00393377 | 1.86574208 | 0.47553008 |
| 1.76 CMV huIFNα2 T2A huIFN-β | 0.05854426 | 0.00151915 | 1.00891248 | 0.73015436 |
| 1.76 CMV huIFNα2 T2A huIRF3 S396D | 0.06245151 | 0.0015994 | 18.8868229 | 2.54040139 |
| 1.76 CMV huIRF3 S396D T2A huIFNα2 | 0.06495505 | 0.00023288 | 18.8039985 | 3.32761255 |

**B.** Plasmids containing combinations of human IRF3 S396D or mouse IRF3 S388D, or human or mouse IFNα2, or human or mouse IFN-β, and combinations thereof, where the bi-cistronic constructs contain T2A peptides, were tested for expression by transfection in murine primary bone marrow-derived dendritic cells (BMDCs).

To test these, Golden Ticket murine bone marrow was isolated and flushed into 1.5 mL Eppendorf tubes, and spun at 1200 RPM for 5 minutes, to collect the bone marrow cells. Cells were washed once in RPMI-1640 + 10% FBS, then seeded in 96-well TC-treated plates in RPMI-1640 + 10% FBS with 20 ng/ml GM-CSF. After four days, non-adherent cells were pipetted off the wells and re-seeded at 2e5 cells per well in RPMI-1640 + 10% FBS in a 96-well plate for transfection. Cells were transfected using Viromer® RED, according to the manufacturer's instructions. Briefly, 300 ng of plasmid DNA, as well as a "No DNA" control, were diluted in the provided buffer, and mixed with 0.08 µL of Viromer® RED and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 96-well plate (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator. Supernatants were harvested at 48 hours, and assayed for murine IFNα, IFN-β, CXCL10 (IP-10), and IL-6 using a flow cytometry-based cytokine bead array (CBA), according to the manufacturer's protocol.

Various single expressing plasmids and combination plasmids encoding mouse IRF3 S388D, mouse IFNα2, mouse IFN-β were compared to the hSTING N154S/R284G tazCTT plasmid. As shown in the table below, transfections with constructs mIFNα2, mIFN-β, and mIFNα2 T2A mIFN-β had higher levels of IFN-α and IFN-β than hSTING N154S/R284G tazCTT. mIFN-β and mIFNα2 T2A mIFN-β constructs had higher levels of CXCL10 than hSTING N154S/R284G tazCTT. mIRF3 S388D, mIFNα2 T2A mIRF3 S388D, and mIRF3 S388D T2A mIFNα2 constructs all had lower IL-6 levels than hSTING N154S/R284G tazCTT. These data demonstrate the ability to induce type I IFN signaling through multiple combinations of expressed type I IFN components, alone and in combination, and with minimal

induction of pro-inflammatory IL-6. Expression of IFN-β alone induces the highest type I IFN activity of all the targets tested.

**Cytokines Resulting from Transfection of Mouse IRF3, IFNα2, and IFN-β in BMDCs**

| | IFN-α | | IFN-β | | CXCL10 | | IL-6 | |
|---|---|---|---|---|---|---|---|---|
| | Mean | ± SD | Mean | ± SD | Mean | ± SD | Mean | ± SD |
| No DNA | 3.38 | 1.99 | 5.18 | 1.85 | 34.05 | 0.78 | 5.74 | 1.86 |
| huSTING tazCTT N154S+R284G | 72.07 | 26.35 | 62.18 | 13.68 | 7199.95 | 1468.73 | 183.82 | 50.88 |
| mIFNα2 | 99.91 | 21.01 | 62.48 | 9.97 | 5524.30 | 303.77 | 174.86 | 39.07 |
| mIFN-β | 91.95 | 44.55 | 190.12 | 13.94 | 8178.50 | 1917.96 | 191.92 | 14.28 |
| mIRF3 S388D | 33.36 | 3.19 | 30.42 | 0.88 | 2428.30 | 414.51 | 90.29 | 12.83 |
| mIFNα2 T2A mIFN-β | 72.84 | 12.10 | 77.70 | 3.78 | 5226.50 | 795.07 | 202.70 | 17.23 |
| muIFNα2 T2A muIRF3 S388D | 20.82 | 6.37 | 21.49 | 3.13 | 2077.50 | 356.38 | 95.41 | 10.88 |
| muIRF3 S388D T2A muIFNα2 | 43.72 | 15.15 | 32.72 | 3.39 | 3187.75 | 9.12 | 134.63 | 0.66 |

### C. Mouse IFNα2 T2A IFN-β is Expressed *in vivo* in a Murine Orthotopic Breast Cancer Model

For this experiment, 6-8 week-old female BALB/c mice (5 mice per group) were inoculated in the left mammary fat pad with EMT6 tumor cells (ATCC # CRL-2755) ($1x10^6$ cells in 100 µL PBS). Mice bearing 9 day-old established mammary tumors (~100 $mm^3$ in volumne) were IV injected with a single dose of $2 x10^7$ CFUs of the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(large\ clean)}$ strain containing a bicistronic plasmid encoding mIFNα2 T2A mIFN-β, and compared to PBS control.

The level of tumor mIFNα2 and mIFN-β expression, relative to actin, was determined in order to assess tumor-specific payload delivery. On day 4 post-IV injection, tumors were excised and processed for RNA extraction using the TissueLyser II (Qiagen) in 1.2 mL of RLT plus lysis buffer. The homogenates were collected for RNA isolation using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA concentration was measured using a NanoDrop™ 2000 UV-Vis Spectrophotometer (Thermo Scientific). The purity of each sample also was assessed from the $A_{260}/A_{230}$ absorption ratio. Synthesis of cDNA was performed from 0.5-1 µg of template RNA using a CFX96™ Real-Time System (Bio-Rad) and iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad) in a 20

μL reaction, according to the manufacturer's instructions. qPCR was performed with a CFX96 Real-Time System (Bio-Rad). PrimePCR™ Probe Assay for mIFNα2 (qMmuCEP0043629), mIFNβ1 (qMmuCEP0058870) were purchased from Bio-Rad. The qPCR reaction (20 μL) was conducted per protocol, using either the SsoAdvanced Universal SYBR Green Supermix or the iQ Multiplex Powermix (Bio-Rad). The standard thermocycling program on the Bio-Rad CFX96 Real-Time System consisted of a 95 °C denaturation for 150 sec, followed by 39 cycles of 95 °C for 15 sec and 60 °C for 55 sec. Quantification of the target mRNA was normalized using Actin reference mRNA (Bio-Rad, qMmuCEP0039589). $\Delta C_q$ was calculated as the difference between the target and reference gene.

The values are shown in the table below, as the average of five mice (PBS group) or three mice (mIFNα2 + mIFN-β group). Compared to the PBS control group, YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(\text{large clean})}$ strain containing plasmids encoding mIFNα2 and mIFN-β significantly increased expression of mIFNα2 and mIFN-β genes in EMT6 tumors.

| Treatments | Ave Fold Expression Relative to PBS Control | |
|---|---|---|
| | IFNα2 | IFNβ1 |
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(\text{large clean})}$ strain + mIFNα2 + mIFN-β | 5.37E+03 | 7.60E+00 |

In order to measure plasmid delivery to the tumor, and subsequent heterologous gene expression and protein secretion, the amount of mIFNα2 and mIFN-β protein was measured in these tumors. For this, lysates were harvested from homogenized tumors and assessed for protein expression using a flow cytometry-based cytokine bead array (CBA), according to the manufacturer's protocol.

As shown in the table below, the YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(\text{large clean})}$ strain containing plasmids encoding mIFNα2 and mIFN-β demonstrated high levels of mIFNα2 and mIFN-β protein per gram of tumor, as compared to the PBS control tumors, demonstrating potent delivery of mIFNα2 and mIFN-β into tumors following IV administration.

| treatments | pg/ml per gram of tumor | |
|---|---|---|
| | IFNα2 | IFN-β |
| PBS | 20497.6 ±6249 | 75.8 ±21 |

| | | |
|---|---|---|
| YS1646Δ*asd*/ΔFLG/Δ*pagP*/Δ*ansB*/Δ*csgD*/Δ*purI*$_{(large\ clean)}$ strain + mIFNα2 + mIFN-β | 37538.5 ±3843 | 307.7 ±49 |

**Example 41**

**Expression of Membrane Bound Human and Mouse IL-12 in Mouse and Human Cells**

Mouse membrane bound IL-12p70 (SEQ ID NO:466) was constructed with mouse IL-12p40 (residues 3009-4010 of SEQ ID NO:399) followed by a 15 amino acid linker (SEQ ID NO:480; GGGGSGGGGSGGGGS), and IL-12p35 (residues 4056-4634 of SEQ ID NO:399). The transmembrane and cytosolic portions of murine CD80 (SEQ ID NO:481; PPEDPPDSKNTLVLFGAGFGAVITVVVIVVIIKCFCKHRSCFRRNEASRETNNS LTFGPEEALAEQTVFL) follow this sequence. Human membrane bound IL-12p70 (SEQ ID NO:467) was constructed with human IL-12p40 (SEQ ID NO: 482; MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCD TPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLL HKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSV KSSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDT WSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDR YYSSSWSEWASVPCS) followed by a 15 amino acid linker sequence (GGGGSGGGGSGGGGS, SEQ ID NO: 480) and human IL-12p35 (RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDI TKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIY EDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQ KSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS, SEQ ID NO:483). The transmembrane and cytosolic portions of human CD80 (SEQ ID NO:484; DNLLPSWAITLISVNGIFVICCLTYCFAPRCRERRRNERLRRESVRPV) follow this sequence.

HEK cells were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, and incubated overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 300 ng of each plasmid DNA, and 40 ng of a CMV-GFP vector (*i.e.*, a vector encoding green fluorescent protein under the control of a CMV promoter), were diluted in serum-free media and added to FuGENE®

transfection reagent (Promega), at the proper reagent:DNA ratio, with untransfected wells serving as negative controls (in duplicates). Cell culture supernatants from each sample were collected 48 hours post-transfection.

The activity of the constructs were evaluated using a co-culture of transfected HEK cells with HEK-Blue IL-12 (InvivoGen) cells. 24 hours post transfection, 5e4 HEK cells were detached and seeded in a 96 well TC plate. Additionally, 5e4 HEK-Blue IL-12 cells were seeded in the same wells. 48 hours post transfection, cell culture supernatants from each sample were collected 48 hours post-transfection. Human and mouse IL-12 bioactivity was determined by measuring the SEAP reporter, which measures activation of the STAT-4 pathway downstream of IL-12 binding to its receptor. IL-12 bioactivity was determined by measuring ISRE-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), at an absorbance wavelength of 650 nm.

The expression results of the constructs are provided in the table below. Each of the murine and human membrane bound IL-12p70 activated the STAT4-SEAP reporter in HEK-Blue IL-12 cells to at least the same extent as soluble murine and human IL-12p70, thereby confirming the functionality of constructs.

**Mouse and Human Membrane Bound IL-12p70 Expression Normalized by Transfection Efficiency**

| | **STAT4-SEAP (Absorbance / %GFP)** | |
|---|---|---|
| | Mean | ± SD |
| Untransfected | ND | ND |
| muIL-12p70 | 0.1104 | 0.0024 |
| huIL-12p70 | 0.1098 | 0.0031 |
| muIL-12p70.B7 | 0.1166 | 0.0005 |
| huIL-12p70.B7 | 0.2368 | 0.0057 |

Membrane bound IL-12p70 constructs were tested for expression on the surface of transfected HEK cells by flow cytometry. HEK wildtype cells transfected with murine or human IL-12p70, using the same conditions as above, and were harvested 48 hours post transfection. Cells were detached by PBS and seeded in wells of a V-bottom 96-well plate. The cells were washed once with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were then resuspended in 50 uL PBS + 2% FBS containing either anti-human IL-12p70 APC (Miltenyi) diluted 1:10

or anti-mouse IL-12p70 APC (Miltenyi) diluted 1:10, and incubated on ice for 30 minutes in the dark. The cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes and resuspended in DAPI diluted 1:6000 in PBS + 2% FBS. Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.). Only 1.67% of untransfected cells were APC positive, while the membrane bound human IL-12p70 was 40.4% positive and the membrane bound murine IL-12p70 was 59.9%. Therefore, these membrane bound IL-12 constructs are well expressed on human cells.

Plasmids containing human or murine membrane bound IL-12p70 were tested for expression by transfection in murine primary bone marrow-derived dendritic cells (BMDCs). Wild type murine bone marrow was isolated and flushed into 1.5 mL Eppendorf tubes, and spun at 1200 RPM for 5 minutes, to collect the bone marrow cells. Cells were washed once in RPMI-1640 + 10% FBS, then seeded in 96-well TC-treated plates in RPMI-1640 + 10% FBS with 20 ng/ml GM-CSF. On Day 3, additional RPMI-1640 + 10% FBS with 20 ng/ml GM-CSF was added to the cells. After six days, non-adherent cells were pipetted off the wells and re-seeded at 2e5 cells per well in RPMI-1640 + 10% FBS in a 96-well plate for transfection. Cells were transfected using Viromer® RED transfection reagent, according to the manufacturer's instructions. Briefly, 300 ng of plasmid DNA, as well as a "No DNA" (transfection reagent only) control, were diluted in the provided buffer, and mixed with 0.08 µL of Viromer® RED transfection reagent and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 96-well plate (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator. Cells were harvested 48 hours post transfection and were assayed for IL-12 surface expression using flow cytometry.

The cells were washed once with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were then resuspended in 50 µL PBS + 2% FBS containing either anti-human IL-12p70 APC (Miltenyi) diluted 1:10 or anti-mouse IL-12p70 APC (Miltenyi) diluted 1:10, CD11b PE diluted 1:200, ClassII APC-Cy7 diluted 1:200, and incubated on ice for 30 minutes in the dark. The cells were washed

twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes and resuspended in DAPI stain diluted 1:6000 in PBS + 2% FBS. Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.). In the dendritic cell population, only 0.205% ± 0.021% of untransfected cells and 0.685% ± 0.262% of VCIP IRES CMV NanoLuc transfected cells were APC positive while the membrane bound human IL-12p70 was 6.14% ± 1.27% positive and the membrane bound murine IL-12p70 was 2.64% ± 0.87% positive. These results show that the membrane bound IL-12 constructs were detected on the surface of mouse bone marrow dendritic cells above background.

Plasmids containing murine membrane bound IL-12p70 also were tested for expression by transfection in murine primary bone marrow-derived macrophages (BMMs). Wild type murine bone marrow was isolated and flushed into 1.5 mL Eppendorf tubes, and spun at 1200 RPM for 5 minutes, to collect the bone marrow cells. Cells were washed once in RPMI-1640 + 10% FBS, then seeded in 24-well TC-treated plates in RPMI-1640 + 10% FBS with 20 ng/ml M-CSF. After three days, media was aspirated and replenished with 20 ng/ml M-CSF. Cells were transfected using Viromer® RED transfection reagent, according to the manufacturer's instructions. Briefly, 750 ng of plasmid DNA, as well as a "No DNA" control, were diluted in the provided buffer, and mixed with Viromer® RED transfection reagent and incubated at room temperature for 15 minutes to allow the DNA/Viromer® RED complexes to form. The DNA/Viromer® RED complexes were then slowly added to each well of the 96-well plate (in duplicates), and the plate was incubated at 37 °C in a $CO_2$ incubator. Cells were harvested 48 hours post transfection and were detached for IL-12 surface expression using flow cytometry.

The cells were detached with 10mM EDTA and washed once with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were then resuspended in 50 uL PBS + 2% FBS containing either anti-human IL-12p70 APC (Miltenyi) diluted 1:10 or anti-mouse IL-12p70 APC (Miltenyi) diluted 1:10, CD11b PE diluted 1:200, ClassII APC-Cy7 diluted 1:200, and incubated on ice for 30 minutes in the dark. The cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes and resuspended in DAPI diluted 1:6000 in PBS + 2% FBS. Flow cytometry

data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.). Only 0.01% ± 0.003% of untransfected cells and 0.36% ± 0.028% of VCIP IRES CMV NanoLuc transfected cells were APC positive while the membrane bound murine IL-12p70 was 1.945% ± 0.106%. Therefore, these membrane bound IL-12 constructs were detected on the surface of mouse bone marrow macrophages above background.

## EXAMPLE 42

### Expression of DLL3xCD3 Bispecific T-cell Engagers from Human Cells

DLL3xCD3 bispecific T-cell engagers (sold under the trademark BiTE®), which targets delta-like ligand 3, which is selectively expressed in small-cell lung cancer, and CD3+ T cells, containing a FLAG tag, were designed using the amino acid sequences of anti-DLL3 antibodies SC16.15, SC16.34, and SC16.56, see *e.g.*, International Patent Publication No. WO 2017/031458 A2. The anti-CD3 arm of this BiTE construct was designed from clone 145-2C11. These antibodies are fully murine sequences cloned into the pATI-1.76 vector under the control of a CMV promoter and with a 3' HPRE. For human administration, the antibodies can be humanized as appropriate. The sequences were confirmed by Sanger sequencing.

The sequence of the resulting constructs are as follows:

**SC16.56 DLL3HLxCD3HL (SEQ ID NO:485)**

The construct includes, in the following order: mouse IgGK leader, SC16.56 VH, 15 amino acid GS linker, SC16.56 VL, 5 amino acid linker, 145-2C11 VH, 15 amino acid GS linker, 145-2C11 VL, and Flag tag, as set forth in SEQ ID NO: 485, and below:

| | |
|---|---|
| METDTLLLWVLLLWVPGSTGD | mouse IgGK leader |
| QIQLVQSGPELKKPGETVKISCKASGYTFTNYGM NWVKQAPGKGLKWMAWINTYTGEPTYADDFKG RFAFSLETSASTASLQIINLKNEDTATYFCARIGDS SPSDYWGQGTTLTVSS | SC16.56 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVVW YQQKPGQSPKLLIYYASNRYTGVPDRFAGSGYGTD FSFTISTVQAEDLAVYFCQQDYTSPWTFGGGTKLEIR | SC16.56 VL |
| GGGGS | 5 amino acid GS linker |
| EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGM | 145-2C11 VH |

| | |
|---|---|
| HWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWD KNYWGQGTMVTVSS | |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNW YQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRD SSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIK | 145-2C11 VL |
| DYKDDDDK | Flag tag |

**SC16.56 DLL3LHxCD3HL (SEQ ID NO: 486)**

The construct includes, in the following order: mouse IgGK leader, SC16.56 VL, 15 amino acid GS linker, SC16.56 VH, 5 amino acid linker, 145-2C11 VH, 15 amino acid GS linker, 145-2C11 VL, and Flag tag, as set forth in SEQ ID NO: 486, and below:

| | |
|---|---|
| METDTLLLWVLLLWVPGSTGD | mouse IgGK leader |
| SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVVW YQQKPGQSPKLLIYYASNRYTGVPDRFAGSGYGTD FSFTISTVQAEDLAVYFCQQDYTSPWTFGGGTKLEIR | SC16.56 VL |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| QIQLVQSGPELKKPGETVKISCKASGYTFTNYGM NWVKQAPGKGLKWMAWINTYTGEPTYADDFKG RFAFSLETSASTASLQIINLKNEDTATYFCARIGDS SPSDYWGQGTTLTVSS | SC16.56 VH |
| GGGGS | 5 amino acid linker |
| EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGM HWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWD KNYWGQGTMVTVSS | 145-2C11 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNW YQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRD SSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIK | 145-2C11 VL |
| DYKDDDDK | Flag tag |

**SC16.15 DLL3HLxCD3HL** (SEQ ID NO:487)

The construct includes, in the following order: mouse IgGK leader, SC16.15 VH, 15 amino acid GS linker, SC16.15 VL, 5 amino acid linker, 145-2C11 VH, 15 amino acid GS linker, 145-2C11 VL, and Flag tag, as set forth in SEQ ID NO: 487,

and below:

| | |
|---|---|
| METDTLLLWVLLLWVPGSTGD | mouse IgGK leader |
| QVQLQQSGAELAKPGASVKMSCKASGYTFTRYWIH WIKQRPGQGLEWIGYINPTTVYTEFNQNFKDKATLT ADKSSTTASMQLSSLTSEDSAVYYCARGGSNFFDYW GQGTTLTVSS | SC16.15 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPASLAASVGETVAITCRASENIYYNLAWY QQKQGKSPQLLIYTANSLEDGVPSRFSGSGSGTQYSL KINSMQPEDSATYFCKQAYDVPPTFGGGTKLEIK | SC16.15 VL |
| GGGGS | 5 amino acid linker |
| EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGM HWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWD KNYWGQGTMVTVSS | 145-2C11 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNW YQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRD SSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIK | 145-2C11 VL |
| DYKDDDDK | Flag tag |

**SC16.15 DLL3LHxCD3HL (SEQ ID NO:488)**

The construct includes, in the following order: mouse IgGK leader, SC16.15 VL, 15 amino acid GS linker, SC16.15 VH, 5 amino acid linker, 145-2C11 VH, 15 amino acid GS linker, 145-2C11 VL, and Flag tag, as set forth in SEQ ID NO: 488, and below:

| | |
|---|---|
| METDTLLLWVLLLWVPGSTGD | mouse IgGK leader |
| DIQMTQSPASLAASVGETVAITCRASENIYYNLAWY QQKQGKSPQLLIYTANSLEDGVPSRFSGSGSGTQYSL KINSMQPEDSATYFCKQAYDVPPTFGGGTKLEIK | SC16.15 VL |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| QVQLQQSGAELAKPGASVKMSCKASGYTFTRYWIH WIKQRPGQGLEWIGYINPTTVYTEFNQNFKDKATLT ADKSSTTASMQLSSLTSEDSAVYYCARGGSNFFDYW GQGTTLTVSS | SC16.15 VH |
| GGGGS | 5 amino acid linker |
| EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGM HWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWD | magenta highlight is 145-2C11 VH |

| | |
|---|---|
| KNYWGQGTMVTVSS | |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNW YQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRD SSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIK | 145-2C11 VL |
| DYKDDDDK | Flag tag |

**SC16.34 DLL3HLxCD3HL (SEQ ID NO:489)**

The construct includes, in the following order: mouse IgGK leader, SC16.34 VH, 15 amino acid GS linker, SC16.34 VL, 5 amino acid linker, 145-2C11 VH, 15 amino acid GS linker, 145-2C11 VL, and Flag tag, as set forth in SEQ ID NO: 489, and below:

| | |
|---|---|
| METDTLLLWVLLLWVPGSTGD | mouse IgGK leader |
| QIQLVQSGPELKRPGETVKISCKASGYTFTNYGMN WVKQAPGKGLKWMGWINTYTGDPTYADDFKGRF AFSLETSASTAYLQINNLKNEDTATYFCARIGGNSPS DYWGQGTSLTVSS | SC16.34 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVAW YQQKPGQSPKLLIYYASNRYSGVPDRFTGSGYGTDF TFTISTVQAEDLAVYFCQQDYSSPWTFGGGTKLEIK | SC16.34 VL |
| GGGGS | 5 amino acid linker |
| EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGM HWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWD KNYWGQGTMVTVSS | 145-2C11 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNW YQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRD SSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIK | 145-2C11 VL |
| DYKDDDDK | |

**SC16.34 DLL3LHxCD3HL (SEQ ID NO: 490)**

The construct includes, in the following order: mouse IgGK leader, SC16.34 VL, 15 amino acid GS linker, SC16.34 VH, 5 amino acid linker, 145-2C11 VH, 15 amino acid GS linker, 145-2C11 VL, and Flag tag, as set forth in SEQ ID NO: 490, and below:

| | |
|---|---|
| METDTLLLWVLLLWVPGSTGD | mouse IgGK leader |
| SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVAW | SC16.34 VL |

| | |
|---|---|
| YQQKPGQSPKLLIYYASNRYSGVPDRFTGSGYGTDF TFTISTVQAEDLAVYFCQQDYSSPWTFGGGTKLEIK | |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| QIQLVQSGPELKRPGETVKISCKASGYTFTNYGMN WVKQAPGKGLKWMGWINTYTGDPTYADDFKGRF AFSLETSASTAYLQINNLKNEDTATYFCARIGGNSPS DYWGQGTSLTVSS | SC16.34 VH |
| GGGGS | 5 amino acid linker, |
| EVQLVESGGGLVQPGKSLKLSCEASGFTFSGYGM HWVRQAPGRGLESVAYITSSSINIKYADAVKGRFT VSRDNAKNLLFLQMNILKSEDTAMYYCARFDWD KNYWGQGTMVTVSS | 145-2C11 VH |
| GGGGSGGGGSGGGGS | 15 amino acid GS linker |
| DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNW YQQKPGKAPKLLIYYTNKLADGVPSRFSGSGSGRD SSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIK | 145-2C11 VL |
| DYKDDDDK | Flag tag |

1.5 x $10^6$ HEK293T cells were plated one day prior on 6-well plates coated with poly-L-lysine, to achieve 80% confluency. On the day of transfection, 3 µg of DNA was diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios. Cell culture supernatants from each sample were collected after 48 hours of incubation. Some supernatant was concentrated in a 10 kDa spin column (Millipore).

The functionality of these cells transfected with the DLL3xCD3 BiTE encoding plasmids was demonstrated by binding of the DLL3xCD3 bispecific T-cell engagers on the cells to SHP77 cells (ATCC), which have DLL3 on the surface. 500,000 SHP77 cells were seeded in wells of a V-bottom 96-well plate. The cells were washed once with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were resuspended in 50 uL PBS + 2% FBS containing concentrated HEK supernatant corresponding to either untransfected cells or cells transfected with a BiTE. After 30 minutes, the cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes. The cells were then resuspended in 50 uL PBS + 2% FBS containing either anti-Flag biotin (Sigma) at 1:100 dilution or Protein L biotin (Genscript) at 1:200 dilution, and incubated on ice for 30 minutes. The cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes.

Subsequently, the cells were stained with Streptavidin APC (Biolegend) at 1:200 dilution and incubated on ice for 30 minutes in the dark.

The cells were washed twice with PBS + 2% FBS by centrifugation at 1300 RPM for 3 minutes and resuspended in DAPI diluted 1:6000 in PBS + 2% FBS. Flow cytometry data were acquired using the ACEA NovoCyte® flow cytometer (ACEA Biosciences, Inc.), and analyzed using the FlowJo™ software (Tree Star, Inc.).

The table below provides the percent of positive cells gated as APC positive, corresponding to cells stained with BiTE and detected either by Protein L or anti-Flag antibody. SC16.56 DLL3LHxCD3HL, SC16.34 DLL3HLxCD3HL, and SC16.34 DLL3LHxCD3HL BiTEs all have similar detection on SHP77 cells while the other BiTEs are not detected. Therefore, SC16.56 DLL3LHxCD3HL, SC16.34 DLL3HLxCD3HL, and SC16.34 DLL3LHxCD3HL bind to DLL3.

**Binding of cells expressing the DLL3xCD3 bispecific T-cell engagers DLL SHP77 cells**

| | | % Binding to SHP77 Detected by Protein L | % Binding to SHP77 Detected by anti-Flag |
|---|---|---|---|
| Untransfected | Mean (%) | ND | ND |
| SC16.56 DLL3HLxCD3HL BiTE | Mean (%) | ND | ND |
| SC16.56 DLL3LHxCD3HL BiTE | Mean (%) | 36.4 | 20.9 |
| SC16.15 DLL3HLxCD3HL BiTE | Mean (%) | ND | ND |
| SC16.15 DLL3LHxCD3HL BiTE | Mean (%) | ND | ND |
| SC16.34 DLL3HLxCD3HL BiTE | Mean (%) | 36.6 | 19.6 |
| SC16.34 DLL3LHxCD3HL BiTE | Mean (%) | 36.4 | 20.1 |

The expressed DLL3xCD3 bispecific T-cell engagers also were evaluated for binding to DLL3 and to CD3 with an ELISA. This ELISA was developed to detect correctly folded DLL3xCD3 bispecific T-cell engager binding to DLL3 and CD3 in the same assay. Human DLL3 (R&D Systems) was coated overnight at 4 °C on a high protein-binding 96-well plate, at a concentration of 1 ug/ml. The wells then were washed three times with PBS 0.05% Tween-20, and the wells were blocked with ELISA blocking buffer for 1 hour at room temperature. The wells then were washed three times with PBS 0.05% Tween-20. HEK293T cell culture supernatants, containing the DLL3xCD3 bispecific T-cell engagers, were added to the wells and incubated for 2 hours at room temperature. The wells were washed three times with PBS 0.05% Tween-20, and human IgG Fc conjugated CD3e was added to the wells (Acro Biosystems), and incubated for one hour at room temperature. The wells again

were washed three times with PBS 0.05% Tween-20, and horseradish peroxidase (HRP)-conjugated anti-human antibody was added for a 1 hour incubation. The wells were then washed three times with PBS 0.05% Tween-20, and detection reagent (3,3′,5,5′-tetramethylbenzidine (TMB), Thermo Fisher Scientific) was added to the wells. The enzymatic reaction was stopped with sulfuric acid (BioLegend), and the optical densities were read at 450 nm.

The table below provides the results of this ELISA, shown as the absorbance at 450 nm. All of the SC16.56 DLL3LHxCD3HL, SC16.34 DLL3HLxCD3HL, and SC16.34 DLL3LHxCD3HL BiTE antibodies were detected, showing that these BiTEs antibodies are fully functional. SC16.34 DLL3LHxCD3HL has the highest binding in this ELISA, but since cell supernatants were used in this assay, it is not possible to distinguish whether this results from a higher concentration of this bispecific T-cell engager, or higher affinity to its targets compared to the other 3 bispecific T-cell engagers. In a separate experiment, the bispecific T-cell engagers were purified using ANTI-FLAG M2 Affinity Gel (Sigma) and run on this ELISA. SC16.34 DLL3LHxCD3HL was the only purified BiTE to show ELISA signal above background.

**BiTE binding by ELISA**

| | Absorbance at 450 nm | |
|---|---|---|
| | **Mean** | ± SD |
| Untransfected | 0.010 | 0.001 |
| SC16.56 DLL3HLxCD3HL BiTE | 0.007 | 0.002 |
| SC16.56 DLL3LHxCD3HL BiTE | 0.175 | 0.051 |
| SC16.15 DLL3HLxCD3HL BiTE | 0.002 | 0.001 |
| SC16.15 DLL3LHxCD3HL BiTE | 0.001 | 0.002 |
| SC16.34 DLL3HLxCD3HL BiTE | 0.253 | 0.026 |
| SC16.34 DLL3LHxCD3HL BiTE | 1.458 | 0.089 |

## Example 43

### Anti-CTLA-4 scFv-Fc Demonstrates Blockade of the CD80/CTLA-4 and CD86/CTLA-4 Interactions and Co-Expresses with human STING N154S/R284G tazCTT

An scFv-Fc specific for human CTLA-4 (see, SEQ ID NOs:427 and 428 for the nucleic acid and protein sequences, respectively) was designed using the amino acid sequence of ipilimumab. Ipilimumab is a fully human IgG1κ monoclonal antibody that specifically binds human CTLA-4 (see, *e.g.*, the antibody designated

10D1 in U.S. Patent Publication No. 2002/0086014 and in U.S. Patent No. 6,984,720), blocking CTLA-4's immune inhibiting interaction with CD80 (also known as B7.1 or B7-1) and CD86 (also known as B7.2 or B7-2).

To generate the ipilimumab scFv antibody fragment (see, SEQ ID NO:429), the variable light chain ($V_L$) and variable heavy chain ($V_H$) of ipilimumab were linked with a 20 amino acid long glycine-serine (GS) linker ($(GGGGS)_4$). To generate the scFv-Fc antibody fragment (see, SEQ ID NO:428), the variable heavy chain of the ipilimumab scFv was linked to a human IgG1 Fc, containing a mutation of the free cysteine in the hinge region to a serine (at position 272 in SEQ ID NO:428). The leader sequence (METPAQLLFLLLLWLPDTTG; corresponding to residues 1-20 in SEQ ID NO:428) was derived from the sequence of the human immunoglobulin kappa variable 3-20 (IGKV3-20) protein. The sequence was codon optimized for human using the GenScript GenSmart™ Codon Optimization tool (ATGGAGACACCTGCCCAGCTGCTGTTCCTGCTGCTGCTGTGGCTGCCCGA CACCACCGGC); the sequence is set forth in SEQ ID NO:491.

The neutralizing ability of the anti-CTLA-4 scFv-Fc was determined using competitive ELISAs to measure the ability of the antibody fragment to block the interactions between CTLA-4 and its ligands, CD80 and CD86. HEK293T cells were transfected with 3 micrograms of DNA encoding the anti-CTLA-4 scFv-Fc antibody fragment construct, using the FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios. Forty-eight hours post-transfection, HEK293T cell-free culture supernatants were harvested, filtered, and used in a competitive ELISA to assess the blockade activity of the anti-CTLA-4 antibody fragment.

For the competitive ELISAs, human CD80 or CD86 recombinant proteins (R&D Systems) were coated overnight at 4 °C on a high protein-binding 96-well plate, at a concentration of 100 ng/ml. The wells were then washed three times with PBS 0.05% Tween-20, and the wells were blocked with ELISA blocking buffer for 1 hour at room temperature. The wells were then washed three times with PBS 0.05% Tween-20. HEK293T cell culture supernatants, containing each of the anti-CTLA-4 antibody fragments, were mixed with 10 ng/ml of a recombinant human CTLA-4-human IgG1 Fc chimera (R&D Systems), and added to the wells and incubated for 2 hours at room temperature. The wells were then washed three times with PBS 0.05%

Tween-20, and horseradish peroxidase (HRP)-conjugated anti-human IgG1 antibody was added to the wells (Jackson ImmunoResearch), and incubated for one hour at room temperature. The wells were then washed three times with PBS 0.05% Tween-20, and detection reagent (3,3′,5,5′-tetramethylbenzidine (TMB), Thermo Fisher Scientific) was added to the wells. The enzymatic reaction was stopped with sulfuric acid (BioLegend), and the optical densities were read at 450 nm.

The results of the competitive ELISAs are summarized in the table below. The anti-CTLA-4 scFv-Fc blocked the binding of CTLA-4 to CD86 by 94.14% and blocked the binding of CTLA-4 to CD80 by 83.46%. The CTLA4 scFv-Fc had a higher degree of CD86/CTLA-4 blocking activity compared with CD80/CTLA-4 blocking activity.

**Competitive ELISA Results**

| | | **% CD86 Blockade Activity** | **% CD80 Blockade Activity** |
|---|---|---|---|
| Human Anti-CTLA-4 scFv-Fc | Mean (%) | 94.14 | 83.46 |
| | ± SD | 0.25 | 0.32 |

Human anti-CTLA4 scFv-Fc (SEQ ID NO:427) was cloned into expression vectors with huSTING N154S/R284G tazCTT either containing or not containing the Vascular Endothelial Growth Factor and Type 1 Collagen Inducible Protein (VCIP) IRES. HEK293T STING Null Cells (293-Dual™ Null Cells; InvivoGen) were used, which do not contain endogenous STING, and express secreted embryonic alkaline phosphatase (SEAP), placed under the control of the endogenous IFN-stimulated response element (ISRE) promoter, where the coding sequence of ISRE is replaced by the SEAP ORF using knock-in technology. STING activity, thus, is assessed by monitoring type I IFN induced SEAP production. The 293-Dual™ Null cells also express Lucia™ luciferase, a secreted luciferase, placed under the control of the endogenous IFN-β promoter; the coding sequence of IFN-β has been replaced by the Lucia™ luciferase ORF using knock-in technology. This allows for the assessment of STING activity by monitoring the expression of IFN-β. Using these cells, STING activity can be assessed by monitoring ISRE-induced SEAP production and/or IFN-β-dependent expression of Lucia™ luciferase. The two reporter proteins, SEAP and Lucia™ Luciferase, can be measured in the cell supernatant using standard assays

and detection reagents, such as the QUANTI-Blue™ and QUANTI-Luc™ detection reagents (InvivoGen), respectively.

The cells were seeded in 24-well plates coated with poly-L-lysine at 200,000 cells per well, and incubated overnight at 37 °C in a 5% $CO_2$ incubator, to achieve 80% confluency. The following day, 300 ng of each plasmid DNA, and 40 ng of a CMV-GFP vector (*i.e.*, a vector encoding green fluorescent protein under the control of a CMV promoter), were diluted in serum-free media and added to FuGENE® transfection reagent (Promega), at the proper reagent:DNA ratios, with untransfected wells serving as negative controls (in duplicates). Cell culture supernatants from each sample were collected 48 hours post-transfection.

The STING activity of the huSTING N154S/R284G tazCTT variant was evaluated using the ISRE-SEAP and IFN-β-Lucia™ reporter systems. The type I interferon (IFN) activity (induced by STING) was assessed by monitoring type I IFN-stimulated SEAP production in the cell supernatants. 20 µL of cell culture supernatant was added to 180 µL of QUANTI-Blue™ reagent (InvivoGen), which is used for measuring SEAP. Type I interferon activation was determined by measuring ISRE-induced SEAP activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), at an absorbance wavelength of 650 nm. The type I interferon (IFN) activity (induced by STING) also was assessed by monitoring the type I IFN-stimulated Lucia™ luciferase production in the cell supernatants. 20 µL of cell culture supernatant was added to 50 µL of QUANTI-Luc™ reagent (InvivoGen), which is used for measuring Lucia™ luciferase activity. Type I interferon activation was determined by measuring IFNβ-induced Lucia™ luciferase activity on a SpectraMax® M3 Spectrophotometer (Molecular Devices), on the luminescence setting.

Cell culture supernatants also were assessed for the expression of human CTLA4 scFv-Fc. Human CTLA4 (R&D Systems) was coated overnight at 4 °C on a high protein-binding 96-well plate, at a concentration of 1 ug/ml. The wells were then washed three times with PBS 0.05% Tween-20, and the wells were blocked with ELISA blocking buffer for 1 hour at room temperature. The wells were then washed three times with PBS 0.05% Tween-20. HEK293T cell culture supernatants, containing anti-CTLA4 scFv-Fc were added to the wells and incubated for 2 hours at

room temperature. The wells were washed three times with PBS 0.05% Tween-20, and horseradish peroxidase (HRP)-conjugated anti-human antibody was added for a 1 hour incubation. The wells were then washed three times with PBS 0.05% Tween-20, and detection reagent (3,3′,5,5′-tetramethylbenzidine (TMB), Thermo Fisher Scientific) was added to the wells. The enzymatic reaction was stopped with sulfuric acid (BioLegend), and the optical densities were read at 450 nm.

The expression results of the human anti-CTLA4 scFv-Fc + hSTING N154S/R284G tazCTT constructs are shown below. Constructs containing both payloads retained expression of each protein. The human anti-CTLA4 scFv-Fc T2A hSTING N154S/R284G tazCTT construct had better STING activity without the VCIP IRES. Expression of anti-CTLA4 scFv-Fc was higher by ELISA from the construct without VCIP IRES, demonstrating that individual targets can require unique elements for improved expression.

**Human anti-CTLA4 scFv-Fc + STING Expression Normalized by Transfection Efficiency**

| | **ISRE-SEAP (Absorbance / %GFP)** | | **IFNβ-Lucia (Luminesence / %GFP)** | | **Hu anti-CTLA4 scFv-Fc (Absorbance / %GFP)** | |
|---|---|---|---|---|---|---|
| | Mean (%) | ± SD | Mean (%) | ± SD | Mean (%) | ± SD |
| Untransfected | ND | ND | ND | ND | ND | ND |
| hu anti-CTLA4 scFv-Fc | 0.00006 | 0.00003 | 0.13 | 0.029 | 0.129 | 0.006 |
| VCIP hu anti-CTLA4 scFv-Fc | 0.0001 | 0.00008 | ND | ND | 0.080 | 0.009 |
| huSTING N154S/R284G tazCTT | 0.095 | 0.005 | 17.567 | 2.859 | ND | ND |
| hu anti-CTLA4 scFv-Fc T2A hSTING N154S/R284G tazCTT | 0.063 | 0.004 | 6.698 | 0.222 | 0.028 | 0.001 |
| VCIP hu anti-CTLA4 scFv-Fc T2A hSTING N154S/R284G tazCTT | 0.050 | 0.005 | 5.213 | 0.191 | 0.017 | 0.00003 |

## Example 44

## Bacterial Strain Infection of Macrophages Allows the Presentation of Immunogenic Peptide by MHC-I Molecules

This Example shows that the bacteria provided herein bactofect antigen presenting cells with either mRNA delivery or plasmid DNA delivery, and the resulting expressed product is presented by antigen presenting cells. The anti-SIINFEKL/H-2Kb antibody, used for this demonstration, binds specifically to the

ovalbumin SIINFEKL [SEQ ID NO:492] peptide only when presented by MHC-I (H-2Kb) at the surface of IC21 cells. This demonstrates that the vaccines strains provided herein, such as those that encode STING + IL-15 receptor complex + cancer antigen, can treat immune desert tumors, also can could be used as a cancer vaccine in the neoadjuvant setting, also can be used as a platform to present pathogenic antigens for prophylactic vaccination, and can be used as a treatment for pathogen infection, such as, for example, MRSA, chronic viral hepatitis infection, chronic p.gingivalis, HIV, and other chronic infections, as well as acute infection.

In demonstrating the above, the experiment in this Example, shows how the infection of antigen-presenting cells by the exemplary strain, designated *YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD*, leads to presentation of immunogenic peptides by the major histocompatibility complex class-I (MHC-I) to CD8+ T cells. YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD strains encoding the chicken ovalbumin under the eukaryotic CMV promoter and the prokaryotic MTL promoter followed or not with the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) were generated. The chicken ovalbumin is a model antigen, and the immune-dominant SIINFEKL peptide derived from the chicken ovalbumin was monitored for its presentation by the murine H-2Kb MHC-I molecules at the surface of antigen-presenting cells. The murine peritoneal macrophages cell line IC21 (ATCC) was used as antigen-presenting cell in the assay.

Fifty thousand IC21 cells per well of a 96 well flat-bottom plate were seeded in 100ul RPMI 10% FBS and left in a 37°C and 5% $CO_2$ incubator over-night. The next day, IC21 cells were infected with *YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD* strains containing plasmids encoding the chicken ovalbumin under the regulation of the CMV or MTL promoters. The bacterial strains were added to the cultured cells and the plate was centrifugated at 500g for 5 minutes at room temperature. Cells were then incubated for 1h at 37°C and culture media containing gentamycin was added for a final concentration of 50ug/ml. Infected cells were left in at 37°C and 5% $CO_2$ incubator for seven hours. Seven hours after the infection, IC21 cells were detached using PBS 10mM EDTA and stained with anti-murine F4/80 APC-conjugated antibodies and anti-SIINFEKL/H-2Kb PE-conjugated antibodies (both from Biolegend), and the data was acquired using the ACEA Novocyte flow-cytometer.

The anti-SIINFEKL/H-2Kb antibody binds specifically to the ovalbumin SIINFEKL peptide only when presented by MHC-I (H-2Kb) at the surface of IC21 cells. The mean fluorescence intensity for the H-2Kb/SIINFEKL presentation by IC21 cells seven hours post-infection was measured. The results are presented in the table below, which shows the net mean fluorescence intensity (Net MFI) observed in the groups treated with the ovalbumin encoding strains over the uninfected cells.

| Treatment | Net Mean Fluorescence Intensity over uninfected (Net MFI) | SEM* |
|---|---|---|
| Uninfected | 0 | NA |
| CMV-Ovalbumin | 606 | 54 |
| MTL-IRES-Ovalbumin | 632 | 3.5 |
| MTL-Ovalbumin | 384.5 | 42 |

*SEM = standard error of the mean

This Example shows the ability of strains, such as *YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD*, to deliver antigens that are processed by the antigen-presenting cells and presented to CD8+ T cells upon delivery, to mount a T cell specific response against antigens relevant to or from pathogens or cancer cells.

Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

**WHAT IS CLAIMED:**

1. An immunostimulatory bacterium, comprising a plasmid that encodes a therapeutic product, wherein the bacterium comprises genome modifications, whereby the bacterium does not produce active thymidylate synthase, and requires supplementation for growth.

2. The immunostimulatory bacterium of claim 1, wherein the supplementation for growth comprises thymine, thymine derivatives, thymidine, thymidine derivatives, thymine precursor(s), thymidine precursor(s), or thymidine monophosphate precursor(s).

3. The immunostimulatory bacterium of claim 1 or claim 2, wherein the bacterium comprises genome modifications that reduce or eliminate activation of TLR2, and optionally TLR4 and/or TLR5.

4. An immunostimulatory bacterium, comprising a plasmid that encodes a therapeutic product, wherein;

the bacterium comprises genome modifications, whereby the bacterium does not secrete active asparaginase; and

the bacterium comprises genome modifications that reduce or eliminate activation of TLR2, and optionally TLR4 and/or TLR5.

5. The immunostimulatory bacterium of any of claims 1-4, wherein the genome of the immunostimulatory bacterium is modified by deletion or disruption or modification of all or of a sufficient portion of the gene *ansB* encoding L-asparaginase II, whereby the bacterium is *ansB*$^-$ and does not express active L-asparaginase II.

6. An immunostimulatory bacterium, comprising genome modification(s) that reduce or eliminate activation of TLR2, whereby induction of type I IFN is not inhibited by TLR2, wherein:

the immunostimulatory bacterium comprises genome modification(s) whereby it cannot replicate *in vivo*, but can replicate when grown *in vitro* with nutritional supplementation; and

the genome modification(s) eliminate(s) or inactivate(s) thymidylate synthase, whereby the bacterium is *thyA*$^-$.

7. An immunostimulatory bacterium, comprising genome modification(s) that reduce or eliminate activation of TLR2, whereby induction of type I IFN is not

inhibited by TLR2, wherein the immunostimulatory bacterium comprises a plasmid that encodes an interferon, or encodes a modified STING protein that constitutively induces type I interferon, and encodes an antigen or protein from a pathogen or tumor.

8. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product, wherein:

the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has been modified to generate lipopolysaccharide (LPS) with penta-acylated lipid A;

lipopolysaccharide with hexa-acylated lipid A is substantially reduced, by at least 10-fold, compared to the wild-type bacterium, or is absent;

the genome of bacterium is modified to be auxotrophic for an essential nutrient;

the genome of the bacterium is modified, whereby the bacterium itself does not inhibit or prevent induction of type I interferon (IFN) in an infected immune cell; and

the genome of the bacterium is modified, whereby it does not encode or produce active asparaginase and/or thymidylate synthase.

9. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product, wherein:

the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has been modified to generate lipopolysaccharide (LPS) with penta-acylated lipid A;

lipopolysaccharide with hexa-acylated lipid A is substantially reduced, by at least 10-fold, compared to the wild-type bacterium, or is absent; and

the genome of bacterium is modified whereby it does not produce active thymidylate synthase, whereby the bacterium is *thyA*$^-$.

10. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product, wherein:

the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium lacks flagella;

the unmodified immunostimulatory bacterium has flagella; and

the genome of the bacterium is modified, whereby it does not produce active thymidylate synthase.

11. The immunostimulatory bacterium of claim 9 or claim 10, wherein genome modifications comprise deletions, insertions, and/or replacements whereby the bacterium is *thyA*$^-$.

12. The immunostimulatory bacterium of any of claims 1-11, wherein the genome is modified so that the bacterium lacks curli fimbriae.

13. The immunostimulatory bacterium of any of claims 1-12, wherein the bacterium comprises genome modifications that reduce or eliminate activation of TLR4 and/or TLR5.

14. The immunostimulatory bacterium of any of claims 1-13, wherein:

the genome modifications result in a bacterium that does not have flagella; and

the wild-type of the bacterium has flagella.

15. The immunostimulatory bacterium of any of claims 1-14, wherein the bacterium does not produce curli fimbriae.

16. The immunostimulatory bacterium of any of claims 1-15, wherein genome modifications result in penta-acylated lipopolysaccharides.

17. The immunostimulatory bacterium of any of claims 1-16, wherein the immunostimulatory bacterium lacks flagella and is *msbB*$^-$/*pagP*$^-$.

18. An immunostimulatory bacterium, comprising genome modifications that reduce or eliminate activation of TLR2, whereby induction of type I IFN is not inhibited by TLR2, wherein:

the immunostimulatory bacterium can replicate *in vivo* in a eukaryotic host; and

the immunostimulatory bacterium comprises a plasmid that encodes a tumor-associated antigen, or encodes a tumor antigen and a STING protein, or encodes a tumor-associated antigen and interferon alpha, or encodes a tumor-associated antigen and interferon beta.

19. The immunostimulatory bacterium of any of claims 1-18, wherein the bacterium encodes a STING protein that is a modified STING protein that has increased induction of type I interferon compared to the unmodified human STING protein.

20. The immunostimulatory bacterium of claim 19, wherein the modified STING protein is a modified STING protein of any of claims 406-431.

21. The immunostimulatory bacterium of claim 20, wherein the modified STING protein comprises the replacement N154S/R284G.

22. The immunostimulatory bacterium of any of claims 1-21, wherein a therapeutic product encoded in the plasmid is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), or the encoded product is interferon-alpha or is interferon-beta, or the encoded product is both IFN-alpha and IFN-beta.

23. The immunostimulatory bacterium of claim 22, wherein the therapeutic product that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN) is a STING protein.

24. The immunostimulatory bacterium of any of claims 1-23, wherein the plasmid encodes an antigen, epitope(s), or protein from a pathogen or a tumor.

25. An immunostimulatory bacterium, comprising a plasmid encoding a combination of heterologous products, wherein:

the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has attenuated recognition by TLR2, and optionally one or both of TLR4 and TLR5;

one product is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN); and

a second product that is an antigen, an epitope or epitopes from is antigen, or is protein for immunization against the pathogen or tumor.

26. The immunostimulatory bacterium of claim 25, wherein the genome of the bacterium is modified whereby the bacterium does not have curli fimbriae.

27. An immunostimulatory bacterium, comprising nucleic acid operatively linked to a prokaryotic promoter, wherein:

the nucleic acid encodes RNA that lacks sequences necessary for translation by a prokaryote, whereby the RNA is produced in the bacterium; and

the encoded RNA lacks a Shine-Dalgarno sequence, and/or comprises an Internal Ribosome Entry Site (IRES), and/or a translational read through 2A peptide.

28. An immunostimulatory bacterium, comprising nucleic acid operatively linked to a prokaryotic promoter, wherein the nucleic acid comprises RNA that lacks sequences necessary for translation by a prokaryote.

29. The immunostimulatory bacterium of claim 28, wherein the RNA lacks a Shine-Dalgarno sequence.

30. The immunostimulatory bacterium of any of claims 27-29, wherein the 2A peptide is one or more of T2A, P2A, E2A, or F2A.

31. The immunostimulatory bacterium of any of claims 1-30, wherein:

the nucleic acid encoding a therapeutic product is operatively linked to a prokaryotic promoter, wherein:

the nucleic acid encodes RNA that lacks sequences necessary for translation by a prokaryote, whereby the RNA is produced in the bacterium, wherein:

the RNA lacks a Shine-Dalgarno sequence, and comprises an Internal Ribosome Entry Site (IRES), or a translational read through 2A peptide.

32. The immunostimulatory bacterium of any of claims 27-31, wherein the prokaryotic promoter is a bacterial promoter or a bacteriophage promoter.

33. The immunostimulatory bacterium of claim 32, wherein the promoter is a bacteriophage promoter.

34. The immunostimulatory bacterium of claim 32 or claim 33, wherein the immunostimulatory bacterium encodes a phage RNA polymerase.

35. The immunostimulatory bacterium of claim 32, wherein the promoter is selected from a promoter comprising any of SEQ ID NOs: 393-396, respectively:

attatgtcttgacatgtagtgagtgggctggtataatgcagcaag, or

ttatgcttgacgctgcgtaaggtttttgttataatacaccaag, or

attatgtcttgacatgtagtgagtgggctggtaaatgcagcaag, or

gatcccggagttcatgcgtgatgcaatgaaagtgccgttctacttcggtgggacctcactgcttatcgttgttgtcgtgattatggact
ttatggctcaagtgcaaactctgatgatgtccagtcagtatgagtctgcattgaagaaggcgaacctgaaaggctacggccgttaa
ttggtcgcctgagaagttacggagagtaaaaatgaaagttcgtgcttccgtcaagaaattatgccgtaactgcaaaatcgttaagc
gtgatggtgtcatccgtgtgatttgcagtgccgagccgaagcataaacagcgccaaggctgatttttttcgcatatttttcttgcaaag
ttgggttgagctggctagattagccagccaatcttttgtatgtctgtacgtttccatttgagtatcctgaaaacgggcttttcagcatgg
tacgtacatattaaatagtaggagtgcatagtggcccgtatagcaggcattaacattcctgatcagaaacacgccgtgatcgcgtta
acttcgatctacggtgtcggcaagacccgttctaaagccatcctggctgcagcgggtatcgctgaaaatgttaagatcctctagatt
taagaaggagatatacat (*Salmonella* rpsm promoter).

36. The immunostimulatory bacterium of any of claims 27-35, wherein the immunostimulatory bacterium comprises genomic modifications whereby the bacterium infects tissue-resident myeloid cells, and does not infect epithelial cells.

37. The immunostimulatory bacterium of claim 36, wherein:

the bacterium lacks flagella; and

the bacterium without the genomic modifications comprises flagella.

38. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product, wherein infection of a macrophage by the bacterium converts a human M2 macrophage to an M1 or M1-like phenotype macrophage.

39. The immunostimulatory bacterium of claim 38, wherein the therapeutic product is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN).

40. The immunostimulatory bacterium of claim 39, wherein the expression of the type I IFN is constitutive.

41. The immunostimulatory bacterium of claim 39 or claim 40, wherein the therapeutic product is a gain-of-function (GOF) variant of the therapeutic product that is part of the cytosolic DNA/RNA sensor pathway, wherein the variant GOF product does not require cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides to result in expression of type I IFN.

42. The immunostimulatory bacterium of any of claims 39-41, wherein the product is a variant STING protein.

43. The immunostimulatory bacterium of any of claims 38-42, wherein infection by the bacterium converts human M2 macrophages into M1-like type I IFN producing cells.

44. The immunostimulatory bacterium of any of claims 1-43 that lacks flagella, wherein the wild-type bacterium has flagella, and is *pagP*$^-$/*msbB*$^-$.

45. The immunostimulatory bacterium of claim 44 that is a *Salmonella* strain.

46. The immunostimulatory bacterium of any of claims 1-45, wherein the therapeutic product is an anti-cancer therapeutic.

47. The immunostimulatory bacterium of any of claims 1-45, wherein the therapeutic product is an anti-viral therapeutic or an anti-pathogenic bacterial therapeutic.

48. The immunostimulatory bacterium of claim 47, wherein the anti-viral therapeutic inhibits a viral enzyme or inhibits viral replication.

49. The immunostimulatory bacterium of claim 47, wherein the therapeutic is an anti-viral therapeutic is a viral antigen whose expression results in an immune-protective response against a virus, or the anti-viral therapeutic is an antibody that binds to or interacts with a viral antigen, whereby a virus is inhibited or blocked, or anti-viral immunity results.

50. The immunostimulatory bacterium of claim 47, wherein the therapeutic is an anti-bacterial therapeutic that is a bacterial antigen whose expression results in an immune-protective response against the bacterial pathogen, or the anti-bacterial therapeutic is an antibody that binds to or interacts with a bacterial antigen, whereby the pathogenic bacterium is inhibited or blocked, or anti-pathogenic bacterium immunity results.

51. The immunostimulatory bacterium of any of claims 47-50, wherein the immunostimulatory bacterium encodes an anti-viral therapeutic for treating a virus or infectious agent that causes persistent infection.

52. The immunostimulatory bacterium of any of claims 47-49 and 51, wherein the anti-viral therapeutic is a viral antigen or epitope of an antigen, and the antigen comprises a viral surface protein, or a viral nucleocapsid protein, or a viral nonstructural protein, or a virus open reading frame protein.

53. The immunostimulatory bacterium of claim 51, wherein the virus or infectious agent causes chronic infection.

54. The immunostimulatory bacterium of claim 51, wherein the virus or infectious agent causes latent infection.

55. The immunostimulatory bacterium of claim 51, wherein the virus or infectious agent causes slow infection.

56. The immunostimulatory bacterium of claim 51, wherein the virus or infectious agent is selected from among T-Cell leukemia viruses, Epstein-Barr virus, cytomegalovirus, herpesviruses, varicella zoster virus, measles virus, papovaviruses, prions, hepatitis virus type A, B, C, D and E, adenoviruses, parvoviruses, human immunodeficiency virus (HIV), coronaviruses, smallpox virus, poliovirus, influenza virus, rotavirus, yellow fever virus, mumps virus, rubella virus, and papillomaviruses.

57. The immunostimulatory bacterium of claim 51, wherein the virus is HIV or is a hepatitis virus.

58. The immunostimulatory bacterium of claim 51, wherein the infectious agent is a prion or a protozoan.

59. The immunostimulatory bacterium of any of claims 47-58, wherein the therapeutic product is a viral surface antigen or portion thereof sufficient to produce an immune response in a host.

60. The immunostimulatory bacterium of any of claims 51-59, wherein the therapeutic product interferes with viral gene expression or replication.

61. The immunostimulatory bacterium of any of claims 1-60, wherein the bacterium encodes an immunostimulatory protein.

62. The immunostimulatory bacterium of claim 61, wherein the immunostimulatory protein is a Stimulator of Interferon Genes (STING) protein, a modified STING protein, a cytokine, a chemokine, or a co-stimulatory receptor or ligand.

63. The immunostimulatory bacterium of any of claims 1-62, wherein the bacterium comprises a genomic modification whereby it lacks flagella.

64. The immunostimulatory bacterium of any of claims 1-63, wherein the bacterium comprises a genomic modification whereby it is *pagP*$^-$ or *msbB*$^-$/*pagP*$^-$.

65. The immunostimulatory bacterium of any of claims 1-64, wherein:

the bacterium comprises a bacterial genomic modification whereby it does not express asparaginase or activate the synthesis of secreted asparaginase; and/or

the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of the gene *ansB* encoding L-asparaginase II, whereby the bacterium is *ansB*$^-$ and does not express active L-asparaginase II.

66. The immunostimulatory bacterium claim 65, wherein the immunostimulatory bacterium is an anti-cancer therapeutic that colonizes tumors and/or the tumor microenvironment, whereby the *ansB*$^-$ phenotype reduces or eliminates production of active asparaginase

67. An immunostimulatory bacterium, comprising nucleic acid operatively linked to a prokaryotic promoter, wherein:

the nucleic acid comprises RNA that lacks sequences necessary for translation by a prokaryote, whereby the RNA is produced in the bacterium, but cannot be translated by the bacterium;

the bacterium has genomic modifications whereby infection is restricted to myeloid cells; and

the RNA encodes a therapeutic product or is a therapeutic product.

68. An RNA delivery system, comprising an immunostimulatory bacterium that primarily or solely infects myeloid cells and that comprises RNA encoded by the bacterium under control of a prokaryotic promoter, wherein:

the RNA lacks regulatory sequences necessary for translation by the bacterium; and

the RNA encodes a therapeutic product or is a therapeutic product.

69. The immunostimulatory bacterium of claim 67 or the RNA delivery system of claim 68, wherein the transcribed RNA lacks a Shine-Dalgarno sequence.

70. The immunostimulatory bacterium of claim 67 or the RNA delivery system of claim 68, wherein the transcribed RNA lacks a Shine-Dalgarno sequence and comprises a Kozak consensus sequence.

71. The immunostimulatory bacterium or RNA delivery system of claim 70, wherein the Kozak consensus sequence is ACCAUGG (SEQ ID NO: 397).

72. The immunostimulatory bacterium or RNA delivery system of any of claims 67-70, wherein the bacterium lacks flagella and contains a genome modification, whereby it is *pagP*$^-$/*msbB*$^-$.

73. The immunostimulatory bacterium or RNA delivery system of any of claims 67-72, wherein the RNA encoding the therapeutic product or that is the therapeutic product is encoded on a plasmid.

74. The immunostimulatory bacterium or RNA delivery system of any of claims 67-73 wherein the nucleic acid encoding the RNA is operatively linked to a prokaryotic promoter that is inducible.

75. The immunostimulatory bacterium or RNA delivery system of any of claims 67-73, wherein the nucleic acid encoding the RNA is operatively linked to a prokaryotic promoter that is constitutive.

76. The immunostimulatory bacterium or RNA delivery system of any of claims 67-75, wherein the nucleic acid encoding the RNA comprises an Internal Ribosome Entry Site (IRES).

77. An immunostimulatory bacterium or RNA delivery system of any of claims 1-76, wherein the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of the gene *ansB*, encoding L-asparaginase II, whereby the bacterium is *ansB*$^-$ and does not express active L-asparaginase II.

78. An immunostimulatory bacterium or RNA delivery system of any of claims 1-77, wherein the genome of the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of the gene *ansB*, encoding L-

asparaginase II, and by deletion or disruption of all or of a sufficient portion of the gene *csgD*, whereby the bacterium is *ansB*$^-$ and does not express active L-asparaginase II, and is *csgD*$^-$ and does not activate the synthesis of curli fimbriae.

79. The immunostimulatory bacterium or RNA delivery system of any of claims 1-78, wherein the bacterial genome further comprises deletion of or disruption of all or of a sufficient portion of the genes encoding the flagella, whereby the bacterium is flagellin$^-$ and does not produce flagella, wherein the wild-type bacterium has flagella.

80. The immunostimulatory bacterium or RNA delivery system of claim 79, wherein the genome of the bacterium is further modified by deletion or disruption of all or of a sufficient portion of the gene *csgD*, whereby the bacterium is *csgD*$^-$ or has another or additional modification in the genome whereby biofilm formation is impaired.

81. The immunostimulatory bacterium or RNA delivery system of claim 79, wherein the genome of the bacterium is further modified by deletion or disruption of all or of a sufficient portion of genes, whereby the bacterium is *csgD*$^-$/*msbB*$^-$/*pagP*$^-$.

82. The immunostimulatory bacterium or RNA delivery system of any of claims 69-81, wherein the bacterium comprises modification of the genome, whereby the bacterium lacks flagella.

83. An immunostimulatory bacterium, comprising genome modifications whereby the bacterium lacks flagella, and is *lppA*$^-$/*lppB*$^-$, and optionally is *csgD*$^-$.

84. The immunostimulatory bacterium of any of claims 1-83, wherein the bacterium is auxotrophic for purines.

85. The immunostimulatory bacterium of any of claims 1-83, wherein the bacterium is auxotrophic for adenosine or is auxotrophic for adenosine, adenine, and ATP.

86. The immunostimulatory bacterium of any of claims 1-85, wherein the bacterium is *purI*$^-$.

87. The immunostimulatory bacterium of any of claims 1-86, wherein the bacterium is *pagP*$^-$.

88. The immunostimulatory bacterium of any of claims 1-87, wherein the bacterium is *asd*$^-$ or *thyA*$^-$ or both.

89. The immunostimulatory bacterium of any of claims 1-88 that is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$), wherein the bacterium is *asd*$^-$ by virtue of disruption of or deletion of all or a portion of the endogenous gene encoding aspartate-semialdehyde

dehydrogenase (*asd*), whereby endogenous *asd* is not expressed or functional enzyme is not produced, or is *thyA*$^-$ by virtue of the disruption of or deletion of all or a portion of the endogenous gene or genes, whereby endogenous thymidylate synthase is not expressed or functional enzyme is not produced.

90. An immunostimulatory bacterium that is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$), wherein:

the bacterium is *asd*$^-$ by virtue of disruption of or deletion of all or a portion of the endogenous gene encoding aspartate-semialdehyde dehydrogenase (*asd*), whereby endogenous *asd* is not expressed or functional enzyme is not produced; and

the bacterium is *thyA*$^-$ by virtue of the disruption of or deletion of all or a portion of the endogenous gene or genes, whereby endogenous thymidylate synthase is not expressed or functional enzyme is not produced.

91. The immunostimulatory bacterium of claim 90, wherein the unmodified bacterium is a *Salmonella* bacterium.

92. The immunostimulatory bacterium of any of claims 1-91 that encodes aspartate-semialdehyde dehydrogenase (*asd*) on the plasmid under control of a bacterial promoter.

93. The immunostimulatory bacterium of any of claims 1-92, wherein the bacterium is *msbB*$^-$.

94. The immunostimulatory bacterium of any of claims 1-93 that is *asd*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ and *pagP*$^-$.

95. The immunostimulatory bacterium of any of claims 1-94 that is *asd*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ and *pagP*$^-$; or *thyA*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ and *pagP*

96. The immunostimulatory bacterium of any of claims 1-95 that is *ansB*$^-$, *asd*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ and *pagP*$^-$; or *ansB*$^-$, *thyA*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ and *pagP*$^-$

97. The immunostimulatory bacterium of any of claims 1-96, wherein an encoded therapeutic product is an anti-cancer therapeutic.

98. The immunostimulatory bacterium of any of claims 1-97, wherein the plasmid encodes a modified STING and IL-15 or IL-15/IL-15R alpha chain complex, wherein the STING constitutively induces type I IFN in the absence of cGAS and/or any STING ligands.

99. The immunostimulatory bacterium any of claims 1-98, wherein the bacterium is *ansB*$^-$, *thyA*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$ and *pagP*$^-$.

100. The immunostimulatory bacterium of any of claims 1-99, wherein nucleic acid encoding the therapeutic product is operably linked to nucleic acid encoding a secretion signal, whereby, when expressed, the therapeutic product is secreted.

101. The immunostimulatory bacterium of any of claims 1-100, wherein an encoded therapeutic product is an anti-viral product.

102. The immunostimulatory bacterium of any of claims 1-100, wherein the encoded therapeutic product is a viral antigen.

103. The immunostimulatory bacterium of any of claims 100-102, wherein the encoded therapeutic product is an anti-viral antibody.

104. The immunostimulatory bacterium of any of claims 100-103, wherein:

the encoded therapeutic product is an anti-viral product; and

the virus that is selected is one that causes chronic infections or latent infections.

105. The immunostimulatory bacterium of any of claims 100-104, wherein:

the encoded therapeutic product is an anti-viral product; and

the virus is selected from among a hepatitis virus, a herpes virus, a varicella zoster virus, a poxvirus, a measles virus, and a retrovirus.

106. The immunostimulatory bacterium of any of claims 1-105, wherein the number of copies of the plasmid is greater than 150.

107. The immunostimulatory bacterium of any of claims 1-105, wherein the number of copies of the plasmid is 150 copies or fewer, or is less than or equal to 150.

108. The immunostimulatory bacterium of any of claims 1-105, wherein the plasmid is present in low copy number, and low copy number is less than 25 or less than 20 or less than about 25 or less than about 20 copies.

109. The immunostimulatory bacterium of any of claims 1-108, wherein an encoded therapeutic product is a nucleic acid or a protein.

110. The immunostimulatory bacterium of claim 109, wherein the encoded therapeutic product is a protein.

111. The immunostimulatory bacterium of any of claims 1-110, wherein the plasmid encodes two or more therapeutic products.

112. The immunostimulatory bacterium of any of claims 109-111, wherein the encoded therapeutic product(s) on the plasmid is/are anti-cancer treatment(s).

113. The immunostimulatory bacterium of any of claims 109-111, wherein the encoded therapeutic product(s) on the plasmid is/are anti-viral treatment(s).

114. The immunostimulatory bacterium of claim 112 or claim 113, wherein the bacterium encodes two or more products selected from among a cytokine, a protein that constitutively induces a type I IFN, and a co-stimulatory receptor or molecule.

115. The immunostimulatory bacterium of claim 114, wherein the co-stimulatory molecule lacks a cytoplasmic domain.

116. The immunostimulatory bacterium of any of claims 1-115, wherein the nucleic acid encoding one or more of the therapeutic product or products comprises nucleic acid encoding a signal for secretion of the therapeutic product(s) from a cell comprising the bacterium.

117. The immunostimulatory bacterium of any of claims 1-116, wherein the nucleic acid encoding the product on the plasmid is operatively linked to regulatory sequences recognized by a eukaryotic host.

118. The immunostimulatory bacterium of any of claims 1-117, wherein the immunostimulatory bacterium encodes two or more products, and expression of each product is under control of a separate promoter, or expression of all is under control of a single promoter, and each product is separated by nucleic acid encoding a 2A peptide to effect separate translation of each encoded therapeutic product.

119. The immunostimulatory bacterium of claim 118, wherein the nucleic acid encodes a T2A, F2A, E2A, or P2A peptide to effect separate expression of therapeutic products expressed under control of a single promoter.

120. The immunostimulatory bacterium of any of claims 1-119, wherein the eukaryotic promoter is an RNA polymerase II promoter or an RNA polymerase III promoter.

121. The immunostimulatory bacterium of claim 120, wherein the promoter is an RNA polymerase II promoter that is a viral promoter or a mammalian RNA polymerase II promoter.

122. The immunostimulatory bacterium of claim 121, wherein the promoter is a viral promoter selected from among a cytomegalovirus (CMV) promoter, an SV40 promoter, an Epstein Barr virus (EBV) promoter, a herpes virus promoter, and an adenovirus promoter.

123. The immunostimulatory bacterium of any of claims 1-121, wherein the promoter that controls expression of one or more of the encoded therapeutic products or heterologous proteins on the plasmid is an elongation factor-1 (EF-1) alpha promoter, or an MND promoter, or a UBC promoter, or a PGK promoter, or a CAG promoter.

124. The immunostimulatory bacterium of any of claims 1-121, wherein the promoter that controls expression of one or more of the encoded therapeutic products or heterologous proteins on the plasmid is an EF-1 alpha, an adenovirus 2 or 5 late, a CMV, an SV40, an MND, a PGK, an EIF4A1, a CAG, or a CD68 promoter.

125. The immunostimulatory bacterium of any of claims 1-121, wherein the promoter that controls expression of one or more of the encoded therapeutic products or heterologous proteins on the plasmid is a viral promoter that is a late promoter.

126. The immunostimulatory bacterium of any of claims 1-125, wherein the plasmid comprises regulatory sequences that comprise a terminator and/or promoter(s) selected from among SV40, hGH, BGH, MND, chicken beta-globulin, and rbGlob (rabbit globulin) genes, to control expression of the therapeutic product(s).

127. The immunostimulatory bacterium of any of claims 1-126, wherein the encoded therapeutic product(s) is/are operatively linked to a signal sequence for secretion from a cell containing the plasmid.

128. The immunostimulatory bacterium of any of claims 1-127, wherein the plasmid that encodes the therapeutic product comprises a construct that includes an enhancer, a promoter, the open reading frame encoding the therapeutic product or heterologous protein, and a polyA tail.

129. The immunostimulatory bacterium of any of claims 1-128, wherein the plasmid comprises a construct that includes an enhancer, a promoter, an IRES, the open reading frame encoding the therapeutic product or heterologous protein, and a polyA tail.

130. The immunostimulatory bacterium of any of claims 1-129, wherein the plasmid comprises a construct that includes an enhancer, a promoter, an IRES, a localization sequence, the open reading frame encoding the therapeutic product or heterologous protein, and a polyA tail.

131. The immunostimulatory bacterium of claim 130, wherein the construct on the plasmid encoding the therapeutic product or heterologous protein comprises a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) or a Hepatitis B virus Posttranscriptional Regulatory Element (HPRE).

132. The immunostimulatory bacterium of any of claims 1-131, wherein the plasmid contains nucleic acid encoding a therapeutic product that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), or a variant of the therapeutic product.

133. The immunostimulatory bacterium of claim 132, wherein the therapeutic product in its unmodified form senses or interacts directly or indirectly with cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides, to induce expression of type I IFN, and the variant protein induces expression of type I IFN in the absence of the sensing or interacting with the cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides.

134. The immunostimulatory bacterium of claim 132, wherein the therapeutic product is a variant that, when expressed in a subject, leads to constitutive expression of type I IFN.

135. The immunostimulatory bacterium of claim 132, wherein the therapeutic product is a gain-of-function (GOF) variant that does not require cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides to result in expression of type I IFN.

136. The immunostimulatory bacterium of any of claims 1-135, wherein the therapeutic product is selected from among STING, RIG-I, MDA-5, IRF-3, IRF-5, IRF-7, IRF-8, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200.

137. The immunostimulatory bacterium of any of claims 1-135, wherein the therapeutic product is selected from among TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200.

138. The immunostimulatory bacterium of any of claims 132-137, wherein the therapeutic product is a variant that has increased activity, or that results in constitutive expression of type I interferon (IFN).

139. The immunostimulatory bacterium of any of claims 1-138, wherein the plasmid encodes a gain-of-function, constitutively active variant of a protein that, in humans, promotes or causes interferonopathies.

140. The immunostimulatory bacterium of any of claims 132-139, wherein the therapeutic product is a variant that comprises a mutation that eliminates a

phosphorylation site in the protein to thereby reduce nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling.

141. The immunostimulatory bacterium of any of claims 132-140, wherein the therapeutic product that induces type I IFN is STING, RIG-I, IRF-3, or MDA5; or wherein the therapeutic product that induces type I IFN is IRF-5, or IRF-8.

142. The immunostimulatory bacterium of any of claims 132-141, wherein:

the therapeutic product that induces expression of type I IFN is a variant thereof that has increased activity or constitutive activity; and

the therapeutic product is STING, RIG-I, IRF-3, IRF-5, IRF-8, or MDA5.

143. The immunostimulatory bacterium of any of claims 132-142, wherein the therapeutic product is a variant of STING, RIG-I, IRF-3, IRF-5, IRF-8, or MDA5 that comprises a gain-of-function mutation resulting in increased expression of type I IFN.

144. The immunostimulatory bacterium of any of claims 132-143, wherein the therapeutic product is a variant of STING, RIG-I, IRF-3, IRF-5, IRF-8, or MDA5 in which one or more serine (S) or threonine (T) residue(s) that is/are phosphorylated as a consequence of viral infection, is/are replaced with an aspartic acid (D), whereby the resulting variant is a phosphomimetic that constitutively induces type I IFN.

145. The immunostimulatory bacterium of any of claims 132-144, wherein:

the therapeutic product is IRF-3 that has one or more replacement(s) at residues at positions 396, 398, 402, 404 and 405, with reference to SEQ ID NO:312; and

the residues are replaced with aspartic acid residues.

146. The immunostimulatory bacterium of claim 145, wherein IRF-3 comprises the replacement S396D with reference to SEQ ID NO:312.

147. The immunostimulatory bacterium of claim 145, wherein IRF-3 comprises the replacements S396D/S398D/S402D/T404D/S405D with reference to SEQ ID NO:312.

148. The immunostimulatory bacterium of any of claims 132-147, wherein the therapeutic product is selected from among STING, RIG-I, MDA-5, IRF-3, IRF-7, IRF-5, IRF-8, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200.

149. The immunostimulatory bacterium of any of claims 132-148, wherein:

the therapeutic product that senses cytosolic DNA/RNA is a variant STING, MDA5, RIG-I or IRF-3; and

unmodified STING has the sequence set forth in any of SEQ ID NOs: 305-309, unmodified MDA5 has the sequence set forth in SEQ ID NO: 310, unmodified RIG-I has the sequence set forth in SEQ ID NO: 311, and unmodified IRF-3 has the sequence set forth in SEQ ID NO: 312.

150. The immunostimulatory bacterium of any of claims 132-149, wherein the therapeutic product is selected from among STING, MDA5, IRF-3, and RIG-I, and comprises a gain-of-function mutation(s) that renders the STING, MDA5, IRF-3, IRF-5, IRF-8, or RIG-I constitutively active, whereby expression of type I IFN is constitutive.

151. The immunostimulatory bacterium of claim 149 or claim 150, wherein the mutations are selected as follows:

a) in STING, with reference to SEQ ID NOs: 305-309, one or more selected from among: S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E, S102P/F279L, F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R197A, D205A, R310A, R293A, T294A, E296A, R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q, S272A, R293A/T294A/E296A, D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, S358A, E360A, S366A, R238A, R375A, N154S/R284G, and S324A/S326A;

b) in MDA5, with reference to SEQ ID NO:310, one or more of: T331I, T331R, A489T, R822Q, G821S, A946T, R337G, D393V, G495R, R720Q, R779H, R779C, L372F, and A452T;

c) in RIG-I, with reference to SEQ ID NO:311, one or both of E373A and C268F; and

d) in IRF-3, with reference to SEQ ID NO:312, S396D.

152. The immunostimulatory bacterium of any of claims 132-151, wherein the therapeutic product is a variant STING that contains one or more amino replacement(s) selected, with reference to SEQ ID NOs: 305-309, from among: S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E, S102P/F279L, F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R197A, D205A, R310A, R293A, T294A, E296A, R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q, S272A, R293A/T294A/E296A, D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, S358A, E360A, S366A, R238A, R375A, N154S/R284G, and S324A/S326A, and conservative replacements thereof.

153. The immunostimulatory bacterium of any of claims 114 and 132-152, wherein the type I IFN is an interferon-α or interferon-β.

154. The immunostimulatory bacterium of any of claims 1-153 that encodes a bi-specific antibody.

155. The immunostimulatory bacterium claim 154, wherein the bi-specific antibody is a bi-specific T-cell engager.

156. The immunostimulatory bacterium of any of claims 1-155, wherein the plasmid encodes a bi-specific T-cell engager antibody that binds DLL3 and CD3.

157. The immunostimulatory bacterium of claim 156, wherein the bi-specific T-cell engager antibody comprises a heavy chain and light chain of an anti-DLL3 antibody and of an anti-CD3 antibody.

158. The immunostimulatory bacterium of claim 156 or 157, wherein the bi-specific T-cell engager antibody is selected from among those encoded in the constructs designated SC16.15, SC16.34, and SC16.56, which encode variable heavy and variable light chains of antibodies that bind each of DLL3 and CD3, and whose sequences are set forth in SEQ ID NOs.485-491, or humanized variants thereof, and variants that have at least 95% sequence identity thereto.

159. The immunostimulatory bacterium of claim 157 or claim 158, wherein the bi-specific T-cell engager antibody comprises combinations of a) - f), whereby the resulting construct can bind to each of DLL3 and CD3:

a) a light chain that comprises amino acid residues 154-260 of SEQ ID NO: 487, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

b) a heavy chain that comprises the sequence of amino acid residues set forth as amino acid residues 22-138 of SEQ ID NO: 487, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

c) a light chain that comprises a sequence of amino acid residues set forth as amino acid residues 155-261 of SEQ ID NO: 489, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

d) a heavy chain that comprises a sequence of amino acid residues set forth as amino acid residues 22-139 of SEQ ID NO: 489, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

e) a heavy and light chain, wherein:

the light chain comprises a sequence of amino acid residues set forth as amino acid residues 155-261 of SEQ ID NO: 485, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

the heavy chain comprises a sequence of amino acid residues set forth as amino acid residues 22-139 of SEQ ID NO: 485, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

f) a heavy and light chain of an anti-CD3 antibody, wherein:

the light chain of the anti-CD3 antibody comprises a sequence of amino acid residues set forth as amino acid residues 398-504 of SEQ ID NO: 485, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto; and

the heavy chain of the anti-CD3 antibody comprises a sequence of amino acid residues set forth as amino acid residues 267-382 of SEQ ID NO: 485, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto.

160. The immunostimulatory bacterium of any of claims 156-159, wherein the encoded bi-specific T-cell engager antibody construct comprises a leader sequence.

161. The immunostimulatory bacterium of claim 160, wherein the leader sequence is an IgGK leader sequence.

162. The immunostimulatory bacterium of any of claims 156-161, wherein:

the bispecific antibody comprises a Gly-Ser linker linking one or more light and heavy chains; and

a Gly-Ser linker linking the anti-DLL3 and anti-CD3 portions of the bi-specific T-cell engager antibody.

163. The immunostimulatory bacterium of claim 162, wherein the linker comprises the sequence of amino acids set forth as residues 383-397 of SEQ ID NO: 485.

164. The immunostimulatory bacterium of any of claims 156-163, wherein the bi-specific T-cell engager antibody comprises a flag tag.

165. The immunostimulatory bacterium of claim 164, wherein the flag tag comprises the sequence of amino acids set forth as residues 505-512 of SEQ ID NO: 485.

166. The immunostimulatory bacterium of any of claims 156-165, comprising a nucleic acid construct encoding a leader sequence, the heavy and light chain of an anti-

DLL antibody, and the heavy and light chain of an anti-CD3 antibody, and optionally one or more peptide linkers, and optionally a flag tag.

167. The immunostimulatory bacterium of any of claims 156-166, wherein the encoded bi-specific T-cell engager antibody construct comprises the sequence of amino acid residues set forth in any of SEQ ID NOs: 485-491, or a humanized variant thereof, or a variant having at least 95 % sequence identity thereto, and sequences having at least 95% sequence identity thereto.

168. The immunostimulatory bacterium of any of claims 1-167, wherein the plasmid encodes a tumor-associated antigen.

169. The immunostimulatory bacterium of any of claims 1-168, wherein the immunostimulatory bacterium encodes a modified STING protein that constitutively induces type I interferon.

170. The immunostimulatory bacterium of claim 169, wherein the STING protein comprises the replacements N154S, or R284G, or N154S/R284G.

171. The immunostimulatory bacterium of any of claims 1-170, wherein the plasmid encodes IL-15 or an IL-15/IL-15R alpha chain complex.

172. The immunostimulatory bacterium of any of claims 1-171, wherein the plasmid encodes the combination of a modified STING, IL-15/IL-15R alpha chain complex, a tumor-associated antigen and or a bi-specific T-cell engager antibody.

173. The immunostimulatory bacterium of any of claims 1-172, wherein the plasmid encodes an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment that is selected from among one or more of: IL-2, IL-7, IL-12p70 (IL-12p40 + IL-12p35), IL-15, IL-2 that has attenuated binding to IL-2Ra, IL-15/IL-15R alpha chain complex (IL-15Rα-IL-15sc), IL-18, IL-21, IL-23, IL-36γ, IL-2 that is modified so that it does not bind to IL-2Ra, CXCL9, CXCL10, CXCL11, interferon-α, interferon-β, interferon-γ, CCL3, CCL4, CCL5, proteins that are involved in or that effect or potentiate recruitment and/or persistence of T cells, CD40, CD40 ligand (CD40L), CD28, OX40, OX40 ligand (OX40L), 4-1BB, 4-1BB ligand (4-1BBL), members of the B7-CD28 family, CD47 antagonists, an anti-IL-6 antibody or IL-6 binding decoy receptor, TGF-beta polypeptide antagonists, and members of the tumor necrosis factor receptor (TNFR) superfamily.

174. The immunostimulatory bacterium of claim 173, wherein:

the immunostimulatory protein is a co-stimulatory molecule selected from among CD40, CD40 ligand (CD40L), CD28, OX40, OX40 ligand (OX40L), 4-1BB, and a 4-1BB ligand (4-1BBL) that optionally is truncated and lacking a cytoplasmic domain for expression on an antigen-presenting cell (APC); and

the truncated gene product is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement and is unable to counter-regulatory signal to the antigen-presenting cell (APC) due to a deleted cytoplasmic domain.

175. The immunostimulatory bacterium of claim 173 or claim 175, wherein the immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment is a cytokine or a chemokine.

176. The immunostimulatory bacterium of any of claims 173-175, wherein the immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment is a co-stimulatory molecule or cytoplasmic domain-deleted form thereof.

177. The immunostimulatory bacterium of claim 176, wherein the immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment is selected from among 4-1BBL, CD80, CD86, CD27L, CD24L, B7RP1, and OX40L.

178. The immunostimulatory bacterium of any of claims 1-177, wherein the plasmid encodes a therapeutic product that is a TGF-beta polypeptide antagonist.

179. The immunostimulatory bacterium of any of claims 1-178, wherein the therapeutic product is an antibody or antigen-binding fragment thereof.

180. The immunostimulatory bacterium of claim 179, wherein the antibody or antigen-binding fragment thereof is an antigen-binding fragment that is selected from among a Fab, Fab', $F(ab')_2$, single-chain Fv (scFv), Fv, dsFv, nanobody, diabody fragment, and a single-chain antibody.

181. The immunostimulatory bacterium of claim 179 or claim 180, wherein the antibody or antigen-binding fragment thereof is humanized or is human.

182. The immunostimulatory bacterium of any of claims 179-181, wherein the antibody or antigen-binding fragment thereof is an antagonist of PD-1, PD-L1, CTLA-4, VEGF, VEGFR2, CD24, or IL-6.

183. The immunostimulatory bacterium of any of claims 1-182, wherein the plasmid encodes two or more therapeutic products selected from among:

a) an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment;

b) one or more of a protein that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), or a variant thereof that has increased activity to increase expression of type I IFN, or a variant thereof that results in constitutive expression of a type I IFN; and

c) an anti-cancer antibody or antigen-binding portion thereof.

184. The immunostimulatory bacterium of claim 183, wherein the immunostimulatory protein is a co-stimulatory molecule that lacks a cytoplasmic domain or a sufficient portion thereof, for expression on an antigen-presenting cell (APC), whereby the truncated co-stimulatory molecule is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement and is unable to counter-regulatory signal to the antigen presenting cell (APC).

185. An immunostimulatory bacterium, comprising a plasmid that encodes two or more therapeutic products under control of a single promoter, wherein:

the therapeutic products are selected from among:

a) an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment;

b) one or more of a protein that is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN), or a variant thereof that has increased activity to increase expression of type I IFN, or a variant thereof that results in constitutive expression of a type I IFN; and

c) an anti-cancer antibody or antigen-binding portion thereof; and

the encoding nucleic acids are separated by an IRES sequence or 2A peptides, and each nucleic acid encoding each product is optionally operatively linked to nucleic acid encoding a signal sequence, whereby, upon translation of the encoded mRNA, each product is separately expressed and secreted from a cell comprising the bacterium and/or plasmid.

186. The immunostimulatory bacterium of claim 185, wherein the immunostimulatory protein is a co-stimulatory molecule that lacks a cytoplasmic domain or a sufficient portion thereof, for expression on an antigen-presenting cell (APC),

whereby the truncated co-stimulatory molecule is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement and is unable to counter-regulatory signal to the antigen presenting cell (APC).

187. The immunostimulatory bacterium of any of claims 1-186, wherein the plasmid encodes at least two therapeutic products selected from among a cytokine, a protein that constitutively induces a type I IFN, a co-stimulatory molecule, and an anti-cancer antibody or antigen-binding portion thereof.

188. The immunostimulatory bacterium of any of claims 1-187, wherein the immunostimulatory bacterium comprises a plasmid that encodes at least two therapeutic products selected from among a cytokine, a protein that constitutively induces a type I IFN, a co-stimulatory molecule, and an anti-cancer antibody or antigen-binding portion thereof, and also encodes an antigen or an antigenic protein.

189. The immunostimulatory bacterium of claim 188, wherein the antigen is a tumor-associated antigen.

190. The immunostimulatory bacterium of claim 189, wherein the tumor-associated antigen is an oncofetal antigen, an oncoviral antigen, and overexpressed/accumulated antigen, a cancer-Testis antigen, a linear restricted antigen, a mutated antigen, a post-translationally altered antigen, or an idiotypic antigen.

191. The immunostimulatory bacterium claim 190, wherein the tumor-associated antigen is selected from among the following antigens:

| Category of Tumor-Associated Antigen (TAA) | Name / Family Name of TAA |
|---|---|
| *Oncofetal* | Carcinoembryonic antigen (CEA) |
| | Immature laminin receptor |
| | Tumor-associated glycoprotein 72 (TAG-72) |
| *Oncoviral* | HPV E6, HPV E7 |
| *Overexpressed/Accumulated* | BING-4 |
| | Epidermal Growth Factor Receptor (EGFR) |
| | Wilms' Tumor Protein |
| | Calcium-activated chloride channel 2 |
| | Cyclin-B1 |
| | 9D7 |
| | Epithelial cell adhesion molecule (EpCAM) |
| | Ephrin type-A receptor 3 (EphA3) |
| | Human epidermal growth factor receptor 2 (HER2/Neu) |

| | |
|---|---|
| | Telomerase |
| | Mesothelin |
| | Stomach-cancer–associated protein tyrosine phosphatase 1 (SAP-1) |
| | Survivin |
| *Cancer-Testis* | BAGE (B melanoma antigen) family |
| | CAGE family |
| | GAGE family |
| | MAGE (melanoma antigen) family |
| | SAGE (sarcoma antigen) family |
| | PAGE family |
| | XAGE family |
| | CT9, CT10 |
| | New York esophageal squamous cell carcinoma 1 (NY-ESO-1), LAGE-1 |
| | Preferentially expressed antigen in melanoma (PRAME) |
| | synovial sarcoma/X breakpoint 2 (SSX-2) |
| *Lineage-Restricted* | Melanoma antigen recognized by T-cells 1 (Melan-A/MART-1) |
| | gp100/Pmel17 |
| | Tyrosinase |
| | Tyrosinase related protein (TRP)-1, TRP-2 |
| | P.polypeptide |
| | Melanocortin 1 receptor (MC1R) |
| | Prostate-specific antigen (PSA) |
| *Mutated* | β-catenin |
| | BRCA1, BRCA2 |
| | Cyclin dependent kinase 4 (CDK4) |
| | Chronic Myelogenous Leukemia Tumor Antigen 66 (CML66) |
| | Fibronectin |
| | Melanoma antigen recognized by T-cells 2 (MART-2) |
| | p53 |
| | Ras |
| | TGF-β receptor type II (TGF-βRII) |
| *Post-Translationally Altered* | Mucin 1, Cell Surface Associated (MUC1) |
| *Idiotypic* | Immunoglobulin (Ig), T-cell Receptor (TCR) |

192. The immunostimulatory bacterium any of claims 1-191 that is an anti-cancer therapeutic.

193. The immunostimulatory bacterium of claim 192, wherein the encoded payloads are expressed under control of a eukaryotic promoter.

194. The immunostimulatory bacterium any of claims 1-193, that comprises the genome modifications whereby the bacterium is flagellin$^-$, *asd*$^-$, *msbB*$^-$, *pagP*$^-$, *and csgD*$^-$, *or is ansB*$^-$, *asd*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$, and *pagP*$^-$.

195. The immunostimulatory bacterium of any of claims 194 that comprises genome modification whereby the bacterium is *thyA*$^-$, *asd*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$, and *pagP*$^-$.

196. The immunostimulatory bacterium of any of claims 1-195, wherein the immunostimulatory bacterium is a vaccine for treating or preventing a cancer or infection from a pathogen.

197. The immunostimulatory bacterium of claim 196, wherein the encoded payloads are expressed under control of a prokaryotic promoter, and the nucleic acid encoding the payloads comprise translational regulatory signals that are recognized by eukaryotic ribosomes, and not by bacterial ribosomes.

198. The immunostimulatory bacterium of any of claims 1-197, wherein the plasmid in the immunostimulatory bacterium encodes an antigen or protein or epitope(s) thereof from a pathogen

199. The immunostimulatory bacterium of claim 198, wherein the pathogen causes chronic viral infections, such as infections by hepatitis viruses, herpes viruses, varicella zoster virus (VZV), Epstein-Barr virus, human immunodeficiency virus (HIV), human T-cell leukemia virus (HTLV), Respiratory Syncytial Virus (RSV), and measles virus; or is a virus or other pathogen chronically infect subjects; or is a pathogen that causes acute infections, such as initial infections with chronic influenza and coronaviruses, such as Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory Syndrome coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19).

200. The immunostimulatory bacterium of claim 199, wherein the plasmid encodes an encode an antigen from a pathogen or an epitope or combination of epitopes thereof, such as an antigen from an essential viral protein, such as, for example, in the case of a coronavirus, an antigen from the Nucleocapsid, M and/or S proteins, which can result in neutralizing antibodies, and the enhancement of long-lived circulating and tissue-resident CD8+ T-cells.

201. The immunostimulatory bacterium of any of claims 198-200, wherein nucleic acid encoding the antigen, epitope, or antigenic protein is operatively linked to a prokaryotic promoter recognized by the bacterium; and the encoding sequence includes regulatory sequences for translation that are recognized by eukaryotic ribosomes, whereby the bacterium cannot translate the encoded RNA, or the encoding sequence does not include a Shine Dalgarno sequence that is recognized by the bacterial ribosomes so that the encoded mRNA is not translated; and the mRNA is delivered to the eukaryotic host cell into which the bacterium is delivered.

202. The immunostimulatory bacterium of any of claims 1-201, wherein:

the plasmid encodes two or more therapeutic products under control of a single promoter; and

expression of the nucleic acid encoding at least two or all of the products is under control of a single promoter, and the nucleic acid encoding each product is separated by nucleic acid encoding 2A polypeptides, whereby, upon translation, each product is separately expressed.

203. The immunostimulatory bacterium of any of claims 198-202, wherein nucleic acid encoding one or more of the therapeutic products is operatively linked to nucleic acid encoding a sequence that directs secretion of the expressed product(s).

204. The immunostimulatory bacterium of any of claims 1-203, wherein:

the therapeutic product is a co-stimulatory molecule with a cytoplasmic domain deletion for expression on an antigen-presenting cell (APC); and

the truncated gene product is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement, and is unable to counter-regulatory signal to the APC due to the cytoplasmic domain deletion.

205. The immunostimulatory bacterium of claim 204, wherein the co-stimulatory molecule that lacks a cytoplasmic domain is 4-1BBL, CD80, CD86, CD27L, B7RP1, CD24L, or OX40L.

206. The immunostimulatory bacterium of any of claims 1-205 that encodes two or more therapeutic products, wherein at least one product is selected from a) and at least one is selected from b), wherein:

a) is IL-2, IL-7, IL-12p70 (IL-12p40 + IL-12p35), IL-15, IL-23, IL-36 gamma, IL-2 that has attenuated binding to IL-2Ra, IL-15/IL-15R alpha chain complex (IL-15Rα-IL-15sc), IL-18, IL-2 that is modified so that it does not bind to IL-2Ra, CXCL9,

CXCL10, CXCL11, interferon-α, interferon-β, CCL3, CCL4, CCL5, proteins that are involved in or that effect or potentiate recruitment and/or persistence of T cells, CD40, CD40 Ligand (CD40L), OX40, OX40 Ligand (OX40L), 4-1BB, 4-1BB Ligand (4-1BBL), members of the B7-CD28 family, TGF-beta polypeptide antagonists, or members of the tumor necrosis factor receptor (TNFR) superfamily; and

b) is STING, RIG-I, MDA-5, IRF-3, IRF-5, IRF-7, IRF-8, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, or SNRNP200.

207. The immunostimulatory bacterium of any of claims 1-206 that encodes or further encodes one or more of a TGF-beta inhibitory antibody, a TGF-beta binding decoy receptor, an anti-IL-6 antibody, and an IL-6 binding decoy receptor.

208. The immunostimulatory bacterium of any of claims 1-207 that encodes one or more of the following combinations of therapeutic products:

IL-2 and IL-12p70;

IL-2 and IL-21;

IL-2, IL-12p70, and a STING GOF variant;

IL-2, IL-21, and a STING GOF variant;

IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt), where Δcyt is a deleted cytoplasmic domain;

IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα, and a STING GOF variant;

IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα and IL-12p70;

IL-15/IL-15Rα and IL-21;

IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

IL-15/IL-15Rα, IL-21, and a STING GOF variant;

IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and IL-21;

IL-12p70, IL-21, and a STING GOF variant;

IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and a STING GOF variant;

IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and IL-18;

IL-12p70, IL-18, and a STING GOF variant;

IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-2, and IL-12p70;

a TGF-β decoy receptor, IL-2, and IL-21;

a TGF-β decoy receptor, IL-2, IL-12p70, and a STING GOF variant;

a TGF-β decoy receptor, IL-2, IL-21, and a STING GOF variant;

a TGF-β decoy receptor, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, and a STING GOF variant;

a TGF-β decoy receptor, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-12p70;

a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-21;

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, and a STING GOF variant;

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-12p70, and IL-21;

a TGF-β decoy receptor, IL-12p70, IL-21, and a STING GOF variant;

a TGF-β decoy receptor, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor and IL-12p70;

a TGF-β decoy receptor, IL-12p70, and a STING GOF variant;

a TGF-β decoy receptor, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-12p70, and IL-18;

a TGF-β decoy receptor, IL-12p70, IL-18, and a STING GOF variant;

a TGF-β decoy receptor, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, and IL-12p70;

an anti-CTLA-4 antibody, IL-2, and IL-21;

an anti-CTLA-4 antibody, IL-2, IL-12p70, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, IL-21, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-12p70;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-21;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-12p70, and IL-21;

an anti-CTLA-4 antibody, IL-12p70, IL-21, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody and IL-12p70;

an anti-CTLA-4 antibody, IL-12p70, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-12p70, and IL-18;

an anti-CTLA-4 antibody, IL-12p70, IL-18, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody and a STING GOF variant;

a CD40 agonist, IL-2, and IL-12p70;

a CD40 agonist, IL-2, and IL-21;

a CD40 agonist, IL-2, IL-12p70, and a STING GOF variant;

a CD40 agonist, IL-2, IL-21, and a STING GOF variant;

a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, and a STING GOF variant;

a CD40 agonist, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, and IL-12p70;

a CD40 agonist, IL-15/IL-15Rα, and IL-21;

a CD40 agonist, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

a CD40 agonist, IL-15/IL-15Rα, IL-21, and a STING GOF variant;

a CD40 agonist, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-12p70, and IL-21;

a CD40 agonist, IL-12p70, IL-21, and a STING GOF variant;

a CD40 agonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist and IL-12p70; a CD40 agonist, IL-12p70, and a STING GOF variant;

a CD40 agonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-12p70, and IL-18;

a CD40 agonist, IL-12p70, IL-18, and a STING GOF variant;

a CD40 agonist, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a tumor-associated antigen;

a CD40 agonist and a STING GOF variant.

209. The immunostimulatory bacterium of claim 208, further encoding a tumor-associated antigen.

210. The immunostimulatory bacterium of claim 208 or claim 209, wherein the STING gain-of-function (GOF) variant is/are selected from among any set forth in claims 149-153.

211. An immunostimulatory bacterium or a cell, encoding one or more of the following combinations of therapeutic products:

IL-2 and IL-12p70;

IL-2 and IL-21;

IL-2, IL-12p70, and a STING GOF variant;

IL-2, IL-21, and a STING GOF variant;

IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt), where Δcyt is a deleted cytoplasmic domain;

IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα, and a STING GOF variant;

IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα and IL-12p70;

IL-15/IL-15Rα and IL-21;

IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

IL-15/IL-15Rα, IL-21, and a STING GOF variant;

IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and IL-21;

IL-12p70, IL-21, and a STING GOF variant;

IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and a STING GOF variant;

IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

IL-12p70 and IL-18;

IL-12p70, IL-18, and a STING GOF variant;

IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-2, and IL-12p70;

a TGF-β decoy receptor, IL-2, and IL-21;

a TGF-β decoy receptor, IL-2, IL-12p70, and a STING GOF variant;

a TGF-β decoy receptor, IL-2, IL-21, and a STING GOF variant;

a TGF-β decoy receptor, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, and a STING GOF variant;

a TGF-β decoy receptor, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-12p70;

a TGF-β decoy receptor, IL-15/IL-15Rα, and IL-21;

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, and a STING GOF variant;

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-12p70, and IL-21;

a TGF-β decoy receptor, IL-12p70, IL-21, and a STING GOF variant;

a TGF-β decoy receptor, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor and IL-12p70;

a TGF-β decoy receptor, IL-12p70, and a STING GOF variant;

a TGF-β decoy receptor, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor, IL-12p70, and IL-18;

a TGF-β decoy receptor, IL-12p70, IL-18, and a STING GOF variant;

a TGF-β decoy receptor, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a TGF-β decoy receptor and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, and IL-12p70;

an anti-CTLA-4 antibody, IL-2, and IL-21;

an anti-CTLA-4 antibody, IL-2, IL-12p70, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, IL-21, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-12p70;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, and IL-21;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-12p70, and IL-21;

an anti-CTLA-4 antibody, IL-12p70, IL-21, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody and IL-12p70;

an anti-CTLA-4 antibody, IL-12p70, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody, IL-12p70, and IL-18;

an anti-CTLA-4 antibody, IL-12p70, IL-18, and a STING GOF variant;

an anti-CTLA-4 antibody, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

an anti-CTLA-4 antibody and a STING GOF variant;

a CD40 agonist, IL-2, and IL-12p70;

a CD40 agonist, IL-2, and IL-21;

a CD40 agonist, IL-2, IL-12p70, and a STING GOF variant;

a CD40 agonist, IL-2, IL-21, and a STING GOF variant;

a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, and a STING GOF variant;

a CD40 agonist, IL-15/IL-15Rα, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, and IL-12p70;

a CD40 agonist, IL-15/IL-15Rα, and IL-21;

a CD40 agonist, IL-15/IL-15Rα, IL-12p70, and a STING GOF variant;

a CD40 agonist, IL-15/IL-15Rα, IL-21, and a STING GOF variant;

a CD40 agonist, IL-15/IL-15Rα, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-15/IL-15Rα, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-12p70, and IL-21;

a CD40 agonist, IL-12p70, IL-21, and a STING GOF variant;

a CD40 agonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist and IL-12p70;**

a CD40 agonist, IL-12p70, and a STING GOF variant;

a CD40 agonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist, IL-12p70, and IL-18;

a CD40 agonist, IL-12p70, IL-18, and a STING GOF variant;

a CD40 agonist, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBLΔcyt);

a CD40 agonist and a STING GOF variant;

a bi-specific T-cell engager (BiTe) + a STING protein, a BiTe + IL-15, a BiTe + IL-15 + a STING protein, where the BiTe targets DLL3, EGFR, Her2, CEA, Mesothelin, PSMA, EpCAM, CD74, Folate Receptor, Nectin4, EphA2, CA-IX, B7H3, Siglec-15, Muc1, or Lewis Y antigen;

a tumor antigen(s) + STING gain-of-function variant;

a therapeutic composition of a tumor antigen(s) and IL-15;

a therapeutic composition of a tumor antigen(s) + IL-15 + a STING gain-of-function variant;

one or more antigens and an IFN;

one or more antigens and an IFNα;

one or more antigens, and IFNα2 or an IFNα1-16;

one or more antigens and any of IFNα1-16;

one or more antigens and IFN-β;

one or more antigens, IFNα2, and IFN-β;

one or more antigens and an IRF3 GOF variant with the mutation S396D;

one or more antigens, IFNα2 or an IFNα1-16, and an IRF3 GOF variant with the mutation S396D;

IFNalpha2 + IRF3-S396D;

IFNα1-16 + IRF3-S396D;

IFNalpha2 + IFN-beta;

IFNα1-16 + IFN-beta

FLT-3L, or sialidase, or IL-12p35, or Azurin, or a membrane anchored IL-2, IL-12, IL-12p35, IL-21, IL-15, FLT-3L, alone or in combination with other immunostimulatory proteins; and

a TLR8 agonist, where the agonist is polyU or polyU/G, a microRNA, or miR-21, alone or in combination with any of the immunostimulatory proteins.

212. The immunostimulatory bacterium of any of claims 202-211, further encoding a tumor-associated antigen.

213. The immunostimulatory bacterium of any of claims 1-212, wherein the plasmid encodes a tumor-associated antigen as set forth in claim 191.

214. The immunostimulatory bacterium of any of claims 1-213, wherein an encoded therapeutic product is a bi-specific T-cell engager.

215. The immunostimulatory bacterium claim 214, wherein the bi-specific T-cell engager binds Delta-like ligand 3 (DLL3) and CD3.

216. The immunostimulatory bacterium of any of claims 1-215, encoding a cytokine and a modified or variant STING protein.

217. The immunostimulatory bacterium of claim 215, wherein the STING protein is a STING protein of any of claims 406-436.

218. The immunostimulatory bacterium of claim 215 or claim 216, wherein the cytokine is IL-15/IL-15R alpha chain complex, or IL-15, or IL-12.

219. The immunostimulatory bacterium of any of claims 215-218, wherein the cytokine is IL-15/IL-15R alpha chain complex, or IL-15.

220. The immunostimulatory bacterium any of claims 215-219, wherein the STING protein is a chimeric STING protein that comprises a human STING protein with the CTT from Tasmanian devil, or is a chimeric STING that comprises a human STING protein with the CTT from Tasmanian devil and having one or more gain-of-function mutations.

221. The immunostimulatory bacterium of any of claims 215-220, wherein the STING protein comprises a gain-of-function mutation that is N154S, or R284G, or N154S/R284G.

222. The immunostimulatory bacterium or cell of any of claims 214-221, wherein the STING gain-of-function variant is selected from among any set forth in any of claims 405-430.

223. The immunostimulatory bacterium of any of claims 1-191, wherein an encoded therapeutic product comprises an Fc domain.

224. The immunostimulatory bacterium of any of claims 1-221, wherein an encoded therapeutic product comprises the B7 protein transmembrane domain.

225. The immunostimulatory bacterium any of claims 1-224 wherein a encoded therapeutic product is a tumor-associated antigen.

226. The immunostimulatory bacterium of any of claims 1-225, wherein an encoded product is a bi-specific T-cell engager antibody.

227. The immunostimulatory bacterium of any of claims 1-226, wherein an encoded therapeutic product is GPI-anchored.

228. The immunostimulatory bacterium of any of claims 1-227, wherein an encoded therapeutic product comprises human serum albumin or its derivatives thereof that increase serum half-life of the encoded product.

229. The immunostimulatory bacterium of any of claims 1-228, wherein an encoded therapeutic product comprises a fusion to collagen.

230. An immunostimulatory bacterium of any of claims 1- 229 that is a strain designated as YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI, or YS1646Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD/F-ΔpurI/ΔthyA.

231. A genome modified bacterium, comprising genome modifications, whereby the response by toll-like receptors (TLRs) 2, 4, and 5 is reduced compared to the bacterium without the genome modifications, wherein:

the bacterium comprises further genomic modifications whereby it is auxotrophic for a required nutrient or factor so that it is unable to replicate in a eukaryotic host, but can replicate *in vitro* when supplied with the nutrient or factor;

the bacterium comprises a plasmid containing nucleic acid encoding a product, or comprises RNA encoding the product;

the product encoded by the nucleic acid or RNA is an antigenic sequence or sequences from pathogen that is a pathogenic virus, bacterium, or parasite, or is a tumor antigen, whereby, upon expression of the encoded antigen in the host, the host develops an immune-protective response or immunizing response against the pathogenic virus, bacterium, parasite, or tumor antigen, or the product is a therapeutic product;

expression of the antigenic sequence(s) is/are under control of a prokaryotic promoter so that RNA encoding the antigen(s) is produced in the bacterium;

nucleic acid encoding the antigen comprises regulatory sequences that inhibit or prevent translation of encoded RNA by bacterial ribosomes, but that does not inhibit or prevent translation of the encoded RNA by eukaryotic host ribosomes, whereby translation is de-coupled from transcription in the bacterium;

the resulting bacterium is selective for infecting phagocytic cells when administered to a eukaryotic subject, and delivers the nucleic acid into the phagocytic cells, wherein the RNA is translated.

232. The bacterium of claim 231, wherein the nucleic acid encoding the antigenic sequence(s) comprises an internal ribosomal entry site (IRES) sequence, whereby host cell translation is facilitated or enhanced, and bacterial translation is inhibited or prevented.

233. The bacterium of claim 232, wherein the IRES is a Vascular Endothelial Growth Factor and Type 1 Collagen Inducible Protein (VCIP) IRES.

234. The bacterium of claim 232 or claim 233, wherein the nucleic acid encoding the antigen(s) comprises a VCIP or other IRES that inhibits or reduces translation in the bacterium, and permits and optionally promotes or enhances translation in a eukaryotic host.

235. The bacterium of any of claims 331-233, wherein the IRES or the VCIP IRES is included in the plasmid, at a position that is 3' of the promoter and 5' of the antigen(s) coding sequence.

236. The bacterium of any of claims 233-235, wherein the sequence of the VCIP IRES is set forth in SEQ ID NO:434.

237. The bacterium of any of claims 231-236, wherein the pathogen is a bacterium or a virus.

238. The bacterium of any of claims 231-237, wherein the encoded antigen is a tumor antigen.

239. The bacterium of any of claims 231-238, wherein the bacterium is a vaccine to prevent or treat a viral infection or a bacterial infection.

240. The bacterium of claim 236, wherein the pathogen is selected from among viruses that causes chronic viral infections.

241. The bacterium of claim 240, wherein the infections are selected from among infections by hepatitis viruses, herpes viruses, varicella zoster virus (VZV), Epstein-Barr virus, human immunodeficiency virus (HIV), human T-cell leukemia virus (HTLV), Respiratory Syncytial Virus (RSV), measles virus, and other viruses that chronically infect subjects.

242. The bacterium of any of claims 239-241, wherein the infection is an acute infection.

243. The bacterium of claim 242, wherein the infection is an infection by Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), Middle East Respiratory

Syndrome coronavirus (MERS-CoV), or Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2, which causes COVID-19).

244. The bacterium of any of claims 231-243, wherein the pathogen is a species of *Escherichia, Staphylococcus, Pseudomonas*, *Actinobacteria*, *Archaeobacteria*, *Mycobacteria* or *Porphyromonas*.

245. The bacterium of any of claims 231-243, wherein the pathogen is *P. gingivalis*, SARS-CoV, or *E. coli*, or Haemophilus influenza.

246. The bacterium of any of claims 231-245, wherein the plasmid in the bacterium further encodes an immunostimulatory protein or other adjuvant.

247. The bacterium of claim 246, wherein the plasmid encodes a combination of immunostimulatory proteins or other therapeutic proteins.

248. The bacterium of claim 247 wherein the immunostimulatory protein is a STING protein.

249. The bacterium of claim 248, wherein the STING protein comprises a gain-of-function mutation and/or is a chimeric STING protein.

250. The bacterium of claim 248 or claim 249, wherein the STING protein is the STING protein of any of claims 406-436.

251. The bacterium of any of claims 231-250, wherein the bacterium comprises a plasmid that encodes a combination of therapeutic products.

252. The bacterium of claim 250, that is an immunostimulatory bacterium as described in any of claims 1-251.

253. The bacterium of any of claims 231-252, wherein:

the immunostimulatory protein(s) and/or other therapeutic proteins are encoded in the plasmid as part of a polycistronic sequence with expression of the antigen under control of a prokaryotic promoter recognized by the bacterium; or

the immunostimulatory protein(s) and/or other therapeutic proteins are encoded on the plasmid under control of a eukaryotic promoter recognized by the eukaryotic host.

254. The bacterium of any of claims 231-253, wherein the bacterium comprises mRNA encoding the antigen(s) and any other proteins expressed under control of a prokaryotic promoter that are produced by culturing the bacterium *in vitro*.

255. The bacterium of any of claims 231-254 that comprises genome modifications whereby the bacterium lacks flagella and produces LPS with pent-acylated lipid A.

256. The bacterium of any of claims 231-255 that is *asd*$^-$ or *thyA*$^-$ or both.

257. The bacterium of any of claims 231-256 that is one or both o adenosine auxotroph, and *csgD*$^-$, and is optionally *ansB*$^-$.

258. The bacterium of any of claims 231-257 that comprises nucleic acid encoding a TLR8 agonist.

259. The bacterium of claim 258, wherein the TLR8 agonist is polyU, polyU/G, a microRNA, or miR-21.

260. The bacterium of any of claims 231-259 that is *msbB*$^-$/*pagP*$^-$, lacks flagella, and is *asd*$^-$ or *thyA*$^-$ or both *asd*$^-$ and *thyA*$^-$.

261. The bacterium of any of claims 1-260 that is a species or strain of *Escherichia*, *Listeria*, *Mycobacteria,* or *Salmonella.*

262. The bacterium of any of claims 1-261 that is a strain of *Salmonella.*

263. The bacterium of claim 262 that is a *Salmonella typhimurium* strain.

264. The bacterium of claim 262 or claim 263, wherein the unmodified *Salmonella* is a wild-type strain, or the unmodified *Salmonella* strain is attenuated.

265. The bacterium of any of claims 1-264, wherein the bacterium is derived from strain VNP20009 or YS1646, or from strain ATCC 14028, or from a strain having all of the identifying characteristics of strain ATCC 14028.

266. The bacterium of any of claims 231-265, wherein the genome modification is one or more of a deletion, insertion, disruption, and other modification in a gene, whereby the product encoded by the gene is not produced or, as produced, is inactive.

267. The bacterium of any of claims 231-266, wherein the prokaryotic promoter is a bacterial promoter or a bacterial phage promoter.

268. The bacterium of any of claims 231-267, wherein the eukaryotic host is a human.

269. A vaccine, comprising the bacterium of any of claims 1-269 in an amount and in a vehicle for administration into a subject to elicit an adaptive immune response in a subject.

270. The vaccine of claim 269 that is formulated as an aerosol, or as a powder, or as a tablet, or a suppository.

271. The vaccine of any of claims 269 or claim 270 that formulated for oral administration, nasal administration, inhalation administration, rectal administration,

vaginal administration, intraocular administration, intracranial administration, intradermal administration, or intramuscular administration.

272. A vaccine, comprising nucleic acid encoding an antigen from a protein from a virus or a protein from a virus corona virus formulated for nasal or pulmonary inhalation, wherein the vaccine does not sufficiently activate TRL2, whereby the vaccine induces type I IFN.

273. The vaccine of claim 272, wherein the virus is a corona virus.

274. The vaccine of claim 273, wherein the virus is a corona virus that is SARS-COV2.

275. The vaccine of any of claims 272-274 that also does not activate a TLR4 and/or TRL5 response sufficient to decease or inhibit type I IFN.

276. A vaccine, comprising nucleic acid that encodes an antigen or protein or epitope from a pathogen or tumor, wherein:

the vaccine elicits an immune response against the pathogen or tumor;

the pathogen is a respiratory pathogen that infects the respiratory system including the lungs and/or naso-pharynx;

the tumor is a lung tumor;

the vaccine is formulated for inhalation through the nose or lungs;

the vaccine delivers the nucleic acid to phagocytic macrophages to convert the immunosuppressive phagocytic macrophages to immunostimulatory, phagocytic macrophages that are capable of in situ antigen cross-presentation to CD8+ T-cells, and of migration to lymph nodes to prime CD4+ and CD8+ T-cells.

277. The vaccine of claim 276 that also does not activate a TLR4 and/or TRL5 response sufficient to decease or inhibit type I IFN.

278. The vaccine of claim 276 or claim 277 that does not activate TLR2/4/5 sufficiently to decrease or inhibit type IFN.

279. The vaccine of any of claims 272-278 wherein the vaccine elicits an immune response against a virus.

280. The vaccine of claim 279, wherein the pathogen is a virus.

281. The vaccine of claim 269-280, wherein the pathogen is an RNA virus.

282. The vaccine of claim 281, wherein the virus is a corona virus or influenza virus.

283. The vaccine of claim 282, wherein the virus is a corona virus.

284. The vaccine of claim 283, wherein the virus is a SARS virus.

285. The vaccine of claim 284, wherein the virus is a SARS-COV2 virus

286. The vaccine of claim 269-285, wherein the antigen, protein, or epitope is a viral antigen, protein, or epitope.

287. The vaccine of claim 286, wherein the protein is a capsid or nucleoprotein.

288. The vaccine of any of claims 269-287, wherein the virus is SARS-COV2, and the protein or epitope is or is from a protein designated or encoded by S1, S2, Envelope (E), Membrane (M), Nucleocapsid (N), ORF3a, ORF6, ORF7a, ORF7b, and ORF8.

289. The vaccine of any of claims 269-288, wherein the virus is SARS-COV2, and the protein or epitope is or is from or is a spike protein.

290. The vaccine of any of claims 269-289, wherein the vaccine comprises mRNA encoding the protein or antigen.

291. The vaccine of claim 290, wherein the mRNA is modified to increase stability of the mRNA or the stability of the encoded protein or antigen or epitope.

292. The vaccine of claim 290, wherein the mRNA encodes a modified viral protein, wherein the modification alters the structure of the protein to alter interaction with host cell proteins.

293. The vaccine of any of claims 269-292 that is a delivery vehicle that comprises nucleic acid that encodes an antigen or protein from a pathogen, or encodes a tumor antigen.

294. The vaccine of any of claims 269-293 that comprises an immunostimulatory bacterium, wherein the bacterium comprises genome modifications, whereby it has penta-acylated lipopolysaccharides (LPS), and lacks flagella, wherein wild-type bacterium has flagella.

295. The vaccine of claim 294, wherein the immunostimulatory bacterium has a genome modification(s), whereby it does not produce curli fimbriae.

296. The vaccine of claim 294 or claim 295, wherein the immunostimulatory bacterium comprises a plasmid that encodes an antigen against a pathogen or a tumor antigen.

297. The vaccine of claim 296, wherein the plasmid encodes a therapeutic product is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN).

298. The vaccine of claim 297 wherein the product is STING, IRF3,IRF5, IRF7, IRF8, MDA5, RIG-I or a modified form thereof that comprises a gain-of-function mutation, whereby expression of the type I interferon is constitutive.

299. The immunostimulatory bacterium or vaccine of any of claims 1-298 that is an attenuated bacterium or is a Gram-negative bacterium, or is a Gram-positive bacterium.

300. The immunostimulatory bacterium or vaccine of any of claims 1-299, wherein the bacterium is a strain of *Salmonella*, *Shigella*, *E. coli*, *Bifidobacteriae*, *Rickettsia*, *Vibrio*, *Listeria*, *Klebsiella*, *Bordetella*, *Neisseria*, *Aeromonas*, *Francisella*, *Cholera*, *Corynebacterium*, *Citrobacter*, *Chlamydia*, *Haemophilus*, *Brucella*, *Mycobacterium*, *Mycoplasma*, *Legionella*, *Rhodococcus*, *Pseudomonas*, *Helicobacter*, *Bacillus*, or *Erysipelothrix*, or *Archaeobacteria,* an attenuated strain thereof or a modified strain thereof of any of the preceding list of bacterial strains.

301. The immunostimulatory bacterium or vaccine of any of claims 1-299 wherein the bacterium is *Rickettsia rickettsiae*, *Rickettsia prowazekii*, *Rickettsia tsutsugamuchi*, *Rickettsia mooseri*, *Rickettsia sibirica*, *Bordetella bronchiseptica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Aeromonas eucrenophila*, *Aeromonas salmonicida*, *Francisella tularensis*, *Corynebacterium pseudotuberculosis*, *Citrobacter freundii*, *Chlamydia pneumoniae*, *Haemophilus somnus*, *Brucella abortus*, *Mycobacterium intracellulare*, *Mycobacterium tuberculosis, Staphylococcus aureus, Legionella pneumophila*, *Rhodococcus equi*, *Pseudomonas aeruginosa*, *Helicobacter mustelae*, *Vibrio cholerae*, *Bacillus subtilis*, *Erysipelothrix rhusiopathiae*, *Yersinia enterocolitica*, *Rochalimaea quintana*, or *Agrobacterium tumerfacium*.

302. The immunostimulatory bacterium or vaccine of any of claims 1-301 that is a strain of *Salmonella.*

303. The immunostimulatory bacterium or vaccine of claim 302 that is a *Salmonella typhimurium* strain.

304. The immunostimulatory bacterium or vaccine of claim 302 or claim 303, wherein the unmodified *Salmonella* is a wild-type strain.

305. The immunostimulatory bacterium or vaccine of claim 302 or claim 303, wherein the unmodified *Salmonella* strain is attenuated.

306. The immunostimulatory bacterium or vaccine of any of claims 1-305, wherein the immunostimulatory bacterium is derived from strain VNP20009 or YS1646, or strain ATCC 14028, or a strain having all of the identifying characteristics of strain ATCC 14028.

307. The immunostimulatory bacterium or vaccine of any of claims 301-305 that is *ansB*$^-$, *asd*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$, and *pagP*$^-$ or that is *ansB*$^-$, *thyA*$^-$, *csgD*$^-$, *purI*$^-$, *msbB*$^-$, flagellin$^-$, and *pagP*$^-$.

308. The immunostimulatory bacterium or vaccine of any of claims 1-307, wherein the bacterium encodes and expresses the gene resistance to complement killing (*rck*).

309. The immunostimulatory bacterium or vaccine of claim 308, wherein the *rck* gene is a *Salmonella rck* gene.

310. The immunostimulatory bacterium or vaccine of claim 308 or claim 309 that is an *E. coli* strain.

311. A pharmaceutical composition, comprising the immunostimulatory bacterium or bacterium of vaccine of any of claims 1-310 in a pharmaceutically acceptable vehicle.

312. The pharmaceutical composition of claim 311 that is formulated for systemic administration.

313. The pharmaceutical composition of claim 311 or claim 312 that is formulated for parenteral administration, or intravenous administration, or intramuscular administration, or intratumoral administration, or intraperitoneal administration, or oral administration, or rectal administration, or vaginal administration, or intraocular administration, or intradermal administration, or intracranial administration, or mucosal administration, or administration by inhalation into the mouth or nose

314. A method of treatment of cancer that comprises a solid tumor or a hematological malignancy in a subject, comprising administering the immunostimulatory bacterium, bacterium or vaccine of any of claims 1-310, or the pharmaceutical composition of any of claims 311-313.

315. A method of immunization, comprising administering the vaccine of any of claims 269-298.

316. Use of the immunostimulatory bacterium, bacterium, virus, or vaccine of any of claims 1-310 or the pharmaceutical composition of any of claims 311-313, for the treatment of a cancer that comprises a solid tumor or a hematological malignancy in a subject.

317. The immunostimulatory bacterium, bacterium, or virus, or vaccine of any of claims 1-310 or the pharmaceutical composition of any of claims 311-313 for use for the treatment of or immunization against cancer that comprises a solid tumor or a hematological malignancy in a subject, or for immunization against a pathogen.

318. The method, use, immunostimulatory bacterium, or pharmaceutical composition, or vaccine of any of claims 314-317, wherein the subject is a human.

319. The method, use, immunostimulatory bacterium, or pharmaceutical composition, or vaccine of any of claims 314-318, wherein the subject has a cancer that comprises a solid tumor or comprises a hematological malignancy.

320. The method, use, immunostimulatory bacterium, pharmaceutical composition, or vaccine of any of claims 314-319, wherein the treatment comprises combination therapy in which a second anti-cancer agent or treatment is administered.

321. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of claim 320, wherein the second anti-cancer agent or treatment is administered before, concomitantly with, after, or intermittently with, the immunostimulatory bacterium, or pharmaceutical composition.

322. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of claim 320 or claim 321, wherein the second anti-cancer agent or treatment is an immunotherapy.

323. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-322, wherein administration of the immunostimulatory bacterium, or pharmaceutical composition or vaccine is parenteral.

324. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-322 wherein administration of the immunostimulatory bacterium, or pharmaceutical composition, or vaccine is oral, or rectal, or by aerosol into the lung and/or nose, or mucosal, or intracranial, or intradermal, or intratumoral.

325. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-324, wherein administration of the immunostimulatory bacterium, or vaccine or pharmaceutical composition is intravenously, intramuscularly, intranasally, or subcutaneously.

326. The method, use, immunostimulatory bacterium, pharmaceutical composition, or vaccine of any of claims 314-325, wherein the cancer is selected from among leukemia; lymphoma; gastric cancer; and cancer of the breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head and neck, colorectum, ovary, prostate, brain, pancreas, skin, bone, bone marrow, blood, thymus, uterus, testicles, cervix, and liver.

327. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claim 314-326, wherein the immunotherapy comprises administration of an anti-PD-1, or anti-PD-L1, or anti-CTLA-4 antibody.

328. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-327, wherein the immunostimulatory bacterium is a *Salmonella*, *Shigella*, *Listeria*, or *E. coli* species.

329. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-328, wherein the immunostimulatory bacterium is a *Salmonella* species.

330. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-329, wherein the immunostimulatory bacterium is a *Salmonella typhimurium* strain.

331. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-330, wherein administration of the immunostimulatory bacterium is by intraperitoneal or intratumoral administration.

332. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-331, wherein the subject has metastatic cancer.

333. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-332, comprising administering a second anti-cancer treatment, wherein the second treatment is selected from among anti-PD-1, anti-CTLA-4, anti-PD-L1, anti-IL-6, anti-Siglec-15, anti-VEGF, anti-CD73, and anti-CD38 antibodies.

334. The method, use, immunostimulatory bacterium, vaccine, or pharmaceutical composition of any of claims 314-333, comprising administering a second or further anti-cancer treatment, wherein the second or further treatment is selected from among a poly (ADP-ribose) polymerase (PARP) inhibitor, a histone deacetylase (HDAC) inhibitor, a chemotherapy agent, an anti-EGFR antibody, a CAR-T cell, an anti-Her2 antibody, an anti-mesothelin antibody, and an anti-B-cell maturation antigen (BCMA) antibody.

335. A method of treatment of a viral infection or infection with another infectious pathogen, comprising administering the immunostimulatory bacterium or vaccine of any of claims 1-310, wherein the immunostimulatory bacterium or vaccine comprises or encodes an antigen, protein, or epitope thereof from the virus or pathogen.

336. The method of claim 335, wherein the immunostimulatory bacterium encodes the antigen, protein or epitope thereof under control of a promoter recognized by the bacterium; and the immunostimulatory bacterium comprises a genome modification(s), whereby it does not replicate *in vivo*.

337. The immunostimulatory bacterium or vaccine of any of claims 1-310, or method of claim 335 and 336 for use for treating a viral infection or infection with another infectious agent.

338. A method of immunizing to prevent (reduce the risk of) infection by a virus or other infectious agent, comprising administering an immunizing dose of the immunostimulatory bacterium or vaccine of any of claims 1-310, wherein a product encoded by the immunostimulatory bacterium is an anti-viral or anti-pathogen therapeutic or antigen, epitope, or protein.

339. The immunostimulatory bacterium or vaccine of any of claims 1-310 for use for immunizing against, to protect against, or to reduce the severity of or reduce the risk of infection by a virus or another infectious agent, wherein a produced encoded by the immunostimulatory bacterium is an anti-viral or anti-pathogen therapeutic or antigen, epitope, or protein.

340. The immunostimulatory bacterium or vaccine or method of any of claims 335-339, wherein the virus or infectious agent is a virus.

341. The immunostimulatory bacterium, or vaccine, or pharmaceutical composition, or method, or use of any of claims 1-340, wherein the immunostimulatory bacterium comprises a plasmid that encodes RNA operably linked to a promoter

recognized by a polymerase present in the bacterium, wherein, upon infection of a eukaryotic host cell, the RNA is released into the cytoplasm of the eukaryotic host.

342. The immunostimulatory bacterium, or vaccine, or pharmaceutical composition, or method, or use of claim 341, wherein the RNA released into the cytoplasm of the eukaryotic host is mRNA.

343. The immunostimulatory bacterium, or vaccine, or pharmaceutical composition, or method, or use of claim 341 or claim 342, wherein the promoter is an inducible promoter.

344. The immunostimulatory bacterium, or vaccine, or pharmaceutical composition, or method, or use of any of claims 341-343, wherein the immunostimulatory bacterium also encodes a polymerase that recognizes the promoter.

345. The immunostimulatory bacterium, or vaccine, or pharmaceutical composition, or method, or use of any of claims 341-344, wherein the polymerase is a T7 RNA polymerase.

346. The immunostimulatory bacterium, or vaccine, or pharmaceutical composition, or method, or use of any of claims 341-345, wherein the immunostimulatory bacterium is *asd*$^-$ or is *thyA*$^-$ or both.

347. The immunostimulatory bacterium or vaccine of any of claims 1-310, for use for treating a tumor or a viral infection, wherein the bacterium encodes an anti-cancer or anti-viral therapeutic product or antigen, protein, or epitope or combinations thereof form the virus or tumor.

348. A method of treatment of cancer or a viral infection, comprising administering the RNA delivery system of any of claims 68-80 to a subject with cancer and/or a viral infection, or the immunostimulatory bacterium or vaccine of any of claims 1-310 that encodes a tumor-associated antigen or viral antigen, protein or epitope.

349. A method of converting an M2 macrophage to an M1 or M1-like phenotype, comprising administering the immunostimulatory bacterium of any of claims 38-82 to a subject with a condition, disease, or disorder treated by enhancing an anti-viral or anti-tumor immune response.

350. Use of the immunostimulatory bacterium of any of claims 38-82, or the immunostimulatory bacterium of any of claims 38-82, to convert an M2 macrophage to an M1 or M1-like phenotype macrophage in a subject with a condition, disease, or disorder treated by enhancing an anti-viral or anti-tumor immune response

351. The method of claim 349 or use or bacterium of claim 350, wherein the disease, disorder, or condition is a cancer and/or a viral or other pathogen infection.

352. A method of delivering RNA encoding a therapeutic product, comprising administering an immunostimulatory bacterium of any of claims 16-25 for treatment of a disease, condition, or disorder.

353. Use of an immunostimulatory bacterium of any of claims 68-82, or the immunostimulatory bacterium of any of claims 68-82, for use for delivering RNA encoding a therapeutic product for treatment of a disease, condition, or disorder.

354. The method of claim 352 or the use or bacterium of claim 353, wherein the disease, disorder, or condition is a cancer and/or a viral or other pathogen infection.

355. A pharmaceutical composition, comprising the bacterium of any of claims 231-310 in a pharmaceutically acceptable vehicle.

356. The pharmaceutical composition of claim 355 that is formulated as vaccine, as a liquid, powder, or tablet.

357. The bacterium or pharmaceutical composition of any of claims 231-313, 355, and 356, for use for treating or preventing (reducing the risk of developing) a disease or condition or infection or cancer.

358. A method for treating or preventing (reducing the risk of developing) a disease or condition or infection or cancer, comprising administering the bacterium or pharmaceutical composition of any of claims 231-313, 355 and 356.

359. A method of delivering RNA, comprising administering the bacterium or pharmaceutical composition of any of claims 231-310, and 355-357.

360. The bacterium of any of claims 231-310 and 355-357, for use for delivering RNA.

399. The method of claim 359 or the bacterium of claim 360, wherein the RNA is mRNA.

400. The bacterium of any of claims 231-310 and 355-357, wherein the product is a therapeutic product, and the plasmid in the bacterium encodes the product to produce mRNA that is not translated by the bacterium.

401. A method of delivering RNA to a subject, comprising administering the bacterium of claim 400.

402. The bacterium of claim 400, for use for delivering RNA to a subject.

403. A bacterium for use for delivering RNA to a subject, comprising a plasmid encoding a heterologous product, wherein:

the nucleic acid encoding the heterologous product is linked to a promoter recognized by the bacterium; and

the nucleic acid encoding the product comprises eukaryotic sequences for translation that are not recognized by the bacterium, whereby the bacterium produces RNA, but does not translate the RNA.

404. The bacterium of claim 403 that is an immunostimulatory bacterium of any of claims 1-310, wherein the nucleic acid encoding the RNA encodes a therapeutic product.

405. The bacterium of claim 403 or claim 404, wherein the bacterium encodes an antigen or protein from a pathogen or tumor for eliciting an immune response against the antigen or protein.

406. A modified Stimulator of Interferon Genes (STING) protein from a non-human species, where the non-human STING is one that has lower NF-κB signaling activity compared to human STING, and, optionally, higher type I interferon (IFN) pathway signaling activity compared to human STING, wherein:

the non-human STING protein is modified to include a mutation or mutations so that it has increased activity or acts constitutively in the absence of cytosolic nucleic acids;

the mutations are insertions, deletions, and/or replacements of amino acids; and

the STING protein optionally has a deletion or disruption of the TRAF6 binding site.

407. A modified Stimulator of Interferon Genes (STING) protein from a non-human species, or a chimeric human STING protein and modifications thereof, comprising one or more mutation(s) associated with gain-of-function (GOF) that result in the constitutive activation of the encoded STING protein and/or enhanced sensitivity, or increased affinity or binding to endogenous ligands, whereby the STING protein is modified by one or more of an insertion, deletion, and replacement of an amino acid or amino acids;

the STING protein has IFN-beta signaling activity, and attenuated nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling activity, compared to human STING; and

the mutation or mutations result in increased STING activity or constitutive activity in inducing IFN-beta production.

408. The modified STING protein of claim 406 or claim 407, wherein the human STING protein comprises the sequence set forth in any of SEQ ID NOs:305-309, or is a human allelic variant thereof with at least 98% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOs:305-309.

409. The modified STING protein of any of claims 406-408, wherein:

the STING protein is a chimera comprising replacement of a C-terminal tail (CTT) region in a STING protein from a first species, with the CTT of a STING protein from a second species;

the STING protein of the second species has lower NF-κB signaling activity than the NF-κB signaling activity of human STING; and

the TRAF6 binding site in the CTT optionally is deleted.

410. The modified STING protein of any of claims 406-409, wherein the mutation or mutations is/are any that correspond to those associated with the auto-inflammatory disease STING-associated vasculopathy (SAVI).

411. A modified Stimulator of Interferon Genes (STING) protein that is a chimera, comprising replacement of the CTT (C-terminal tail) region in a STING protein from a first species, with the CTT of a STING protein from a second species, wherein:

the STING protein of the second species has lower NF-κB signaling activity than the NF-κB signaling activity of human STING; and

the TRAF6 binding site in the CTT optionally is deleted.

412. The modified STING protein of any of claims 406-411, wherein the human STING protein comprises the sequence set forth in any of SEQ ID NOs:305-309, or is a human allelic variant thereof with at least 98% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOs:305-309.

413. The modified STING protein of any of claims 406-411, wherein the first species is human, and the second species is selected from among Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, and ghost shark.

414. The modified STING protein of any of claims 406-413, wherein the type I IFN signaling activity is at least or at least about 30% that of a wild type human STING protein.

415. The modified STING protein of any of claims 406-414, wherein the NF-κB signaling activity is less than 30%, less than 20%, less than 15%, less than 10%, or less than 5% that of wild type human STING NF-κB signaling activity.

416. The modified STING protein of any of claims 406-415, wherein the non-human species or second species is selected from among Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, and ghost shark.

417. The modified STING protein of any of claims 406-416, wherein the modification of STING is a mutation or mutations that correspond, by reference to and alignment with human STING, to a mutation that occurs in an interferonopathy, wherein the sequence of human STING with which alignment is effected is set forth in any of SEQ ID NOs:305-309.

418. The modified STING protein of any of claims 406-417 that comprises replacement of the C-terminal tail (CTT) with the CTT from a STING protein that has reduced NF-κB signaling activity compared to the NF-κB signaling activity of human STING.

419. The modified STING protein of claim 418, wherein the replacing CTT is from a Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, or ghost shark STING protein.

420. The modified STING protein of claim 418, wherein the replacing CTT is from a Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, or ghost shark STING protein, and it replaces the human STING CTT.

421. The modified STING protein of claim 419 or claim 420, wherein the replacing CTT is selected from among the following species, and has a sequence:

Tasmanian devil RQEEFAIGPKRAMTVTTSSTLSQEPQLLISGMEQPLSLRTDGF SEQ ID NO:371,
Marmoset EEEEVTVGSLKTSEVPSTSTMSQEPELLISGMEKPLPLRSDLF SEQ ID NO:372,
Cow EREVTMGSTETSVMPGSSVLSQEPELLISGLEKPLPLRSDVF SEQ ID NO:373,
Cat EREVTVGSVGTSMVRNPSVLSQEPNLLISGMEQPLPLRTDVF SEQ ID NO:374,
Ostrich RQEEYTVCDGTLCSTDLSLQISESDLPQPLRSDCL SEQ ID NO:375,
Boar EREVTMGSAETSVVPTSSTLSQEPELLISGMEQPLPLRSDIF SEQ ID NO:376,
Bat EKEEVTVGTVGTYEAPGSSTLHQEPELLISGMDQPLPLRTDIF SEQ ID NO:377,
Manatee EREEVTVGSVGTSVVPSPSSPSTSSLSQEPKLLISGMEQPLPLRTDVF SEQ ID NO:378,
Crested ibis CHEEYTVYEGNQPHNPSTTLHSTELNLQISESDLPQPLRSDCF SEQ ID NO:379,
Coelacanth
(variant 1) QKEEYFMSEQTQPNSSSTSCLSTEPQLMISDTDAPHTLKRQVC SEQ ID NO:380,

Coelacanth
(variant 2) QKEEYFMSEQTQPNSSSTSCLSTEPQLMISDTDAPHTLKSGF SEQ ID NO:381,
Ghost shark LTEYPVAEPSNANETDCMSSEPHLMISDDPKPLRSYCP SEQ ID NO:383, and
Mouse EKEEVTMNAPMTSVAPPPSVLSQEPRLLISGMDQPLPLRTDLI SEQ ID NO:384,

or allelic variants of each of these sequences, having at least 98% sequence identity thereto.

422. The modified STING protein of claim 420 or claim 421, wherein the human CTT comprises the sequence EKEEVTVGSLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS (SEQ ID NO:370), or is an allelic variant having at least 98% sequence identity thereto.

423. The modified STING protein of any of claims 411-422, wherein the modified STING protein is a chimera in which the human STING CTT is replaced with a CTT from the Tasmanian devil STING.

424. The modified STING protein of claim 423, wherein the C-terminal tail (CTT) from the Tasmanian devil STING comprises the sequence: RQEEFAIGPKRAMTVTTSSTLSQEPQLLISGMEQPLSLRTDGF (SEQ ID NO:371), or is an allelic variant having at least 98% sequence identity thereto.

425. The modified STING protein of any of claims 406-424, comprising a deletion or disruption of the TRAF6 binding site.

426. The modified STING protein of claim 425, wherein the STING protein is a human STING protein, and the TRAF6 binding site comprises the amino acid residues DFS at the C-terminus.

427. The modified STING protein of any of claims 406-426, comprising a modification that increases type I interferon signaling activity, or that renders the activity constitutive in the absence of cytosolic nucleic acids.

428. The modified STING protein of claim 427, wherein the modification corresponds, by reference to and alignment with human STING, to a mutation that occurs in an interferonopathy, wherein the human STING protein has the sequence set forth in any of SEQ ID NOs:305-309.

429. The modified STING protein of any of claims 406-428, wherein the modification is one or more amino acid replacements that correspond(s) to one or more of S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E, S102P/F279L, F279L, R281Q, R284G, R284S, R284M, R284K, R284T, R197A, D205A, R310A, R293A, T294A, E296A, R197A/D205A, S272A/Q273A, R310A/E316A, E316A, E316N, E316Q,

S272A, R293A/T294A/E296A, D231A, R232A, K236A, Q273A, S358A/E360A/S366A, D231A/R232A/K236A/R238A, S358A, E360A, S366A, R238A, R375A, and S324A/S326A, with reference to the sequence of human STING, as set forth in any of SEQ ID NOs:305-309.

430. The modified STING protein of claim 429 that comprises a replacement corresponding to C206Y or R284G, with reference to the sequence of human STING, as set forth in any of SEQ ID NOs:305-309.

431. The modified STING protein of claim 430 that is a Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, or ghost shark STING protein.

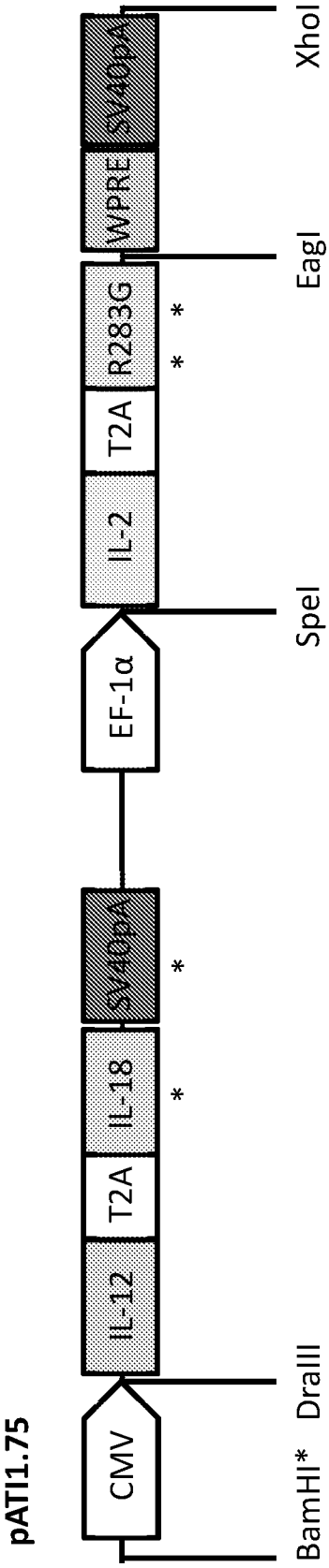

pATI1.75
CMV
IL-12
T2A
IL-18
SV40pA
EF-1α
IL-2
T2A
R283G
WPRE
SV40pA
BamHI*
DraIII
SpeI
EagI
XhoI

FIG. 1A

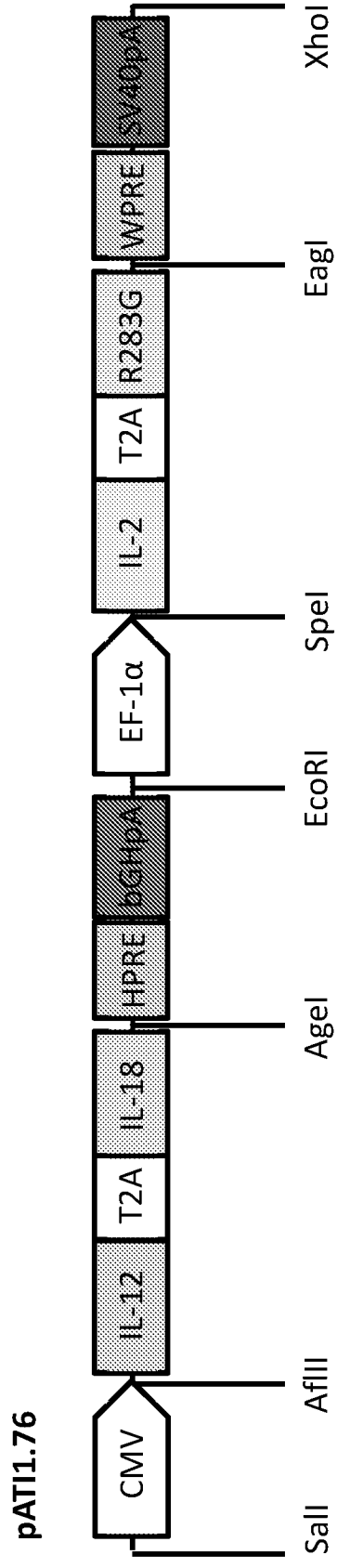

pATI1.76
CMV
IL-12
T2A
IL-18
HPRE
bGHpA
EF-1α
IL-2
T2A
R283G
WPRE
SV40pA
SalI
AflII
AgeI
EcoRI
SpeI
EagI
XhoI
EES26

FIG. 1B

ADN-457 pATI1.76 CMV muIL-36γ DelCyt T2A muIL-12p70 P2A muOX40L HPRE bGHpA

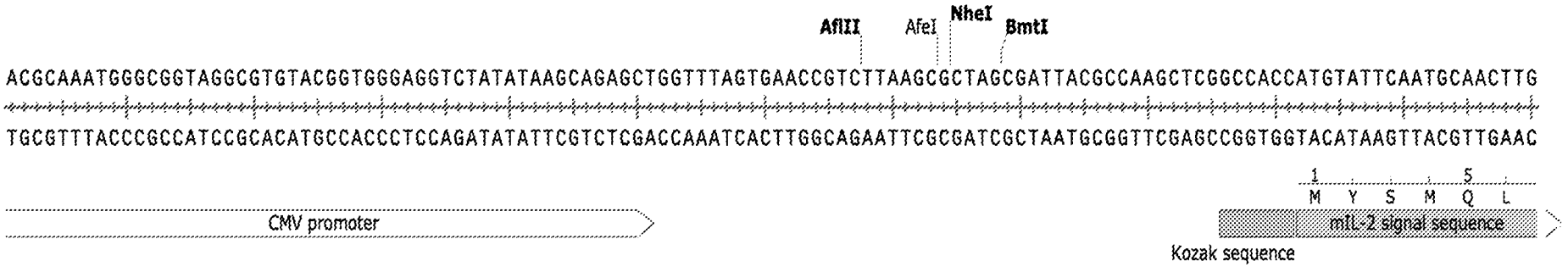



**FIG. 1C**

**Human STING (SEQ ID NO:306) and Tasmanian devil STING (SEQ ID NO:349):**

```
Human             1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                    ||.|.|||||||.|||.|||||||.||||.|:..:::.|||....||::|:.:
Tasmanian         1 MPRSHLHPSIPQPRGWGAQKAACVLLSLCMAAVYILGESAAFTLQWLLFY   50

Human            51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                    ..::|:||||.|...||||||.||.||::||||:.::||:..|.:|.:|||
Tasmanian        51 FTTVQVGLLLKGAYCLAEELYHIQSRHQGSYWQAIQACIHYPFQRISLLL  100

Human           101 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK  150
                    ||.|||.:|.|...|..||..|||||||.||:.:|||:.|..|||||.||||
Tasmanian       101 LSGYFYVTLSNKPNPSLTWTFALLGLSHALSFILGLQNLTSAEISEVCEK  150

Human           151 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI  200
                    .:.|||||||||||||:||||.||.||:.|||...::||.....||:|
Tasmanian       151 RHLNVAHGLAWSYYIGYLKLILPGLQTRIQIYNQFNKDVLRSPEGHRLHI  200

Human           201 LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN  250
                    |:||||.||||||.|||||:||.:|||...|.||||.|:|:|||||::.|:
Tasmanian       201 LIPLDCSVPDNLSQADPNIQFLQELPQHKLDRAGIKGRIYTNSIYKISED  250

Human           251 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA  300
                    ||.|..||||:||||||||||||.::|||||||||||||||||||||||.
Tasmanian       251 GQPAEVCVLEFATPLQTLFAMSQDARAGFSREDRLEQAKLFCRTLEDILE  300

Human           301 DAPESQNNCRLIAYQEPADD--SSFSLSQEVLRHLRQEEKEEVTVGSLKT  348
                    ||||:::.||||:.|||||.|.  |:|||||:|||||||.:||..:|..:.
Tasmanian       301 DAPEARDCCRLVVYQEPEDKAVSNFSLSKEILRHLRQERQEEFAIGPKRA  350

Human           349 SAVPSTSTMSQEPELLISGMEKPLPLRTD-FS   379
                    ..|.::||:||||:||||||||:||.|||| |
Tasmanian       351 MTVTTSSTLSQEPQLLISGMEQPLSLRTDGF-   381
```

**FIGURE 2**

**Human STING (SEQ ID NO:306) and Marmoset STING (SEQ ID NO:359):**

```
Human              1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                     |||||||||||.|||||||:|||||||.||||||.|.|.|||..|||:||||
Marmoset           1 MPHSSLHPSIPHPRGHGAQEAALVLLSVCLVTLWWLREAPEDILRFLVLH   50

Human             51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                     ||||||||||.:|||||||||:|:|||:||||||.|||||||||:|.|||.||
Marmoset          51 LASLQLGLLLNRLCSLAEELRHVHTRYQGSYWRAVRACLGCPIRLGAQLL   100

Human            101 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK  150
                     ||||||..|||  |.||||||||||.|||||||||||||||||||||||||
Marmoset         101 LSIYFYCFLPN---GRPFTWMLALLGFSQALNILLGLKGLAPAEISAVCEK  148

Human            151 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI  200
                     .|||||||||||||||||||..||||||||||.|||:|||..|||||||
Marmoset         149 RNFNVAHGLAWSYYIGYLRLILPGFQARIRTYNQHNNNVLRGPASQRLYI  198

Human            201 LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN  250
                     |.||||||||||.|||||||||||||:|.|.|||||.|||:||||||||||
Marmoset         199 LFPLDCGVPDNLSTADPNIRFLDKLPQETIDRAGIKGRVYTNSIYELLEN  248

Human            251 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA  300
                     |||||.||||||:||||||||||.||||||||||||||||||||||||||
Marmoset         249 GQRAGACVLEYASPLQTLFAMSQYGQAGFSREDRLEQAKLFCRTLEDILA  298

Human            301 DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA  350
                     ||||||||||||.|:|||||.|||.||||||:||||||:||||||||||.
Marmoset         299 DAPESQNNCRLIVYEEPADGSSFLLSQEVLQHLRQEEEEEVTVGSLKTSE  348

Human            351 VPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                     |||||||||||||||||||||||||||:|..
Marmoset         349 VPSTSTMSQEPELLISGMEKPLPLRSDLF   377
```

**FIGURE 3**

**Human STING (SEQ ID NO:306) and cattle STING (SEQ ID NO:360):**

```
Human              1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                     ||||||||||.|||..|||||||||||||.||||||||:|||:|||||
Cattle             1 MPHSSLHPSIPQPRGLRAQKAALVLLSACLVALWGLGEPPDYTLKWLVLH   50

Human             51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                     |||.|:||||:.|:||||||||.|:|||||.|||||.|||||...:|.|||||
Cattle            51 LASQQMGLLIKGICSLAEELCHVHSRYHGSYWRAVRACLCSSMRCGALLL  100

Human            101 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK  150
                     ||.|||.||||....|||||||||||||||||||:||||||:|||:|||
Cattle           101 LSCYFYCSLPNMADLPFTWMLALLGLSQALNILLGLQGLAPAEVSAICEK  150

Human            151 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI  200
                     .|||||||||||||||||||||.|.|||:.|||.:||.|:||.|.||:|
Cattle           151 RNFNVAHGLAWSYYIGYLRLILPGLPARIQIYNQFHNNTLQGAGSHRLHI  200

Human            201 LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN  250
                     |.||||||||:|::|||||||||.:|||||:.|.||||.|||:|||||||||
Cattle           201 LFPLDCGVPDDLNVADPNIRFLHELPQQSADRAGIKGRVYTNSIYELLEN  250

Human            251 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA  300
                     |||||.|||||||||||||||||..:|||||||||||||||||||||||||
Cattle           251 GQRAGVCVLEYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRTLEDILA  300

Human            301 DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA  350
                     :|||||||||||.||||||:.||||||||:|:|||||||: |||:||.:||.
Cattle           301 NAPESQNNCRLIVYQEPAEGSSFSLSQEILQHLRQEER-EVTMGSTETSV  349

Human            351 VPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                     :|.:|.:||||||||||:||||||||:|..
Cattle           350 MPGSSVLSQEPELLISGLEKPLPLRSDVF   378
```

**FIGURE 4**

**Human STING (SEQ ID NO:306) and cat STING (SEQ ID NO:356):**

```
Human              1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                     ||.:.|||||.|||.||||||||||:.||..||.|||.|:|||||:||||
Cat                1 MPRTGLHPSIPRPRGMGAQKAALVLLAVCLAALWRLGESPDHTLRWLVLH   50

Human             51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                     |||||||||..|||.|.|||.|:||||:|||||.::|||||.|:|.|||||
Cat               51 LASLQLGLLFTGVCHLTEELCHLHSRYQGSYWRAMKACLGSPVRSGALLL  100

Human            101 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK  150
                     ||.|||.:|| :...|||||||||||||||||||.|:|||||||:|||||
Cat              101 LSCYFYSTLP-STDLPFTWMLALLGLSQALNILLDLQGLAPAEVSAVCEK  149

Human            151 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI  200
                     .|||||||||||||||||||||.|.||:.|.|.||.:|||:|||..|.||:|
Cat              150 KNFNVAHGLAWSYYIGYLRLILPGLPARVLTCNQLHNNILRGTGSHRLHI  199

Human            201 LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN  250
                     |.|||||||::|:|||||||||.:||||:.|.|||||.|||:||:|.||||
Cat              200 LFPLDCGVPDDMSVADPNIRFLYELPQQSADRAGIKGRVYTNSVYALLEN  249

Human            251 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA  300
                     ||:||.|||||||||||||||..:|||||||||||||||||.|||||||
Cat              250 GQQAGICVLEYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRILEDILA  299

Human            301 DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA  350
                     |.||.|||||||.||||.:.|:||||||:||||||||: ||||||:.||.
Cat              300 DTPECQNNCRLIVYQEPEEGSNFSLSQEILRHLRQEER-EVTVGSVGTSM  348

Human            351 VPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                     |.:.|.:||||.|||||||:||||||..
Cat              349 VRNPSVLSQEPNLLISGMEQPLPLRTDVF   377
```

**FIGURE 5**

**Human STING (SEQ ID NO:306) and ostrich STING (SEQ ID NO:361):**

```
Human             1 MPHSSLHPS--------IPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTL   44
                    |.|.|..||        ||..|...||.||.|||:.|...|:..|||..|..
Ostrich           1 MAHESGTPSNPATPLIPKAREGRAQHAAYVLLALCAAALYLAGEPVVHIA   50

Human            45 RYLVLHLASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLR   94
                    |....|..:|||:|.||.|:|.|.||:.|:.:|:|||:.|.:.|||  .||
Ostrich          51 RSFTSHFVALQIGALLKGICYLVEEIFHLETRHRGSFRRALSACL--HLR   98

Human            95 -RGALLLLSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAE  143
                     ...|||:....|.:|.:....|....|.|..|.|.|.:.|||...:..|
Ostrich          99 WHVTLLLVCGSAYVALLDGDEQPLGLHLGLACLCQLLILALGLHKPSAVE  148

Human           144 ISAVCEKGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGA  193
                    ||.:.|:...||||||||||:|||:::||.|:..:....:...|:|...
Ostrich         149 ISEMSERSKQNVAHGLAWSYYVGYLKIVLPRLKESMENIGRTNPNMLAHK  198

Human           194 VSQRLYILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNS  243
                    .:.:|:||:||.|.:.|:|..||.||::...||:.|...||.|.|||.|:
Ostrich         199 ETWKLHILVPLSCNICDDLEKADSNIQYAMDLPETTLTRAGTKKRVYRNT  248

Human           244 IYELLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCR  293
                    :|: :::..:...||:||||||||:|:||||...|.||||||:||||||.|
Ostrich         249 LYK-IKDEDKFRFCVVEYATPLQSLYAMSQDECAAFSREDRIEQAKLFYR  297

Human           294 TLEDILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTV  343
                    |||:||..|.|.....|||.|:|..:.:..||:|:||||:|:.:||.||
Ostrich         298 TLEEILQSAKECAGTYRLIVYEESGEAETHFLSREILRHLQQQRQEEYTV  347

Human           344 --GSLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                      |:|          .|.:..|.||..:.|.|||:|..
Ostrich         348 CDGTL----------CSTDLSLQISESDLPQPLRSDCL   375
```

**FIGURE 6**

**Human STING (SEQ ID NO:306) and crested ibis STING (SEQ ID NO:362):**

```
Human             1 ---MPHSSLHPS----IPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTL   44
                    .|....||:   ||..||..||.|..:||:.|...|:..|||...:.
Crested           1 MSQEPQWLSHPTALLIPKARGGRAQHAVYLLLALCAAVLYLAGEPLVPSA   50

Human            45 RYLVLHLASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLR   94
                    |.|:.|..:|||:|:||.|.|.|||||:.|:.||:.||:||.:.||.  |||
Crested          51 RSLISHFMALQIGVLLKGTCYLAEEIFHLQSRHHGSFWRALSACF--PLR   98

Human            95 RGALLLLSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEI  144
                    ...|:||.....|......|.|....|.|..|.|.|.:.|||...:..|:
Crested          99 WHGLMLLVCGSAYVALLEDGQPLGLHLGLASLCQLLILALGLHKPSAVEM  148

Human           145 SAVCEKGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAV  194
                    |.:.|:...||||||||||:|||:::||.:::.:.:.:::...|:|....
Crested         149 SEMSERSKQNVAHGLAWSYYVGYLKIVLPRVKKSMEEFSRANPNVLARRE  198

Human           195 SQRLYILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSI  244
                    :.:|:|||:||.|.|.|:|..||.||::|..||:.|...||.|.|||.:|:
Crested         199 TWKLHILVPLSCDVYDDLEKADSNIQYLMDLPETTLTRAGTKKRVYKHSL  248

Human           245 YELLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRT  294
                    |.:.:.|.:...|.:||||||||:|:||||...|.|||||||||||.||
Crested         249 YTIRDEGNKLWHCAVEYATPLQSLYAMSQDEYAAFSREDRLEQAKLFYRT  298

Human           295 LEDILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVG  344
                    ||:||..:.|.....|||.|:|..:..:.|||:::|.||||:..||.||.
Crested         299 LEEILKGSKECAGTYRLIVYEESGEAETHSLSRDILWHLRQQCHEEYTVY  348

Human           345 SLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                    .......|||:..|.|..|.||..:.|.|||:|.
Crested         349 EGNQPHNPSTTLHSTELNLQISESDLPQPLRSDC-   382
```

**FIGURE 7**

**Human STING (SEQ ID NO:306) and coelacanth (variant with SEQ ID NO:363) STING:**

```
Human             1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                    ....:....||.|||:.|.:.|..:::..|:.||.......:.|..:.||
Coelacanth        1 FGLQNMSAIIPQPRGNRANQMAYFIITVILMLLWIFRNIMDNVLEVIALH   50

Human            51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                    :..||...:::.|:|:.|||:.|:..||.||||:.:.|||.........::
Coelacanth       51 ILLLQGAAIMKGICNFAEEVNHVQPRYGGSYWKALEACLNLSKYDVMKIV  100

Human           101 LS--IYFYYSLPNAVGPPFTWMLALL--GLSQALNILLGLKGLAPAEISA  146
                    .:  ::::::|....|    .|.|.||  .|...||.:|||:...:|.|:|.
Coelacanth      101 FAGVLWWHFSSSLFV-----VWFLHLLVSCLCHLLNNVLGVLKPSPVEVSE  146

Human           147 VCEKGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQ  196
                    :.|:....||||||||||:|||:|:||||:.||::||..:.|||:...:.
Coelacanth      147 IYERNRIGVAHGLAWSYYLGYLKLVLPELEDRIKSYNVSHGNLLKHKETW  196

Human           197 RLYILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYE  246
                    ||:||||||.|.:.|||:..|.||.|||.|.:...:.||||.|.|.:|:|:
Coelacanth      197 RLHILLPLSCSIFDNLADVDSNIEFLDNLSELQINRAGIKGRSYKHSLYQ  246

Human           247 LLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLE  296
                    :|:..:|...|:|||||||::|..||:.:.|.|::||||||.|||.|||:
Coelacanth      247 VLDEDKRPHYCILEYATPLKSLLEMSKEASAEFTKEDRLEQTKLFYRTLK  296

Human           297 DILADAPESQNNCRLIAY--QEPADDSSFSLSQEVLRHLRQEEKEEVTVG  344
                    |||.::.|.:...||:.|  :|.:..|.| ||:|:||||.|::|||..:.
Coelacanth      297 DILDNSQECRGRFRLVIYDGREDSGKSHF-LSKELLRHLNQQQKEEYFMS  345

Human           345 SLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                    ........|||.:|.||:|:||..:.|..|:....
Coelacanth      346 EQTQPNSSSTSCLSTEPQLMISDTDAPHTLKRQVC   380
```

**FIGURE 8**

**Human STING (SEQ ID NO:306) and zebrafish STING (SEQ ID NO:348):**

```
Human              1 ----------MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPE   41
                           :|.:.....:.|..|.|    .|.|..|.|:......|
Zebrafish          1 MSVMGEDALVPRARSRLPVMCAAGLG----FLTLAVAWLLDSDKFSE---   43

Human             42 HTLRYLVLHLASLQLGLLLNG----VCSLAEELR-HIHSRYRGSYWRTVR   86
                         ....:..||:|..    :|.||||||. |...||.|......|
Zebrafish         44 ---------RAGIIAFGLMLERFIYCICLLAEELLFHSRQRYHGRMSEIFR   85

Human             87 ACLGCPLRRGA--LLLLSIYFYYSLPNAVGPPFTWMLALLGLSQAL----  130
                   ||.     ||:  |.:.:|:.               ||.|.|:|.::
Zebrafish         86 ACF------RGSGILGMCAIFL----------------MLMLGGVSFSVKQWS  116

Human            131 --NIL---------LGLKGLAPAEISAVCEKGNFNVAHGLAWSYYIGYLR  169
                       |::          ||:.|.||.|||.:||....||||||||:||||:
Zebrafish        117 HFNLMCAGYMLLNSLGVLGPAPVEISEICEAKKMNVAHGLAWSFYIGYLK  166

Human            170 LILPELQARIRTYNQHYNNLLRGAVSQRLYILLPLDCGVPDNLSMADPNI  219
                   .:||.|:..:|.|::.     ....|.||:||||:..||.....|.|:
Zebrafish        167 FLLPALEVNVREYSRR-----ERLSSPRLHILLPLNARVPSKPEEEDTNV  211

Human            220 RFLDKLPQQTGDHAGIKDRVYSNSIYELLENGQRAGTCVLEYATPLQTLF  269
                   .|.:.||....|.||:.:.|.|:||:|::..|.: ..:|:|||||||.||:
Zebrafish        212 VFHENLPDLKLDRAGVRKRSYTNSVYKITHNNE-TFSCILEYATPLLTLY  260

Human            270 AMSQYSQAGFSREDRLEQAKLFCRTLEDILADAPESQNNCRLIAYQEPAD  319
                   .|||.|.|||...:|.:|..||.|||..||.::.|.:|..|||...:...
Zebrafish        261 QMSQESSAGFGERERKQQVLLFYRTLSQILDNSLECRNRYRLILLNDEHT  310

Human            320 DSSFSLSQEVLRHLRQEE--------------KEEVTVGSLKTSAVPST-  354
                   .....||:|:.::|:|::               .||..||:. ..|:.:|
Zebrafish        311 GDPHYLSRELFQNLKQQDGEIFMDPTNEVHPVPEEGPVGNC-NGALQATF  359

Human            355 --STMSQEPELLISGMEKPLPLR------TDFS---------   379
                       ..||.||.|:.|   :|..||      ||:.
Zebrafish        360 HEEPMSDEPTLMFS---RPQSLRSEPVETTDYFNPSSAMKQN   398
```

**FIGURE 9**

**Human STING (SEQ ID NO:305) and boar STING (SEQ ID NO:365):**

```
Human             1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                    ||:||||||||.|||..||.|||||.|||||.|||||.||:||||:||||
Boar              1 MPYSSLHPSIPQPRGLRAQVAALVLLGACLVALWGLGELPEYTLRWLVLH   50

Human            51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                    |||.|:|||:.|:||||||||.|:||||:.|||||..||||||||:|.||||
Boar             51 LASQQIGLLVKGLCSLAEELCHVHSRYQSSYWRAARACLGCPIRCGALLL  100

Human           101 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK  150
                    ||.|||:|:.:..|.|..||||||||||||||||||:.||||||:||:|||
Boar            101 LSCYFYFSIRDKAGLPLPWMLALLGLSQALNILLGLQHLAPAEVSAICEK  150

Human           151 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI  200
                    .|||||||||||||||||||.|:|||:.|||.:.|:|.|..:.||:|
Boar            151 RNFNVAHGLAWSYYIGYLRLILPGLRARIQAYNQRHKNVLGGIGNHRLHI  200

Human           201 LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYELLEN  250
                    |.|||||||:||:|||||||.:|||||:.|||||.|||:|||||||||
Boar            201 LFPLDCGVPDDLSVADPNIRFLHELPQQSADRAGIKGRVYTNSIYELLEN  250

Human           251 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA  300
                    ||.||.|||.|||||||||||..:|||||||||||||||||||||||||
Boar            251 GQPAGVCVLGYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRTLEDILA  300

Human           301 DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA  350
                    ||||:|||||||.|||||.:..|||||||:|||||||: |||:||.:||.
Boar            301 DAPEAQNNCRLIVYQEPTEGGSFSLSQEILRHLRQEER-EVTMGSAETSV  349

Human           351 VPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                    ||::||:||||||||||||:||||||:|..
Boar            350 VPTSSTLSQEPELLISGMEQPLPLRSDIF   378
```

**FIGURE 10**

**Human STING (SEQ ID NO:305) and bat STING (SEQ ID NO:366):**

```
Human          1 MPHSSLHPSIPCPRGHGAQK--AALVLLSACLVTLWGLGEPPEHTLRYLVL   49
                 |.|||||||:|.|||..|:. ||.|||..||..||..|:|.|:||||.|||
Bat            1 MSHSSLHPSVPRPRGRRARNIAAFVLLIVCLAALWLSGKPSEYTLRCLVL   50

Human         50 HLASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALL   99
                 ||||.||||||.|.||.||||||.|||||:|:|||.||||||.||:|.|.:|
Bat           51 HLASQQLGLLSNRVCYLAEELSHIHSRYQGNYWRAVRACLSCPIRFGVVL  100

Human        100 LLSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCE  149
                 |:|.:||.||||....|.||||||||||:|||||||:||.|||:|.|||
Bat          101 LVSFFFYTSLPNIDDLPLTWMLAHLGLSEALNILLGLRGLTPAEVSTVCE  150

Human        150 KGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLY  199
                 :.:|||||||||||||||||||.|:|||.||||.:.|.|:|..|.|||
Bat          151 QRHFNVAHGLAWSYYIGYLRLILPGLRARIHTYNQLHGNTLQGVGSHRLY  200

Human        200 ILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYELLE  249
                 ||.|||||.|:||.|||||||.:|||:|:.|||||:|||:||:|||||
Bat          201 ILFPLDCGVLDDLSAADPNIRFLRELPRQSSDRAGIKNRVYTNSVYELLE  250

Human        250 NGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDIL  299
                 .|:..||||||||||||||||||.::|||||:|||||||||||||.|||
Bat          251 KGKPVGTCVLEYATPLQTLFAMSQDARAGFSQEDRLEQAKLFCRTLADIL  300

Human        300 ADAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTS  349
                 ||.||||.:||||.||||.::|.||||||.:|:|||||||||||||:|.
Bat          301 ADDPESQKSCRLIVYQEPTEESDFSLSQAILKHLRQEEKEEVTVGTVGTY  350

Human        350 AVPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                 ..|.:||:.||||||||||:|||||||..
Bat          351 EAPGSSTLHQEPELLISGMDQPLPLRTDIF   380
```

**FIGURE 11**

**Human STING (SEQ ID NO:305) and manatee STING (SEQ ID NO:367):**

```
Human             1 ---MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYL   47
                    ||||||||||||..||||.|||||.|||.||||.||.||||||.|||::|
Manatee           1 MVEMPHSSLHPSIPRHRGHGIQKAAFVLLVACLVALWELGEPPGHTLQWL   50

Human            48 VLHLASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGA   97
                    |..||||||||||..:||||||.|:||||:.||||.||||:|||:||||
Manatee          51 VCQLASLQLGLLLKVICSLAEELCHVHSRYQSSYWRAVRACMGCPIRRGA  100

Human            98 LLLLSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAV  147
                    |||||.|||:..||....|.||.|||||||.||:||||.|:||.|||||:
Manatee         101 LLLLSCYFYFCFPNMADLPLTWTLALLGLLQAMNILLSLQGLTPAEISAI  150

Human           148 CEKGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQR  197
                    |||.|.|||||||||||||||||||.||.|||.||..:||:||||..|||
Manatee         151 CEKRNLNVAHGLAWSYYIGYLRLILPGLQDRIRIYNLQHNNMLRGTGSQR  200

Human           198 LYILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYEL  247
                    |:||.|||||||:||:||.||||||.||||||:.|.||||.|||:||:|:|
Manatee         201 LHILFPLDCGVPNDLSVADANIRFLQKLPQQSADCAGIKGRVYTNSVYQL  250

Human           248 LENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLED  297
                    ||:||.||.|||||||||||||||||..:|||||||||||||||||||||
Manatee         251 LEHGQPAGICVLEYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRTLED  300

Human           298 ILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLK  347
                    |||||||.|||||||.|||.|:.||||||||:||||||||:||||||:.
Manatee         301 ILADAPEYQNNCRLIVYQESAEGSSFSLSQEILRHLRQEEREEVTVGSVG  350

Human           348 TSAV-----PSTSTMSQEPELLISGMEKPLPLRTDFS   379
                    ||.|     ||||:||||:||||||||:||||||..
Manatee         351 TSVVPSPSSPSTSSLSQEPKLLISGMEQPLPLRTDVF   387
```

**FIGURE 12**

**Human STING (SEQ ID NO:305) and ghost shark STING (SEQ ID NO:368):**

```
Human             1 MPHSSLHPSIPCPRGHGAQKAALVLLS--ACLVTLWGLGEPPEHTLRYLV   48
                      :..|||.|||..|....|:|:|  |||..:.    .|::.::::|
GhostShark        1 -----MSDSIPRPRGKVATYVGLILMSGLACLYFML----TPDYHIKFIV   41

Human            49 LHLASLQL-----GLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPL   93
                    ..:|. ||     .|.|..:|...|||.|..:||:.|:.|.:.|.|...
GhostShark       42 QQVAQ-QLIVSFFALCLLRLCEFVEELGHKQTRYQNSWVRALNASLDWK-   89

Human            94 RRGALLLLSIYFYYSLPNAVGPPFTWMLALLGLSQALNI---------LL  134
                    :.|..||:|:.|.:.||.....:    |:..:.|.:::          :.
GhostShark       90 KYGFCLLISLLFNWVLPYEKHMSY----AVPTVGQTISLTCFCVLFLKVF  135

Human           135 GLKGLAPAEISAVCEKGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQ  184
                    .|..|:||:||.:.|....||||||||||:|||:::||:|:.:|..|::
GhostShark      136 QLDVLSPAQISEISETNKCNVAHGLAWSYYLGYLKIVLPKLEEKINQYHR  185

Human           185 HYNNLLRGAVSQRLYILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDRAG  234
                    .|.|:|: ...::|||||:|..|.|.|.|...|.||.|...||:...||||
GhostShark      186 EYGNVLQ-QKGKKLYILIPFSCKVLDKLEKVDTNIVFYQNLPELLVDRAG  234

Human           235 IKDRVYSNSIYELL-ENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSRED  283
                    ||.|.|.:|||.:. :..|:...|:|||||||:||.|:..|.|.||:|.
GhostShark      235 IKSRSYKHSIYLIYDQKKQQPHHCILEYATPLRSLFEMTNDSAAAFSKEQ  284

Human           284 RLEQAKLFCRTLEDILADAPESQNNCRLIAYQEPADDSSFS---LSQEVL  330
                    ||:||||||.|||:.||.:.||...:.|||.|.:..:.:...   :|:|:|
GhostShark      285 RLDQAKLFYRTLKSILNNVPEVTGSYRLIPYDDDLEGAELGPHFVSEEIL  334

Human           331 RHLRQEEKEEVTVGSLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS  379
                    :|:|||..|....   :.|....|..||.||.|:||  :.|.|||:...
GhostShark      335 KHMRQELTEYPVA---EPSNANETDCMSSEPHLMIS--DDPKPLRSYCP  378
```

**FIGURE 13**

**Human STING (SEQ ID NO:305) and mouse STING (SEQ ID NO:369):**

```
Human              1 MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH   50
                     ||:|:||||:||.|||||.::..|||:.|.|.|:.||...:||.|||:||.||
Mouse              1 MPYSNLHPAIPRPRGHRSKYVALIFLVASLMILWVAKDPPNHTLKYLALH   50

Human             51 LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL  100
                     |||.:||||||..:|.||||||.|:.|||:|||||:.|||||||||:...|::|
Mouse             51 LASHELGLLLKNLCCLAEELCHVQSRYQGSYWKAVRACLGCPIHCMAMIL  100

Human            101 LSIYFYYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK  150
                     ||.|||: |.|.......:||..||.|.::|::|||||:.|.|||:||||||:
Mouse            101 LSSYFYF-LQNTADIYLSWMFGLLVLYKSLSMLLGLQSLTPAEVSAVCEE  149

Human            151 GNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYI  200
                     ...|||||||||||||||||||.|||||||.:||.:|||:|.||.|:||||
Mouse            150 KKLNVAHGLAWSYYIGYLRLILPGLQARIRMFNQLHNNMLSGAGSRRLYI  199

Human            201 LLPLDCGVPDNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYELLEN  250
                     |.||||||||||:.|||||||.|.|||||..|||||:|||||||:||:|||
Mouse            200 LFPLDCGVPDNLSVVDPNIRFRDMLPQQNIDRAGIKNRVYSNSVYEILEN  249

Human            251 GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA  300
                     ||.||.|:||||||||||||||.::|||||||||||||||||||||:||.
Mouse            250 GQPAGVCILEYATPLQTLFAMSQDAKAGFSREDRLEQAKLFCRTLEEILE  299

Human            301 DAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSA  350
                     |.|||:||||||.|||||.|.:||||||||||||:|||||||||:.:..||.
Mouse            300 DVPESRNNCRLIVYQEPTDGNSFSLSQEVLRHIRQEEKEEVTMNAPMTSV  349

Human            351 VPSTSTMSQEPELLISGMEKPLPLRTDFS   379
                     .|..|.:|||||.|||||||::||||||..
Mouse            350 APPPSVLSQEPRLLISGMDQPLPLRTDLI   378
```

**FIGURE 14**