



(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION

(13) **A1**

(22) Date de dépôt/Filing Date: 2020/11/12
(41) Mise à la disp. pub./Open to Public Insp.: 2021/05/20
(62) Demande originale/Original Application: 3 161 450
(30) Priorités/Priorities: 2019/11/12 (US62/934,503);
2020/01/16 (US62/962,162); 2020/03/16 (US62/990,404)

(51) Cl.Int./Int.Cl. *C12N 15/63* (2006.01),
A61K 35/74 (2015.01), *A61K 38/17* (2006.01),
A61K 38/20 (2006.01), *A61K 39/395* (2006.01),
A61P 35/00 (2006.01), *A61P 37/04* (2006.01),
C07K 14/47 (2006.01), *C07K 14/52* (2006.01),
C07K 14/705 (2006.01), *C07K 16/28* (2006.01),
C07K 19/00 (2006.01), ...

(71) Demandeur/Applicant:
ACTYM THERAPEUTICS, INC., US

(72) Inventeurs/Inventors:
THANOS, CHRISTOPHER D., US;
GLICKMAN, LAURA HIX, US;
...

(54) Titre : PLATEFORMES D'ADMINISTRATION DE BACTERIES IMMUNOSTIMULATRICES ET LEUR UTILISATION
POUR L'ADMINISTRATION DE PRODUITS THERAPEUTIQUES
(54) Title: IMMUNOSTIMULATORY BACTERIA DELIVERY PLATFORMS AND THEIR USE FOR DELIVERY OF
THERAPEUTIC PRODUCTS

(57) **Abrégé/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. The increase in colonization of phagocytic cells improves the delivery of encoded therapeutic products to the tumor microenvironment and tumors, and permits, among other routes, systemic administration of the immunostimulatory bacteria.

(51) Cl.Int./Int.Cl. (suite/continued) *C12N 1/21* (2006.01), *C12N 15/12* (2006.01), *C12N 15/13* (2006.01),
C12N 15/19 (2006.01), *C12N 15/62* (2006.01), *C12N 15/85* (2006.01), *C12Q 1/00* (2006.01), *C12Q 1/68* (2018.01),
G01N 33/48 (2006.01)

(72) Inventeurs(suite)/Inventors(continued): IANNELLO, ALEXANDRE CHARLES MICHEL, US; RAE, CHRIS, US;
KEHOE, HAIXING, US; PETERSON, BRET NICHOLAS, US; CHEUNG, CHINGNAM, US

(74) Agent: SMART & BIGGAR LP

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
5 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. The increase in colonization of phagocytic cells improves the delivery of encoded therapeutic products to the tumor microenvironment and tumors, and permits, among other routes, systemic administration of the immunostimulatory
10 bacteria.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 211

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 211

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

**IMMUNOSTIMULATORY BACTERIA DELIVERY PLATFORMS AND
THEIR USE FOR DELIVERY OF THERAPEUTIC PRODUCTS
RELATED APPLICATIONS**

Benefit of priority is claimed to co-pending U.S. Provisional Application
5 Serial No. 62/990,404, filed on March 16, 2020, entitled “TUMOR-SPECIFIC
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 co-pending U.S. Provisional Application
10 Serial No. 62/962,162, filed on January 16, 2020, entitled “TUMOR-SPECIFIC
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 co-pending U.S. Provisional Application
15 Serial No. 62/934,503, filed on November 12, 2019, entitled “TUMOR-SPECIFIC
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.

This application is related to co-pending International Application No.
20 PCT/US2020/020240, filed on February 27, 2020, 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
25 Michel Iannello, and Haixing Kehoe.

This application also is related to co-pending U.S. Provisional Application
Serial No. 62/962,140, filed on January 16, 2020, entitled
“IMMUNOSTIMULATORY BACTERIA ENGINEERED TO COLONIZE
TUMORS, TUMOR-RESIDENT IMMUNE CELLS, AND THE TUMOR
30 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 co-pending U.S. Provisional Application Serial No. 62/934,478, filed on November 12, 2019, entitled

- 5 “IMMUNOSTIMULATORY BACTERIA ENGINEERED TO COLONIZE TUMORS AND THE TUMOR MICROENVIRONMENT,” to Applicant Actym Therapeutics, Inc., and inventors Christopher D. Thanos, Laura Hix Glickman, Justin Skoble, and Alexandre Charles Michel Iannello.

This application also is related to International Application No.

- 10 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, 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,” and each of which claims priority
15 to U.S. Provisional Application Serial Nos. 62/531,327, filed on July 11, 2017, and 62/648,380, filed on March 26, 2018. Where permitted, the subject matter of each of these applications is incorporated by reference in its entirety.

This application also is related to co-pending International Application No.

- PCT/US2019/041489, filed on July 11, 2019, and published as WO 2020/014543 on
20 January 16, 2020, and to co-pending U.S. Patent Application Serial No. 16/520,155, filed on July 23, 2019, each entitled “ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF,” and each of which claims priority to U.S. Provisional Application Serial No. 62/789,983, filed on January 08, 2019, and to U.S. Provisional Application Serial No. 62/828,990, filed on April 03, 2019.

- 25 This application also is related to U.S. Provisional Application Serial Nos. 62/811,521, filed on February 27, 2019, and 62/828,990, filed on April 03, 2019.

- 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
30 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
5 on November 11, 2020, is 687 kilobytes in size, and is titled 1707SEQPC1.txt.

FIELD OF THE INVENTION

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-
10 resident myeloid cells, by improving 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 improves the delivery of encoded therapeutic products to the tumor microenvironment and tumors, and permits, among other routes, systemic administration of the immunostimulatory
15 bacteria.

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.*
20 *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

30 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
5 response in the subject.

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 contain modifications, such as one, two, or more modifications, selected from among:

10 a) deletion or disruption or inactivation 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
15 has been modified to generate penta-acylated lipopolysaccharide; and/or

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

b) deletion or disruption or inactivation of all or of a sufficient portion of a gene or genes, whereby the bacterium has attenuated recognition by Toll-like
20 Receptors (TLRs): TLR2, TLR4, and TLR5;

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

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

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

g) deletion or disruption or inactivation 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 or inactivation 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 or inactivation 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 a)-i).

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⁻ (*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 *asd*⁻ (aspartate-semialdehyde dehydrogenase⁻), 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⁻ (*fliC*⁻/*fljB*⁻), and *pagP*⁻. In some embodiments, the

immunostimulatory bacteria are *ansB*⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*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 a single promoter. The genome of the immunostimulatory bacteria is modified by deletion of a sufficient portion of, or by the disruption of genes, whereby the bacterium is one or more of *ansB*⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*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 bacteria are *lppA*⁻ and *lppB*⁻. Provided are immunostimulatory bacteria that contain a plasmid encoding an anti-cancer 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⁻ (*fliC*⁻/*fljB*⁻), *pagP*⁻, *lppA*⁻, and *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*, *iapC*, *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.

5 Provided are nucleic acid constructs, which are nucleic acid molecules that encode products, such as proteins, that are designed to be introduced into a cell or into a plasmid for expression of the encoded product. The constructs contain nucleic acid encoding a plurality of anti-cancer products as a polycistronic sequence under control of a single promoter. The promoter can be a eukaryotic promoter. Other eukaryote
10 regulatory sequences, such as enhancers, and nucleic acid encoding protein trafficking signals, such as secretion signals, and other regulatory sequences, such as terminators, including bacterial terminators to prevent read-through from bacterial promoters on the constructs, and particular configurations of elements and the order of the product-encoding nucleic acid open reading frames and/or genes, are provided and described
15 herein. In the constructs the polycistronic sequence can include signals or encoded signals, such as peptides, that result in expression of discrete products encoded by the polycistronic construct. Exemplary of such peptides are the 2A family of viral peptides. The constructs include such peptides or other signal between each open reading frame encoding each product. The 2A peptide include one or more of T2A,
20 P2A, E2A, or F2A.

 Included among the anti-cancer products are any that are used for treatment of cancer or to promote or aid or stimulate or help an anti-cancer response in a subject. Generally the anti-cancer products are proteins. The encoded products include one or more immunostimulatory protein(s) that confer(s) or contribute(s) to an anti-tumor
25 immune response in a tumor microenvironment. Exemplary encoded products is/are immunostimulatory protein(s) that confer(s) or contribute(s) to an anti-tumor immune response in the tumor microenvironment, such as, for example, any 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-18, IL-21,
30 IL-23, IL-36 γ , IL-2 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/persistence of T-cells, co-

stimulatory proteins, such as, CD40, CD40 ligand (CD40L), CD28, OX40, OX40 ligand (OX40L), 4-1BB, 4-1BB ligand (4-1BBL), including forms of the co-stimulatory proteins that are cytoplasmic domain deleted or truncated to eliminate the immunosuppressive reverse signaling, with optional modifications to promote correct orientation (*i.e.*, cytoplasmic domain in the cytoplasm) in a cell; members of the B7-
5 CD28 family, CD47 antagonists, an anti-IL-6 antibodies or IL-6 binding decoy receptors, TGF-beta polypeptide antagonists, including soluble TGF-beta receptors and TGF-beta antagonists, and members of the tumor necrosis factor receptor (TNFR) superfamily.

10 Others of the products include 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: 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, IL-36 gamma, IL-2 that has attenuated binding to IL-2Ra, IL-2 that is
15 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 (4-1BBL Δ cyt) or with a partially deleted cytoplasmic domain, which is deleted or
20 truncated to eliminate the immunosuppressive reverse signaling, members of the B7-CD28 family, and members of the tumor necrosis factor receptor (TNFR) superfamily. For example, the construct can contain nucleic acid encoding 4-1BBL with a deleted, or partially deleted cytoplasmic domain, or a partially deleted cytoplasmic domain and optionally including amino acid modifications, whereby the
25 resulting 4-1BBL assumes the proper orientation when expressed in a cell (see, SEQ ID NOs:389-392 and detailed description below providing exemplary modified 4-1BBL variants with truncated cytoplasmic domains, where residues are replaced with positively charged residues (*i.e.*, K and L) to confer proper orientation when expressed in a cell. The cytoplasmic domain is truncated sufficiently to eliminate or
30 reduce immunosuppressive reverse signaling. Hence, provided are constructs containing nucleic acid encoding a 4-1BBL with a deleted or partially deleted cytoplasmic domain, or a modified 4-1BBL with a truncated and modified

cytoplasmic domain, wherein the sequence of 4-1BBL is set forth in SEQ ID NOs:389-392, and exemplified sequences below in the detailed description and Examples. Constructs also can contain nucleic acid encoding any of the following products and combinations of products:

- 5 one or more of IL-12, or IL-15, or IL12p70, or IL-15/IL-15R alpha chain complex;
 - a cytokine and a STING pathway agonist;
 - a cytokine, a sting pathway agonist, and either a co-stimulatory molecule or an immune checkpoint inhibitor;
- 10 a cytokine and a STING pathway agonist, and a TGF-beta polypeptide antagonist;
 - a cytokine, a STING pathway agonist, a TGF-beta polypeptide antagonist, and either a co-stimulatory molecule (receptor or ligand) or an immune checkpoint inhibitor,
- 15 wherein a STING pathway agonist is any product that increases type I interferon expression via activation of the STING pathway. Exemplary are constructs that encode a Stimulator of Interferon Genes (STING) polypeptide, or a variant thereof or chimera thereof, as described in detail herein. Among the constructs are those that encode a combination of therapeutic products, selected from among the
- 20 following combinations:
 - an anti-CTLA-4 antibody and a STING polypeptide,
 - IL-15 and a STING polypeptide,
 - 4-1BBL and a STING polypeptide,
 - A TGF-beta decoy receptor or polypeptide antagonist, and a STING
 - 25 polypeptide,
 - IL-12 and STING polypeptide
 - an anti-CTLA-4 antibody, IL-15, and a STING polypeptide,
 - 4-1BBL, IL-15, and a STING polypeptide,
 - TGF-beta decoy receptor or polypeptide antagonist, and IL-15, and a STING
 - 30 polypeptide,
 - an anti-CTLA-4 antibody, and IL-12, and a STING polypeptide,
 - 4-1BBL, IL-12, and a STING polypeptide,

- a TGF-beta decoy receptor or polypeptide antagonist, IL-12, and a STING polypeptide,
- an anti-CTLA-4 antibody, IL-15, a TGF-beta decoy receptor or polypeptide antagonist, and STING polypeptide,
- 5 4-1BBL, and IL-15, a TGF-beta decoy receptor or polypeptide antagonist, and a STING polypeptide,
- an anti-CTLA-4 antibody, and IL-12, a TGF-beta decoy receptor or polypeptide antagonist, and STING polypeptide,
- 4-1BBL, and IL-12, a TGF-beta decoy receptor or polypeptide antagonist, and
- 10 a STING polypeptide,
- an anti-CTLA-4 antibody, IL-12, IL-15, and a STING polypeptide,
- 4-1BBL, IL-12, IL-15, and a STING polypeptide,
- a TGF-beta decoy receptor or polypeptide antagonist, IL-12, IL-15, and a STING polypeptide,
- 15 a TGF-beta decoy receptor or polypeptide antagonist, IL-12, IL-15, and a STING polypeptide,
- an anti-CTLA-4 antibody, IL-12, IL-15, a TGF-beta decoy receptor or polypeptide antagonist, and a STING polypeptide,
- 4-1BBL, IL-12, IL-21, a TGF-beta decoy receptor or polypeptide antagonist,
- 20 and a STING polypeptide,
- an anti-CTLA-4 antibody, IL-12, IL-15, and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL, IL-12, IL-21, and a TGF-beta decoy receptor or polypeptide antagonist,
- 25 IL-12, IL-15, and a STING polypeptide,
- IL-15, IL-21, and a STING polypeptide,
- IL-12, IL-21, and a STING polypeptide,
- an anti-CTLA-4 antibody, IL-15, IL-21, and a STING polypeptide,
- an anti-CTLA-4 antibody, IL-12, IL-21, and a STING polypeptide,
- 30 4-1BBL, IL-15, IL-21, and a STING polypeptide,
- 4-1BBL, IL-12, IL-21, and a STING polypeptide,
- an anti-CTLA-4 antibody, and IL-15,

- an anti-CTLA-4 antibody, IL-15, and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL and IL-15,
- 4-1BBL, IL-15, and a TGF-beta decoy receptor or polypeptide antagonist,
- 5 an anti-CTLA-4 antibody and IL-12,
- an anti-CTLA-4 antibody, and IL-12, and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL and IL-12,
- 4-1BBL, IL-12, and a TGF-beta decoy receptor or polypeptide antagonist,
- 10 an anti-CTLA-4 antibody and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL and a TGF-beta decoy receptor or polypeptide antagonist,
- IL-15 and a TGF-beta decoy receptor or polypeptide antagonist,
- IL-12 and a TGF-beta decoy receptor or polypeptide antagonist,
- 15 IL-12, IL-15, and a TGF-beta decoy receptor or polypeptide antagonist, and
- IL-15, and IL-21, and a TGF- decoy receptor or polypeptide antagonist,
- wherein:
- the an anti-CTLA-4 antibody is an scFv or an scFv-Fc;
- STING polypeptides include a wild-type STING, or a variant STING
- 20 polypeptide, or a chimeric STING polypeptide, and a chimeric STING protein with amino acid replacements that confer, for example, a gain-of-function; and
- 4-1BBL is 4-1BBL with a deleted cytoplasmic domain, 4-1BBL with a modified cytoplasmic domain, 4-1BBL with a truncated cytoplasmic domain, or 4-1BBL with a truncated and modified cytoplasmic domain.
- 25 Other constructs include those that encode a combination of therapeutic products selected from among:
- IL-2 and IL-12p70;
- IL-2 and IL-21;
- IL-2, IL-12p70, and a STING gain-of-function (GOF) variant;
- 30 IL-2, IL-21, and a STING GOF variant;
- IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt),
- where Δ cyt is a deleted cytoplasmic domain;

- IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 IL-15/IL-15R α , and a STING GOF variant;
 IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 IL-15/IL-15R α and IL-12p70;
 5 IL-15/IL-15R α and IL-21;
 IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
 IL-15/IL-15R α , IL-21, and a STING GOF variant;
 IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-
 1BBL Δ cyt);
 10 IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-
 1BBL Δ cyt);
 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-
 15 1BBL Δ cyt);
 IL-12p70 and a STING GOF variant;
 IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 IL-12p70 and IL-18;
 IL-12p70, IL-18, and a STING GOF variant;
 20 IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-
 1BBL Δ cyt);
 a TGF- β decoy receptor or antagonist polypeptide, IL-2, and IL-12p70;
 a TGF- β decoy receptor or antagonist polypeptide, IL-2, and IL-21;
 a TGF- β decoy receptor or antagonist polypeptide, IL-2, IL-12p70, and a
 25 STING GOF variant;
 a TGF- β decoy receptor or antagonist polypeptide, IL-2, IL-21, and a STING
 GOF variant;
 a TGF- β decoy receptor or antagonist polypeptide, IL-2, IL-12p70, a STING
 GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 30 a TGF- β decoy receptor or antagonist polypeptide, IL-2, IL-21, a STING GOF
 variant, and 4-1BBL (including 4-1BBL Δ cyt);

- a TGF- β decoy receptor or antagonist polypeptide, IL-15/IL-15R α , and a STING GOF variant;
- a TGF- β decoy receptor antagonist polypeptide, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 5 a TGF- β decoy receptor or antagonist polypeptide, IL-15/IL-15R α , and IL-12p70;
- a TGF- β decoy receptor or antagonist polypeptide, IL-15/IL-15R α , and IL-21;
- a TGF- β decoy receptor or antagonist polypeptide, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- 10 a TGF- β decoy receptor antagonist polypeptide, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- a TGF- β decoy receptor or antagonist polypeptide, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or antagonist polypeptide, IL-15/IL-15R α , IL-21, a
- 15 STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, and IL-21;
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, IL-21, and a STING GOF variant;
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, IL-21, a STING
- 20 GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or antagonist polypeptide and IL-12p70;
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, and a STING GOF variant;
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, a STING GOF
- 25 variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, and IL-18;
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, IL-18, and a STING GOF variant;
- a TGF- β decoy receptor or antagonist polypeptide, IL-12p70, IL-18, a STING
- 30 GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 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-
- 5 1BBL (including 4-1BBL Δ cyt);
 - an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , and a STING GOF variant;
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , a STING GOF variant, and 4-
 - 10 1BBL (including 4-1BBL Δ cyt);
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-12p70;
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-21;
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
 - 15 an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, and a STING GOF variant;
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 - an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 - 20 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 4-1BBL Δ cyt);
 - an anti-CTLA-4 antibody and IL-12p70;
 - 25 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 4-1BBL Δ cyt);
 - an anti-CTLA-4 antibody, IL-12p70, and IL-18;
 - an anti-CTLA-4 antibody, IL-12p70, IL-18, and a STING GOF variant;
 - 30 an anti-CTLA-4 antibody, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 - 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;
- 5 a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL
(including 4-1BBL Δ cyt);
- a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-
1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and a STING GOF variant;
- 10 a CD40 agonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL
(including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and IL-12p70;
- a CD40 agonist, IL-15/IL-15R α , and IL-21;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- 15 a CD40 agonist, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-
1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL
(including 4-1BBL Δ cyt);
- 20 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 4-1BBL Δ cyt);
- a CD40 agonist and IL-12p70;
- 25 a CD40 agonist, IL-12p70, and a STING GOF variant;
- a CD40 agonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-
1BBL Δ cyt);
- a CD40 agonist, IL-12p70, and IL-18;
- a CD40 agonist, IL-12p70, IL-18, and a STING GOF variant;
- 30 a CD40 agonist, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL
(including 4-1BBL Δ cyt); and
- a CD40 agonist and a STING GOF variant, wherein:

4-1BBL is 4-1BBL with a deleted cytoplasmic domain (4-1BBL Δ cyt), 4-1BBL with a modified cytoplasmic domain, 4-1BBL with a truncated cytoplasmic domain, or 4-1BBL with a truncated and modified cytoplasmic domain; and an anti-CTLA-4 antibody is an scFv or an scFv-Fc.

5 Exemplary of the STING polypeptides are those in which the STING polypeptide is modified to result in increased or constitutive expression of a type I interferon, or is a chimeric polypeptide comprising a human STING polypeptide with a C-terminal tail from a different species that has lower NF- κ B signaling activity than the NF- κ B signaling activity of human STING, and where, for example: the TRAF6
10 binding site in the CTT optionally is deleted; and the human STING protein has the sequence set forth in any of SEQ ID NOs:305-309. Particular combinations of encoded products include those where the encoded therapeutic proteins comprise IL-12p70 and a chimeric human STING polypeptide with a CTT from Tasmanian devil and an amino acid replacement that results in increased or constitutive expression of
15 type I interferon, or is a STING polypeptide with an amino acid replacement that results in increased or constitutive expression of type I interferon, where a mutation that results in increased or constitutive expression of type I interferon is a gain-of-function mutation. Exemplary are STING proteins or polypeptides with replacements described in the detailed description, such as where the amino replacement in the
20 STING polypeptide corresponds to R284G or N154S/R284G with reference, for alignment, to any of SEQ ID NOs: 305-309, which set forth human STING proteins. These constructs can additional encode IL-36 γ and/or an immune checkpoint inhibitor antibody. As described herein antibodies include an antigen-binding portions thereof, and any of the various forms of antibodies, such as, but not limited to, scFvs, and
25 scFv-Fc (generally IgG Fc), where the presence of the Fc multimerizes the resulting product so that it has two chains. Immune checkpoints targeted include, but are not limited to, CTLA-4 or PD-1 or PD-L1, and antibody forms of include scFV and is an scFV-Fc two-chain polypeptides.

Provided are plasmids that contain the constructs described above and
30 throughout the disclosure herein. Plasmids include a bacterial plasmid, where the construct is operatively linked to eukaryotic transcriptional regulatory sequences.

Provided are compositions, such as pharmaceutical compositions, that contain the mixture of anti-cancer protein products encoded by the construct or plasmid as the only anti-cancer proteins in the compositions. Hence, among these are provided compositions that contain complementary combinations of therapeutic proteins; the mixtures include unique combinations of agents. These include the combinations of agents provided and described herein.

Also provided are immunostimulatory bacteria that contain any of the constructs and/or plasmids. The constructs and plasmids can be provided in or otherwise introduced into any of the immunostimulatory bacteria provided herein, including any discussed above and below. The constructs and plasmids also can be introduced into suitable bacteria known in the art, such as bacteria described in publications, such as International Application Publication Nos. WO2020/172461 and WO2020/172462, and U.S. Patent Nos. 10,449,237, 10,286,051, and 9,616,114.

The immunostimulatory bacteria provided herein include genome modifications, such as deletions, disruptions, alterations, 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 those that are modified so that the resulting bacteria are *msbB*⁻/*purI*⁻. In some embodiment 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 bacteria lack flagella. This is effected in bacteria that normally express flagella. In such bacteria for example the genes in *Salmonella* or equivalent genes in other species to *fliC*⁻/*fliB*⁻ can be deleted or other 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 *ansB*⁻.

Provided are immunostimulatory bacteria that contain a plasmid encoding a therapeutic product under control of a eukaryotic promoter, where 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 does not activate the

synthesis of secreted asparaginase. Exemplary of such bacteria are those in which the asparaginase is L-asparaginase II encoded by the gene *ansB*.

It shown herein that in parental strain VNP20009, which is *msbB*⁻ and *purI*⁻, the genes are not completely deleted. In immunostimulatory bacteria provided the
5 genome of the bacterium is modified so that the full length of at least the coding portion of the *msbB*⁻ and *purI*⁻ genes is deleted. These strains are more fit, grow faster and/or to a greater extent than the parental strain. In all embodiments herein, the bacteria can be modified so that the native *asd* gene product is inactive or not expressed. To aid in producing the strain, the *asd* gene is encoded on a plasmid under
10 control of a prokaryotic promoter, such as an inducible promoter.

In embodiments, the strains include modifications so that bacteria that lack flagella, and are *pagP*⁻, *ansB*⁻, and *csgD*⁻. In addition the bacteria are *purI*⁻ and *asd*⁻. Thus provided are strains, include modified parental strains that already are *msbB*⁻ and *purI*⁻, and/or have other modifications, particularly those that modify the LPS, that
15 also are $\Delta asd/\Delta FLG/\Delta pagP/\Delta ansB/\Delta csgD$. The strains also can be an adenosine or adenosine and adenine auxotroph.

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 interferon (IFN),
20 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
25 counter-regulatory signal to the antigen-presenting cell (APC), due to the deleted or truncated or otherwise modified cytoplasmic domain or portion thereof. Other products include enzymes that activate therapeutic proteins, such as those that activate prodrugs. As described herein and below, the immunostimulatory bacteria provided herein encode anti-cancer therapeutics, including combinations of therapeutic
30 products that combine to provide a robust anti-cancer response. Among the proteins encoded are each of the products listed above and below, and combinations of products from different classes. Included are the co-stimulatory proteins, such as 4-

1BBL, particularly those with truncated or deleted cytoplasmic domains to eliminate immunosuppressive reverse signaling, and also any compensatory mutations to ensure that the resulting protein is correctly oriented in the cell membrane when expressed in a cell. Other products include STING pathway agonists to induce or result in

5 constitutive expression of type I interferons. These products include STING protein and, particularly, the modified and chimeric STING proteins provided and described herein. One or more cytokines, such as IL-12, IL-15, IL-21, L-12p70 (IL-12p40 + IL-12p35), IL-2 that has attenuated binding to IL-2Ra, IL-15/IL-15R alpha chain complex, and others, such as IL-18, IL-21, IL-23, IL-36 γ , also are encoded on the

10 plasmids. In addition to the co-stimulatory products, the STING pathway agonist proteins, such as STING, and the cytokines, antibodies, such as checkpoint inhibitor antibodies, including anti-CTLA-4 antibodies, can be encoded on the plasmids. The antibodies can be scFvs and also scFvs-Fc two-chain forms, as well as other forms. Additionally, among other products, TGF-beta antagonists and TGF-beta receptor

15 decoys can be included.

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,

20 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

25 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 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

30 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 or 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). Additional regulatory elements, such as bacterial terminators inserted in appropriate loci, as described herein, to reduce or eliminate read-through from bacterial promoters, can be included.

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 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-7, 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), 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, and
- 5 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)
- 10 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,
- 15 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

20 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 modified so that it does not bind to IL-2Ra, CXCL9, CXCL10, CXCL11,

25 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 IL-6 binding decoy receptor, TGF-beta polypeptide antagonists, and

30 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
5 cytoplasmic domain, or partially deleted or truncated cytoplasmic domain, to eliminate the immunosuppressive reverse signaling. Other such proteins are TGF-beta polypeptide antagonists, such as an anti-TGF-beta antibody or antibody fragment, an anti-TGF-beta receptor antibody or antibody fragment, a soluble TGF-beta antagonist polypeptide, or a TGF-beta binding decoy receptor.

10 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), scFv-Fc, 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.

15 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
20 (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-
25 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
30 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
 5 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
 10 complex, IL-18, IL-2 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
 15 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, or SNRNP200. They also can encode one or more of a TGF-beta inhibitory antibody, a TGF-beta binding decoy receptor, an anti-IL-6 antibody, and an
 20 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 4-1BBL Δ cyt, where
 25 Δ cyt is a deleted cytoplasmic domain, and 4-1BBL with a truncated cytoplasmic domain (4-1BBLcyt trunc)); IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt, and 4-1BBL with a truncated cytoplasmic domain); IL-15/IL-15R α , and a STING GOF variant; IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); IL-15/IL-15R α and IL-
 30 12p70; IL-15/IL-15R α and IL-21; IL-15/IL-15R α , IL-12p70, and a STING GOF variant; IL-15/IL-15R α , IL-21, and a STING GOF variant; IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc);

IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); IL-12p70 and a STING GOF variant; IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); a TGF- β decoy receptor, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); a TGF- β decoy receptor, IL-15/IL-15R α , and a STING GOF variant; a TGF- β decoy receptor, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); a TGF- β decoy receptor, IL-15/IL-15R α , and IL-12p70; a TGF- β decoy receptor, IL-15/IL-15R α , and IL-21; a TGF- β decoy receptor, IL-15/IL-15R α , IL-12p70, and a STING GOF variant; a TGF- β decoy receptor, IL-15/IL-15R α , IL-21, and a STING GOF variant; a TGF- β decoy receptor, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); a TGF- β decoy receptor, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); an anti-CTLA-4 antibody, IL-15/IL-15R α , and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBL with a truncated cytoplasmic domain); an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-12p70; an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-21; an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, and a STING GOF variant; an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); a CD40 agonist, IL-15/IL-15R α , and a STING GOF variant; a CD40 agonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); a CD40 agonist, IL-15/IL-15R α , and IL-12p70; a CD40 agonist, IL-15/IL-15R α , and IL-21; a CD40 agonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant; a CD40 agonist, IL-15/IL-15R α , IL-21, and a STING

GOF variant; a CD40 agonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); a CD40 agonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); 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 4-1BBL Δ cyt and 4-1BBLcyt trunc); and a CD40 agonist and a STING GOF variant.

In all combinations including 4-1BBL, the 4-1BBL molecule can be a full-length protein (see, *e.g.*, SEQ ID NOs:389 and 393, for human and mouse 4-1BBL, respectively); a 4-1BBL variant with the cytoplasmic domain deleted (4-1BBL Δ cyt; see *e.g.*, SEQ ID NOs:390 and 394, for human and murine 4-1BBL Δ cyt, respectively); a 4-1BBL variant with a truncated (*i.e.*, not fully deleted) cytoplasmic domain (4-1BBLcyt trunc; see, *e.g.*, SEQ ID NOs:391-392 and SEQ ID NOs:395-396, for exemplary human and mouse 4-1BBLcyt trunc variants); or a 4-1BBL molecule with a modified cytoplasmic domain, in which one or more Ser residues, which act as phosphorylation sites, are replaced at an appropriate locus or loci, such as, for human 4-1BBL, with reference to SEQ ID NO:389, Ser5 and Ser8, with a residue that reduces or eliminates reverse signaling. Additionally, all combinations including an anti-CTLA-4 antibody, can include an anti-CTLA-4 antibody fragment, such as an anti-CTLA-4 scFv (see, *e.g.*, SEQ ID NOs:403 and 404, for exemplary human and mouse anti-CTLA-4 scFv fragments, respectively), or an anti-CTLA-4 scFv-Fc (see, *e.g.*, SEQ ID NOs:402 and 405, for exemplary human and mouse anti-CTLA-4 scFv-Fc fragments, respectively).

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 chimeras, as described herein. These STING proteins that are encoded by the immunostimulatory bacteria are provided herein and described throughout. Provided herein are:

- 5 1. Modified non-human STING proteins, where the non-human STING protein is one that has lower NF- κ B 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
10 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
15 (CTT) of the STING protein is deleted, reducing NF- κ B signaling activity.
2. 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- κ B signaling activity and/or higher type I IFN signaling
20 activity than human STING. Also, the TRAF6 binding site is optionally deleted in these chimeras.
3. The modified STING proteins of 2 that also include the mutations of 1.
4. Delivery vehicles, such as immunostimulatory bacteria, any provided herein or known to those of skill in the art, including, for example, exosomes,
25 nanoparticles, minicells, cells, liposomes, lysosomes, oncolytic viruses, and other viral vectors, that encode the modified STING proteins of any of 1-3.
5. 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
30 viral vectors, that encode unmodified STING from a non-human species whose STING protein has reduced NF- κ B signaling activity compared to that of human

STING, and optionally, increased type I interferon stimulating/signaling activity compared to that of human STING.

6. 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 of any of 1-3.

5 7. Pharmaceutical compositions that contain the STING proteins of any of 1-3, or the delivery vehicles of 4 and 5, or the cells of 6.

8. Uses and methods of treatment of cancer by administering any of 1-7, as described herein for the immunostimulatory bacteria.

Assays and methods to assess NF- κ B activity (signaling activity), and type I
10 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- κ B signaling activities of STING proteins from various species, including human, thereby identifying STING
15 proteins from various species that have lower NF- κ B 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)).

20 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
25 (*Rousettus aegyptiacus*; SEQ ID NO:366), manatee (*Trichechus manatus latirostris*; SEQ ID NO:367), ghost shark (*Callorhynchus 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
30 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 to retain viability for at least a limited time, and/or that 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 to result in expression of type I IFN, can convert macrophages that have the M2 phenotype into M1 or M1-like, with immunosuppressive properties reduced or eliminated, and immune-stimulating, anti-tumor or anti-viral properties enhanced or added, macrophages. 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 bacteria that do not have 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 elimination of the 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).

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. 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 to result in expression of type I IFN, such as the variant and non-human STING proteins as described and provided herein. The immunostimulatory bacteria include any that can be modified as described herein, including the species listed herein, such as *Salmonella* species and strains.

5 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 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
10 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
15 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.*
20 *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
25 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,
30 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*,
 5 *Rickettsia sibirica*, *Bordetella bronchiseptica*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Aeromonas eucrenophila*, *Aeromonas salmonicida*, *Francisella tularensis*, *Corynebacterium pseudotuberculosis*, *Citrobacter freundii*, *Chlamydia pneumoniae*, *Haemophilus sommus*, *Brucella abortus*, *Mycobacterium intracellulare*, *Legionella pneumophila*, *Rhodococcus equi*, *Pseudomonas aeruginosa*, *Helicobacter mustelae*, *Vibrio cholerae*, *Bacillus subtilis*, *Erysipelothrix rhusiopathiae*, *Yersinia enterocolitica*, *Rochalimaea quintana*, and *Agrobacterium tumefaciens*.

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
 15 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. 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,
 20 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 DRAWINGS

25 **Figure 1** depicts the alignment of wild-type human and Tasmanian devil STING proteins.

Figure 2 depicts the alignment of wild-type human and marmoset STING proteins.

Figure 3 depicts the alignment of wild-type human and cattle STING proteins.

30 **Figure 4** depicts the alignment of wild-type human and cat STING proteins.

Figure 5 depicts the alignment of wild-type human and ostrich STING proteins.

Figure 6 depicts the alignment of wild-type human and crested ibis STING proteins.

Figure 7 depicts the alignment of wild-type human and coelacanth (SEQ ID NO:345) STING proteins.

5 **Figure 8** depicts the alignment of wild-type human and zebrafish STING proteins.

Figure 9 depicts the alignment of wild-type human and boar STING proteins.

Figure 10 depicts the alignment of wild-type human and bat STING proteins.

10 **Figure 11** depicts the alignment of wild-type human and manatee STING proteins.

Figure 12 depicts the alignment of wild-type human and ghost shark STING proteins.

Figure 13 depicts the alignment of wild-type human and mouse STING proteins.

15 **Figure 14** depicts an exemplary construct containing the *asd* expression cassette, including the bacterial promoter and any other bacterial regulatory sequence(s), placed in the opposite orientation of the cassette encoding the payload(s) under control of the eukaryotic promoter, and including bacterial terminators flanking the nucleic acid encoding the payload(s), and in the orientation to terminate any
20 readthrough transcripts, from the prokaryotic promoter.

DETAILED DESCRIPTION

OUTLINE

A. DEFINITIONS

25 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

30 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
 2. Nutrient Auxotrophy
 - a. *purI* Deletion/Disruption
 - b. Adenosine 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 for Curli Fimbriae Expression
 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 Modified to be Optimized for Anti-Tumor Therapy, and that Encode Therapeutic Products, Including a Plurality Thereof
 10. 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. Molecules that Activate Prodrugs

3. **Constitutively Active Proteins that Stimulate the Immune Response and/or Type I IFN, Non-Human STING Proteins, 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**
 4. **Antibodies and Antibody Fragments**
 - a. **TGF- β**
 - b. **Bispecific scFvs and T-Cell Engagers**
 - c. **Anti-PD-1/Anti-PD-L1 Antibodies**
 - d. **Anti-CTLA-4 Antibodies**
 - e. **Additional Exemplary Checkpoint Targets**
 5. **Combinations of Immunomodulatory Proteins can have Synergistic Effects and/or Complementary Effects**
 6. **Immunostimulatory Bacteria that Deliver Combination Therapies**
- E. 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**

F. 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****G. 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**3. Administration****4. Monitoring****H. EXAMPLES****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.

5 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
10 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
15 expression), except primarily in immunoprivileged environments. Immunostimulatory bacteria provided herein are modified to encode a product or products or exhibit a trait or property that renders them immunostimulatory. Such products, properties and traits 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
20 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 a checkpoint gene, such as 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
25 inhibitory antibody; other antibody constructs such as bi-specific T-cell engagers (BiTEs®); 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 bacterium auxotrophic for a metabolite that is immunosuppressive or that is in an immunosuppressive pathway,
30 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 refer 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 in *msbB* and *purI*, and was generated from wild-type strain ATCC
5 #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).

10 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
15 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
20 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
25 DxExNPGP, and occur in a large number of viral families. They help break apart polypeptides by causing the ribosome to fail at making a peptide bond. The 2A peptides provide for multicistronic 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,
30 such as any having at 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.

As used herein, an “origin of replication” is a sequence of DNA at which replication is initiated on a chromosome, or 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.

5 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
10 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
15 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,
20 GRO- α , GRO- β , GRO- γ , ENA-78, PBP, CTAP III, NAP-2, LAPF-4, MIG (CXCL9), CXCL10 (IP-10), CXCL11, PF4, SDF-1 α , SDF-1 β , SDF-2, MCP-1, MCP-2, MCP-3, MCP-4, MCP-5, MIP-1 α (CCL3), MIP-1 β (CCL4), MIP-1 γ (CCL9), MIP-2, MIP-2 α , MIP-3 α , MIP-3 β , 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
25 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
30 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, 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 (4-1BBL Δ cyt) 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 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 truncated or deleted 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 (T β RI or TGF β R1) and type II (T β RII or TGF β R2), which
5 form heteromeric complexes in the presence of dimerized ligands, as well as the type III receptor betaglycan (T β RIII or TGF β R3). 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 T β RI, T β RII, or T β RIII (β glycan), which can be fused with other molecules, such as an Fc domain. Additionally, BAMBI (bone
10 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 TGF β R2 (DN-TGF β R2), which comprises the extracellular domain of TGF β R2 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.*,
15 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.

20 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
25 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
30 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 (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
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 and lead to expression of innate immune response mediators, such as type I interferon. The immunomodulatory proteins in the DNA/RNA sensor
10 pathways can be modified so that they have increased activity, or act constitutively in the absence of cytosolic nucleic acids, 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
15 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
20 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- κ B 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
25 NF- κ B 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- κ B activation, can decrease NF- κ B 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
30 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	EKEEVTVGSLKTSAPVSTSTMS 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	RQEEYTVCDGTLCTDLSLQIS 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 STELNLQISEDLQPQLRSDCF	379	LNLQIS	PQPLRS	DCF
Coelacanth (variant 1)	QKEEYFMSEQTQPNSSTSCLS TEPQLMISDTPHPTLKRQVC	380	PQLMIS	PHTLKR	QVC
Coelacanth (variant 2)	QKEEYFMSEQTQPNSSTSCLS TEPQLMISDTPHPTLKSGF	381	PQLMIS	PHTLKS	GF
Zebrafish	DGEIFMDPTNEVHPVPEEGPV GNCNGALQATFHEEPMSDEPT LMFSRPQSLRSEPVEDTDYFNP SSAMKQN	382	PTLMFS	PQSLRS	EPVETTDY
Ghost shark	LTEYPVAEPSNANETDCMSSE PHLMISDDPKPLRSYCP	383	PHLMIS	PKPLRS	YCP
Mouse	EKEEVTMNAPMTSVAPPPSVL SQEPRLISGMDQPLPLRTDLI	384	PRLIS	PLPLRT	DLI

As used herein, a “STING pathway agonist” is any product that increases type I interferon (IFN) expression via activation of the STING pathway. Exemplary of such agonists are the gain-of-function STING polypeptide variants provided herein, as well as gain-of-function variants of other cytosolic DNA/RNA sensors and type I IFN

pathway proteins, such as variants of IRF-3, IRF-7, MDA5, and RIG-I, that increase or render expression of type I IFN constitutive, via the STING pathway.

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
5 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
10 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
15 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
20 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
25 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
30 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
5 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,
10 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-
15 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
20 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
25 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
30 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, “bactofection” refers to the bacteria-mediated transfer of genes or plasmid DNA into eukaryotic cells, such as mammalian cells.

5 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
10 immune responses. Two types of IFN- β are IFN- β 1 (IFNB1) and IFN- β 3 (IFNB3).

As used herein, M1 macrophage phenotype and M2 macrophage phenotype refer to the two broad groups into which macrophage phenotype is divided: M1 (classically activated macrophages) and M2 (alternatively activated macrophages). The role of M1 macrophages is to secrete pro-inflammatory cytokines and
15 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 produces are IL-6, IL-12 and TNF- α . 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
20 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
25 that is converted into a macrophage with an M1 or M1-like phenotype exhibits more pro-inflammatory cytokines/chemokines and receptors, such as CD80 and CCR7, and chemokines, such as IFN γ 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 also can serve as a phenotypic marker. CD163
30 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 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.

5 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.

10 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
15 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
20 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
25 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.

30 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

5 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
10 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),
15 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
20 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).

25 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
30 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
5 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
10 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
15 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
20 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 (dsFv), Fd fragments, Fd’ fragments, single-chain Fvs (scFvs), scFv-Fc fragments (in which the V_H domain in the scFv is linked to an Fc, such as a human IgG1 Fc, for example),
25 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
30 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 lack an Fc part; and (ii) BiTEs® (tandem scFvs), DARTs, diabodies, and single-chain diabodies (scDBs). Thus, an antibody
5 fragment includes a/an: Fab, Fab’, scFab, scFv, scFv-Fc, 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}, dAb (single-domain antibody), minimal recognition unit, single-chain diabody (scDb), BiTE®, and DART. The recited antibody fragments
10 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
15 (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).
20 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
30 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 gene, or 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.

5 For purposes herein, amino acids contained in the antibodies and immunostimulatory proteins provided 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
10 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
15 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. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free
20 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		AMINO ACID
1-Letter	3-Letter	
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

SYMBOL		
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 NH₂, 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

Original Residue	Exemplary Conservative Substitution(s)
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.

- 5 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,
- 10 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-
- 15 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-
- 20 Methylglycine, sarcosine (MeGly), N-Methylisoleucine (Melle), 6-N-Methyllysine

(MeLys), N-Methylvaline (MeVal), Norvaline (Nva), Norleucine (Nle), and Ornithine (Orn).

As used herein, a DNA construct is a single- 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
5 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
10 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
15 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
20 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.

25 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
30 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
5 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
10 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
15 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.

20 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
25 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
30 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

5 “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,

10 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

15 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

20 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 poly-

25 peptide 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

30 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
5 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
10 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
15 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
20 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-
25 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
30 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
5 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
10 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
15 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.

20 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.

25 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
30 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
5 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
10 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
15 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.
20

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
25 agents.
30

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.

5 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
10 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
15 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
20 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
25 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
30 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, 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,
5 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
10 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
15 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
20 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 CANCER THERAPY

25 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,
30 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).

5 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
10 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
15 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),
20 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. 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
25 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.

 The bacteria provided herein are designed and engineered to maintain the
30 beneficial scaffold properties of bacteria, and to have a viral-like immune signature. This is advantageous for use as an anti-cancer therapeutic. The following table summarizes some of the immune and scaffold properties of bacteria and viruses; the

immunostimulatory bacteria provided herein retain the feasibility of the bacterial scaffold, but result in a viral-like immune response in a treated subject (discussed in more detail in section C below).

	Bacteria	Viruses	Immunostimulatory Bacteria Provided Herein
Feasibility as a Therapeutic Scaffold	Easy to manufacture; Stable Shelf Life; Easy to engineer; Reversible with antibiotics; Not immunogenic	Difficult to manufacture; Requires -80 °C for storage; Can be difficult to engineer; Immunogenic; Complement can inactivate	Engineered to retain and improve feasibility properties
Inflammatory Profile	Recognized by TLR2, TLR4, and TLR5; Downstream targets suppress adaptive immunity	Recognized by TLR3, TLR7/8, and STING; Downstream targets promote adaptive immunity	Engineered to produce a viral-like immune response
Chemokine Gradients	Attract neutrophils to clear infection	Attract T-cells to clear infection	
Generation of durable immunity	No	Yes, but only to the virus	

In *Salmonella* species and other bacterial species, the flagella contribute to TLR5-mediated inflammation, the LPS results in TLR4-mediated inflammatory responses, and the adhesive curli fimbriae result in TLR2-mediated inflammatory responses. The genomes of the immunostimulatory bacteria provided herein are modified so that the bacteria lack flagella and adhesive curli fimbriae, and have modified LPS, resulting in the reduction or elimination of TLR4-mediated inflammatory responses. As a result, the immunostimulatory bacteria provided herein induce a viral-like anti-tumor immune response. Elimination or modification of these components confers other advantageous properties, such as those discussed in detail below. The immunostimulatory bacteria deliver therapeutic products, such as anti-cancer therapeutics, and particularly, complementary combinations of products. The immunostimulatory bacteria provided herein deliver encoded genetic payloads in a tumor-specific manner to tumor-resident myeloid cells.

Provided is an anti-cancer therapeutic product, an immunostimulatory bacterium, that delivers a genetic payload encoding one or a plurality of therapeutic products. Included is a truncated co-stimulatory molecule (receptor or ligand; *e.g.*, 4-1BBL, CD80, CD86, CD27L, B7RP1, OX40L), with a complete or a 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 truncated or deleted cytoplasmic domain.

5 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-15R α chain complex, IL-18, IL-21, IL-23, IL-36 γ , 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
10 pathway proteins, such as gain-of-function or constitutively active STING, IRF3, IRF7, MDA5, or RIG-I variants (that induce type I IFN), inhibitors of TGF- β , such as TGF- β inhibitory antibodies, TGF- β polypeptide antagonists, and TGF- β 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-
15 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 partial or complete cytoplasmic domain deletion, for expression on an antigen-presenting cell
20 (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. Combinations of such therapeutic products and agents can be expressed in a single therapeutic composition. By virtue of the modifications of the
25 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
30 immune cells. 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 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 in section C.8., the immunostimulatory bacteria provided herein include 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 adenonsine/adenine auxotrophs, such as *purI*⁻; are asparaginase II⁻ (*ansB*⁻), which improves T-cell quality; are *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.

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.

The immunostimulatory bacteria provided herein include genomic modifications whereby they target or accumulate in tumor-resident immune cells, particularly tumor-resident myeloid cells, such as macrophages, MDSCs (myeloid derived suppressor cells), and DCs (dendritic cells), in which they deliver payloads of encoded therapeutic products expressed under control of regulatory sequences recognized by the host cell (eukaryotic) transcriptional/translational machinery. The encoded products are expressed in the myeloid cells, and, as appropriate, delivered into the tumor microenvironment. The bacteria generate anti-tumor immunity, and also can deliver anti-tumor products that directly treat tumors, and products that can activate prodrugs.

Immunostimulatory bacteria provided herein can exhibit at least about 100,000-fold greater tumor infiltration and enrichment compared to unmodified bacteria. The immunostimulatory bacteria are consumed by tumor-resident immune cells, and deliver the plasmid encoding therapeutic products, which are expressed and produced in the immune cells and tumor microenvironment, to generate anti-tumor immunity.

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) *Biomedicine* 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, 5 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, 10 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 thus far have limited capacity to stimulate durable anti-tumor immunity.

15 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*, 20 *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- 25 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.

30 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. The modifications improve the targeting or accumulation of bacteria in the tumor microenvironment, and in particular, are designed so that the bacteria infect tumor-resident immune cells and not 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

5 *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
10 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
15 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
20 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.

25 *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
30 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; 5 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 10 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 15 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 20 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 25 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, 30 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 χ 4550 (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 *et al.* (2003) *Int. J. Med. Microbiol.* 41(7):3413-3414). 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;

5 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

10 (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

15 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

20 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

25 (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.

30 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

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 TNF α (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
5 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
10 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
15 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
20 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
25 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,
30 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. The immunostimulatory bacteria deliver encoded genetic payloads in a tumor-specific manner, to tumor-resident myeloid cells. The immunostimulatory bacteria, by virtue of genomic
5 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 curl fimbriae and reduced biofilm formation. The immunostimulatory bacteria enhance T-cell function, such as by virtue of the
10 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
15 systemically. The immunostimulatory bacteria provided herein exhibit at least 15-fold improved LD₅₀ 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, the table below in the section F.5. describing dosages and administration).

20 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,
25 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

30 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 as 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
 5 as a starting strain, and modified as described herein.

3. Limitations of Existing Bacterial Cancer Immunotherapies

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 have numerous advantageous properties for use as anti-cancer
 10 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
 15 response to a viral infection is similar to an anti-cancer response. This is summarized in the following table (see, also, the Overview, above):

	Bacteria	Viruses
Innate Recognition by:	TLR2, TLR4 and TLR5	TLR3, TLR7/8, 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
Generation of Immunity:	No	Yes
Immunogenicity:	Not immunogenic	Highly immunogenic

A limitation of 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,
 20 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-
5 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
10 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
15 (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
20 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 and a powerful inducer of type I IFN, and its more stable version, poly ICLC
25 (such as that sold under the trademark Hiltonol®), 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.

30 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).

- 5 STING signaling activates the TANK-binding kinase 1 (TBK1)/interferon regulatory factor 3 (IRF3) axis, and the NF- κ B 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
- 10 (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
- 15 produced from intracellular *L. monocytogenes*, or c-di-GMP from *S. typhimurium*. It was later discovered that 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
- 20 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*
- 25 503(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, RIG-I, TLR3 and TLR7/8, 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
- 30 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- κ B-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-1 β 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 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.

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 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

immunostimulatory 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.

5 Provided herein is a superior microbial anti-cancer 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,
10 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
15 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.

 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,
20 CD80, CD86, CD27L, B7RP1, OX40L), with a full or truncated 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
25 domain. The co-stimulatory molecules also can be modified to include residues (such as positive residues) in the truncated cytoplasmic domain, to ensure that they are expressed in the correct orientation in the cell membrane (the Examples below describe this in more detail; see, *e.g.*, Example 19).

 The bacterial strains provided herein are engineered to deliver therapeutic
30 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.

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 with respect to *Salmonella*, particularly *S. typhimurium*; it is understood that the skilled person can effect similar enhancements/modifications in other bacterial species and other *Salmonella* strains. 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- κ B 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 CARD domains 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.

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

As described above, *msbB* mutants of *S. typhimurium* cannot undergo the terminal myristoylation of LPS, 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- 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 a 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* 5 *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 10 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/m², 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 15 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/m². Higher dosing leads to increased colonization of tumors, tumor-resident immune cells, and the tumor 20 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 (see, *e.g.*, Example 5) that *pagP*⁻/*msbB*⁻ mutant strains have increased resistance to 25 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 of the *msbB* gene, and that further are modified by deletion or disruption of *pagP*. As discussed 30 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 with 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.

5 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*.

10 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
15 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
20 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*
25 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),
30 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 *purI* 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
5 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 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.

10 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
15 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
20 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
25 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 shown herein, VNP20009/YS1646 is also auxotrophic for the immunosuppressive nucleoside adenosine, and for ATP (see, *e.g.*, Example 1).

Corresponding genes, encoding homologs or orthologs of phosphoribosyl-
30 aminoimidazole 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 phosphoribosylformylglycinamide cyclo-ligase in *S. typhi*; *purA*, encoding adenylosuccinate synthetase, *purQ*, encoding phosphoribosylformylglycinamide synthase II, and
 5 *purS*, encoding phosphoribosylformylglycinamide synthase subunit PurS in *L. monocytogenes*; *purM* (BL1122), encoding phosphoribosylformylglycinamide cyclo-ligase in *Bifidobacterium longum*; and NT01CX_RS09765, encoding AIR synthase, and NT01CX_RS07625 (*purM*), encoding
 phosphoribosylformylglycinamide cyclo-ligase in *Clostridium novyi*.

10 **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.
 15 Adenosine decreases T-cell receptor induced activation of NF- κ B, 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
 20 extravasation, decreases phagocytosis, and attenuates levels of superoxide and nitric oxide. Adenosine also decreases the expression of TNF- α , IL-12, and MIP-1 α (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
 25 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
 30 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 micro-environment, also inhibits adenylylase 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 μ M, which is up to about 100-1000 fold higher than the typical extracellular adenosine concentration of approximately 0.1 μ M (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 A₃ 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 A₁ 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 A₃, 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
5 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 damaged, dying, or stressed cells. The NLR family
10 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-1 β 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
15 ATP found in healthy tissues are very low (~1-5 μ M) (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
20 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
25 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.

30 Immunostimulatory bacteria, such as *Salmonella typhi*, can be made auxotrophic for adenosine by, for example, 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 in the tumor microenvironment of some tumors is

immunosuppressive, adenosine auxotrophy eliminates the immunosuppression from adenosine that accumulates in the tumor microenvironment of certain cancers.

3. Plasmid Maintenance and Delivery

a. *asd* Deletion

5 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, 10 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) 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- 15 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* engineered to deliver plasmids encoding genetic payloads/therapeutic products, such as immunostimulatory proteins (*e.g.*, cytokines, chemokines, co-stimulatory molecules); 20 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 25 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 30 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, 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
 5 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
 10 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
 15 dehydrogenase in *Clostridium novyi*.

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
 20 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
 25 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
 30 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
5 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
10 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)
15 *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
20 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
25 resulting in the secretion of cytokines, including IL-1 β , 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.*
30 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 can be similarly engineered to eliminate flagella. For example, as shown herein, a *Salmonella* strain lacking *msbB* and/or *pagP*, which results in reduced TNF- α induction, is combined with *fliC* and *fliB* knockouts. This results in a *Salmonella* strain that has a combined reduction in TNF- α induction and a reduction in TLR5 recognition. These bacterial modifications, *msbB*⁻, *pagP*⁻, *fliC*⁻, and *fliB*⁻, can be combined with an immunostimulatory plasmid, optionally containing CpGs, encoding therapeutic products, such as immunomodulatory proteins, alone or in combinations thereof. 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 example, as exemplified and provided herein, a *fliC* and *fliB* double mutant was constructed in the *asd*-deleted strain of *S. typhimurium*, VNP20009. VNP20009, which is attenuated for virulence by disruption of *purI/purM*, contains a modification of the *msbB* gene (a partial deletion) that results in production of a lipid A subunit that is less toxic 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 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*, *iagP*, *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 *fliB* genes

5 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

10 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 *fliB* results in significantly reduced IL-1 β 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

15 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

20 delete or mutate the flagellin genes *fliC* and *fliB* 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

25 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

30 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 enhances the function of T-cells in the tumor micro-environment. Elimination of L-asparaginase activity can be effected by modifying the

bacterial genome to eliminate expression of active enzyme. Modifications include insertions, deletions, inversions, and replacements of 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*,
5 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.
10 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 genome modifications are deletions, insertions, disruptions, and/or other modifications that eliminate expression of L-asparaginase II.
15 As shown herein, the genome of the immunostimulatory bacteria herein can be modified to delete *ansB*, or to disrupt it or otherwise modify it, to result in inactive encoded L-asparaginase II, or to eliminate the asparaginase, preventing T-cell suppression and enhancing 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
20 infected 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. Thus, immunostimulatory bacteria that combine deletions or disruptions of the *ansB* gene, whereby functional encoded enzyme is not expressed, with other modifications described herein that result in
25 increased accumulation in the tumor microenvironment and/or in tumor-resident immune cells, provide a 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*.
30

6. Deletions/Disruptions in *Salmonella* Genes Required for Curli Fimbriae Expression

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).

In *Salmonella*, such as *S. typhimurium*, biofilm formation is regulated by *csgD*, 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., Tükel *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
 5 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
 10 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.*
 15 59:76-84). It is shown herein that *csgD*-deleted strains demonstrate improved anti-tumor efficacy because of greater bacterial uptake into tumor-resident myeloid cells.

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
 20 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
 25 genes to render the bacteria *csgD*⁻, results in elimination of curli fimbriae and inflammatory cyclic dinucleotides (CDNs), and removes cellulose secretion. This eliminates inflammatory and immunosuppressive elements, prevents TLR4 recognition through altered LPS acylation, eliminates cellulose secretion, and, thus, possible biofilm formation, thereby increasing safety and efficacy.

30 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 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
5 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
10 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 play a role in the antibody-mediated immune response. Activation of the
15 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
20 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 and bacterial lysis (see, *e.g.*, Ramu *et al.* (2007) *FEBS Letters* 581:1716-1720).

To survive, pathogens have developed strategies to prevent deleterious
25 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
30 *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).

5 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
10 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
15 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
20 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
25 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
30 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
5 (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.

10 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
15 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

20 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.
25 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 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
30 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.

5 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
10 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
15 *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.

20 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.

25 **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
30 the most abundant components of the outer membrane in *S. typhimurium*, and leads to TLR2 induction of pro-inflammatory cytokines, such as TNF α , 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; 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, the tumor-resident immune cells, particularly macrophages, significantly was increased. As shown herein, deletion or disruption of these genes (*lppA* and *lppB*), thus, resulted in decreased virulence due to the inability to survive in infected macrophages, but resulted in enhanced plasmid delivery of the immunostimulatory bacteria, thereby increasing expression of encoded therapeutic genes in the targeted cells, *i.e.*, the tumor-resident immune cells, particularly macrophages.

9. Robust Immunostimulatory Bacteria Whose Genomes are Modified to be 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, their functional effects, and some of the effects/benefits achieved herein.

Genotype/Modification	Functional effect	Effect/Benefit
Δasd (in genome)	Plasmid maintenance	Improves plasmid delivery Plasmid maintenance <i>in vivo</i> via <i>asd</i> cassette on plasmid

Genotype/Modification	Functional effect	Effect/Benefit
$\Delta purI$	Purine/adenosine auxotrophy	Tumor-specific enrichment Limited replication in healthy tissue
$\Delta msbB$	LPS surface coat modification	Decreases TLR4 recognition Reduces immunosuppressive cytokine profile (TNF- α) Improves safety Prevents intracellular replication
$\DeltafliC/\DeltafliB$ (ΔFLG)	Flagella knockout	Removes major inflammatory and immune-suppressive element Eliminates TLR5 recognition Reduces immunosuppressive cytokine profile Improves safety Reduces ability to invade non-phagocytic cells (<i>e.g.</i> , stromal and tumor cells)
$\Delta pagP$	LPS surface coat modification	Removes major inflammatory and immunosuppressive element Decreases TLR4 recognition Reduces IL-6 production Improves safety
$\Delta ansB$	L-asparaginase II knockout	Enhances tumor T-cell function
$\Delta csdD$	Removes curli fimbriae, cellulose production, c-di-GMP	Reduces inflammation Prevents possible biofilm formation Enhances phagocytic cell uptake
Plasmid	Expresses gene products under control of host-recognized promoter	Eukaryotic promoter limits expression to cells containing the plasmid Long term expression in the TME (<i>i.e.</i> , <i>asd</i> encoded on plasmid under control of host-recognized promoter) Expression of any combination of therapeutic product(s) with large capacity CpGs to induce proper viral-like innate immune response

Strains provided herein are ΔFLG , and/or $\Delta pagP$, and/or $\Delta ansB$, and/or $\Delta csdD$. Additionally, the strains are one or more of $\Delta purI$ ($\Delta purM$), $\Delta msbB$, and Δasd (in the bacterial genome). In particular, the strains are $\Delta purI$ ($\Delta purM$), $\Delta msbB$, $\Delta pagP$, and $\Delta ansB$, and Δasd . The strains also can be $lppA^-$ and/or $lppB^-$, particularly

5 $lppA^-/lppB^-$. 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 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
 5 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*, *iagP*, *iagB*, *spaO*, *spaP*, *spaQ*, *spaR*, *spaS*, *orgA*, *orgB*, *orgC*, *prgH*, *prgI*,
 10 *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
 15 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, however, is desirable because it protects against inactivation against complement. Bacteria that do not endogenously encode *rck*, can be modified
 20 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
 25 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*.
 30 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*,
- 5 *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 tumefaciens*.
- 10 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
- 15 (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
- 20 strain 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 *purI* and *msbB* are completely deleted are more fit (grow faster) than 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*
- 25 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,
- 30 reducing unwanted inflammatory responses, and preventing them 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. As described below, the plasmid can encode one or a plurality of therapeutic products/genetic payloads (*i.e.*, multiplexed), for delivery of anti-cancer therapeutic products to the tumor/tumor microenvironment. The 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 in tumor-resident myeloid cells.

10. 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 macrophage), and M2-type (alternatively activated macrophage).

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 an immune monitor. M1 macrophages produce pro-

inflammatory cytokines, including IL-6, IL-12, and TNF- α . 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 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 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 receptor IIb). Expression of nitric oxide synthase (iNOS) in M1 macrophages also can 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. Thus, the conversion can be monitored or assessed by virtue of expression of these markers.

Tumor-associated macrophages (TAMs) are associated with an immunosuppressive M2 phenotype. Immunostimulatory bacteria provided herein can convert such macrophages into 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 is 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 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 M1 macrophages to M2 macrophages.

Immunostimulatory bacteria provided herein, that include genome modifications as described herein, such as the elimination of flagella and LPS modification, convert infected M2 macrophages into those that induce cytokine profiles of M1 macrophages. Immunostimulatory bacteria that express a variant

5 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.

The Examples demonstrate this change from an M2 to an M1-like or M1 phenotype. A comparison between the cytokine profiles in uninfected M2

10 macrophage with the induced cytokines in M2 macrophage infected with a salmonella strain that is $\Delta asd/\Delta FLG/\Delta pagP/\Delta ansB/\Delta csgD$ M2 induced high level of IFN γ , CXCL10 and CXCL11 secretions. Infection with the same strain that was transformed with plasmids encoding huSTING tazCTT N154S + R284G variant, WT huIL-12 and huSTING tazCTT N154S + R284G variant or WT huIL-15 induced higher CXCL10

15 and CXCL11 secretions than the untransformed $\Delta FLG/\Delta pagP/\Delta ansB/\Delta csgD$ strain not containing a plasmid. Cytokine profiles, characteristic of M1 or M1-like phenotypes, were induced with a variety of different payloads in the strains. The exemplary results are detailed in the Examples.

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

20

The immunostimulatory bacteria provided herein are modified so that they accumulate in the tumor microenvironment, and in tumor-resident myeloid cells,

25 where 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 control of a separate

30 promoter, or they are under control of one promoter, and can include sequences that result in expression of the discrete products, and, where appropriate, include 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, the plasmid can encode one product or a plurality thereof. Each product can be under control of a different eukaryotic promoter, or multiple encoded products can be expressed under 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. Provided herein is a modified 4-1BBL polypeptide, and encoding nucleic acid, that exhibits improved expression and activity when encoded on a plasmid in the immunostimulatory bacteria provided herein that deliver the plasmids to myeloid cells for expression under control of the host transcriptional and translational machinery.

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, the TME, and 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 in the Examples, the order in which the encoding nucleic acids are arranged on the plasmid can improve overall expression,

and modifications to the plasmids can improve the fitness of the bacteria that contain the plasmids encoding the proteins.

The immunostimulatory protein 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-36 γ , GM-CSF, IFN α , IFN β , 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 (4-1BBL Δ cyt), 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, 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, as described herein, potentiates the activation of the co-stimulatory molecule, without the immunosuppressive reverse signaling. This is exemplified with respect to 4-1BBL as described in the Examples and as follows; the same modifications, including replacement of residues in the truncated cytoplasmic domain to ensure proper orientation in the membrane, can be applied to any of the co-stimulatory molecules, as well as other transmembrane polypeptides.

The full-length sequence of human 4-1BBL (SEQ ID NO:389 see also, Uniprot P41273) is:

MEYASDASLDPEAPWPPAPRARACRVLPWALVAGLLLLLLLLAAACAVFLACPWAVS
GARASPGSAASPRREGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDP
GLAGVSLTGGLSYKEDTKELVVAAGVYYYVFFQLELRRVVAGEGSGSVSLALHLQP
LRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHA
WQLTQGATVLGLFRVTPEIPAGLPSRSE

where the cytoplasmic domain corresponds to amino acids 1-28 (italicized), the transmembrane domain corresponds to amino acids 29-49 (bold), and the extracellular domain corresponds to amino acids 50-254 (underlined). The human 4-1BBL Δ cyt sequence (see, SEQ ID NO:390) is:

MWALVAGLLLLLLLLAAACAVFLACPWAVSGARASPGSAASPRREGPELSPDDPA
GLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVA
AGVYYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARN
SAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSR
SE

which is the same as the full-length protein, but lacking the cytoplasmic domain, so that the transmembrane domain corresponds to amino acid residues 2-22 (bold), and the extracellular domain corresponds to amino acid residues 23-227 (underlined).

An exemplary human 4-1BBL, with a truncated cytoplasmic domain is as follows (see, SEQ ID NO:391):

MRVLPWALVAGLLLLLLLLAAACAVFLACPWAVSGARASPGSAASPRREGPELSPD
DPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELV
AKAGVYYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSE
A

RNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLP
SPRSE

where the truncated cytoplasmic domain corresponds to residues RLVP (in *italics*),
with the initiating M, the transmembrane domain corresponds to residues 6-26 (bold),
5 and the extracellular domain corresponds to residues 27-231 (underlined).

With respect to the full-length 4-1BBL, positively charged amino acids, such
as R and K, tend to be positioned in the cytoplasm (inside/cytoplasmic domain),
which orients the transmembrane domain so that N-terminus is inside. But when the
cytoplasmic domain is truncated, this alters the charge balance, so that there are only
10 positive charges on the outside (in the extracellular domain). This favors a
configuration in which the N-terminus of the protein is on the outside, not towards the
cytoplasm, resulting in an “inside out configuration.” If this is observed, such as by
apparent lower activity or expression or other parameters, the 4-1BBL variant with
the truncated cytoplasmic domain can be modified to include positive residues, to
15 ensure the proper orientation of the protein in the cell membrane upon expression.
Exemplary of possible modifications of 4-1BBL are those in which residues are
replaced with positively charged residues, or a c-myc tag is included. The skilled
person can envision other similar replacements/additions to achieve the same result.

Exemplary modified human 4-1BBL variants with a truncated cytoplasmic
20 domain include the following, in which extra positive residues (Arginine (R), Lysine
(K), italicized) are included in the cytoplasmic domain region, as follows, so that the
resulting protein, when expressed in a cell, is properly oriented (has the correct
configuration and not the “inside out” configuration). See, SEQ ID NOs:391 and 392,
respectively:

25 **Truncated cytoplasmic domain:**

MRVLPWALVAGLLLLLLAAACA VFLACPWAVSGARASPGSAASPRLREGPELSP
DDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELY
VAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLOPLRSAAGAAALALTVDLPASSE
ARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAG
30 LPSRSE

This adds a positive charge back to the N-terminus (*R*), which favors a configuration
in which the N-terminus is correctly oriented inside the cytoplasm. In another
example a MYC tag is added.

Truncated cytoplasmic domain with a MYC tag:

5 MEQKLISEEDLRVLPWALVAGLLLLLLAAACAVFLACPWAVSGARASPGS
AASPRLREGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLA
GVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHL
QPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTE
ARARHAWQLTOGATVLGLFRVTPEIPAGLPSRSE

For sequences of full-length mouse 4-1BBL, and exemplary sequences of
 mu4-1BBL Δ cyt (murine 4-1BBL with a deletion of the cytoplasmic domain), mu4-
 1BBL with a truncated cytoplasmic domain, and mu4-1BBL with a truncated
 cytoplasmic domain and a MYC tag, see Example 19 below; see, also, SEQ ID
 10 NOs:393-396, respectively.

Additional or alternative amino acid replacements can be included in the co-
 stimulatory molecules to ensure proper orientation of the expressed protein in the
 membrane. The skilled person readily can prepare other similar modifications to
 ensure proper orientation of a transmembrane protein with a truncated cytoplasmic
 15 domain.

In addition to deletion or truncation of the cytoplasmic domain, co-stimulatory
 molecules (*e.g.*, 4-1BBL, CD80, CD86, CD27L, B7RP1, and OX40L), for expression
 on an APC, also can be modified by introducing amino acid modifications, such as
 insertions, deletions, and/or replacements, to the cytoplasmic domain, such that the
 20 modified 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 the modifications to the cytoplasmic domain. For example,
 the immunosuppressive reverse (intracellular) signaling can be eliminated by
 modifying the cytoplasmic domain phosphorylation sites, such as by replacing one or
 25 more Ser residues, at an appropriate locus or loci, with a residue that reduces or
 eliminates reverse signaling. For example, for human 4-1BBL, the
 immunosuppressive reverse (intracellular) signaling can be eliminated by modifying
 the cytoplasmic domain phosphorylation sites, including Ser5 and Ser8, with
 reference to the sequence of full-length human 4-1BBL (SEQ ID NO:389). The serine
 30 residues in the cytoplasmic domain can be replaced by any other residue that reduces
 or eliminates reverse signaling.

Additional or alternative amino acid replacements can be included in the co-
 stimulatory molecules to eliminate immunosuppressive intracellular (reverse)

signaling. The skilled person readily can prepare other similar modifications to eliminate immunosuppressive reverse signaling, while still maintaining the co-stimulatory molecule's ability to activate constitutive immuno-stimulatory signaling to a T-cell through co-stimulatory receptor engagement.

5 **a. Cytokines and Chemokines**

In some embodiments, the immunostimulatory bacteria herein are engineered to express 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-18, IL-21, IL-23, IL-36 γ , 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.

15 **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 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).

25 **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 (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-1 α , IL-1 β 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 anti-tumor 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-15R α

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 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 anti-tumor immunity, and has demonstrated significant anti-tumor 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-15 and IL-15 receptor alpha (IL-15R α) are coordinately expressed by antigen-presenting cells, such as monocytes and dendritic cells, and IL-15 is presented *in trans* by IL-15R α to the IL-15R $\beta\gamma_c$ receptor complex expressed on the surfaces of CD8⁺ T-cells and NK cells. Soluble IL-15:IL-15R α complexes have been shown to modulate immune responses via the IL-15R $\beta\gamma_c$ 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-15R α , 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-15R α 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).

5 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
10 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 are involved in mediating immune and inflammatory responses. Chemokines are classified into four subfamilies, based on
15 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
20 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,
25 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 herein can be engineered to encode chemokines, including, but not limited to, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CXCL11.

30 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).

5 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-
10 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
15 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, IFN γ , resulting in the
20 production of CXCL9 and CXCL10 (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
25 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.*
30 (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
5 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
10 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
15 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
20 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).

25 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).

30 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 CXCL 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 (4-1BBL Δ cyt), 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
5 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). It is shown herein that the gene product with a truncated cytoplasmic domain, including a full deletion, provides constitutive
10 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 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
15 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
20 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,
25 with reference to SEQ ID NO:389, 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.*, 4-1BB) to enhance the anti-tumor immune response.

30 **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

5 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

10 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

15 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

20 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

25 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

30 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

- 5 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
- 10 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
- 15 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).
- 20 4-1BBL, a member of the TNF superfamily, is expressed in B-cells, dendritic cells, activated T-cells and macrophages. 4-1BBL binds to its receptor, 4-1BB, and provides a co-stimulatory signal for T-cell activation and expansion. The human 4-1BBL gene encodes a 254 amino acid type II transmembrane protein containing a 28 amino acid cytoplasmic domain, a 21 amino acid transmembrane protein domain, and
- 25 a 205 amino acid extracellular domain (see, SEQ ID NO:389). Deletion of all or of a portion of the cytoplasmic domain of 4-1BBL (corresponding to amino acid residues 1-28 of SEQ ID NO:342 or 389), as described herein, potentiates the activation of 4-1BBL without the immunosuppressive reverse signaling. The portion that is deleted is sufficient to potentiate the activation of 4-1BBL, but without the immunosuppressive
- 30 reverse signaling. As described below, the truncated cytoplasmic domain can include amino acids or replacements to ensure proper orientation of the expressed protein in the cell membrane (similar modifications can be effected in others of the membrane-

spanning proteins in which the cytoplasmic domain is truncated or deleted). Provided herein are nucleic acid molecules encoding a 4-1BBL variant that lacks the cytoplasmic domain, or that has a truncated cytoplasmic domain, to eliminate the immunosuppressive reverse signaling. An example of such nucleic acid and encoded protein is described in the Examples (see, *e.g.*, SEQ ID NOs: 391 and 395). The receptors also can be cytoplasmically truncated or deleted.

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. 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.* 2:299-308). These include prodrugs of 5-fluorouracil, azoxaphosphorines, platinum drugs, and enzymes such as deaminases, nitroreductases, phosphorylases, cytochrome P450 enzymes, and many others.

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

5 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

10 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

15 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

20 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).

25

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.

30 **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-Goutières 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 *POLAI*); 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 *ADARI*); 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).

- 5 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
- 10 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. Patent Publication No. 2018/0311343). Another gain-of-function mutation
- 15 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
- 20 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).
- 25 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
- 30 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

5 *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

10 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

15 STAT1/2 showed diminished activity. Both variants showed similar STAT3 and NF- κ B 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- κ B pathway. Patients with the R232

20 allele and 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,

25 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

30 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
5 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
10 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
15 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
20 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
25 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,
30 E316Q, R310A/E316A, S324A/S326A, S358A, E360A, S366A, S358A/E360A/S366A, N154S/R284G, and R375A, as well as conservative mutations

thereof. In addition, combinations of STING gain-of-function mutations can have significantly boosted STING signaling over their individual mutation counterparts.

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	Δ 247–467
G166E		R822Q		S475D/S477D/S479D
C206Y		G821S		
G207E		A452T		
R281Q		A946T		
R284G		R337G		
R284S		D393V		
R284M		G495R		
R284K		R720Q		
R284T		R779H		
S102P/ F279L		R779C		
S102P		L372F		
F279L				
R197A				
D205A				
R197A/ D205A				
S272A/ Q273A				
R310A/ E316A				
R310A				
E316A				
E316N				
E316Q				
S272A				
R375A				
R293A				
T294A				
E296A				
R293A/ T294A/ E296A				

STING	RIG-I	MDA5	IRF3	IRF7
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, with reference to the sequence of murine STING, as set forth in SEQ ID NO:369.

It is shown herein that the combination of replacements N154S/R284G results in constitutive expression of type I interferon. Also included are conservative substitutions of each of the replacements (see, 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 IFN α 1, 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 IFN α 1, 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 IFN α 1, 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 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- κ B pathways via TRAF6 recruitment. Human STING residue S366
5 (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- κ B signaling axis.
10 For example, the zebrafish CTT, which is responsible for hyperactivation of NF- κ B 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-
15 essential, TRAF6 recruitment is essential for zebrafish STING-induced NF- κ B 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- κ B activation, indicating that this region is necessary and sufficient to direct enhanced NF- κ B signal activation. The addition of the
20 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- κ B signaling is exploited herein to produce modified STING proteins that have reduced NF- κ B signaling, and/or optionally, increased IRF3 signaling, so that when the
25 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- κ B 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
30 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 (*Callorhynchus 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- κ B 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- κ B 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- κ B 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- κ B 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
5 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
10 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
15 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)
20 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- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), and AP-1 (activator protein 1). As a result, the
25 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
 5 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
 10 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- κ B reporter gene activity, and this
 15 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
 20 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
 25 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
 30 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- κ B
5 (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).

10 Gain-of-function (GOF) *IFIH1* variants occur in subjects with autoimmune disorders, including Aicardi-Goutières 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
15 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
20 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*
25 (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
30 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 (see, *e.g.*, Rice *et al.* (2014) *Nat. Genet.* 46(5):503-509).

5 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
10 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
15 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
20 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
25 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.*
30 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 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(Δ 247-467), 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 <i>ISG15</i> , <i>IFIT1/ISG56</i> , <i>CXCL10</i> , <i>OASL</i> and <i>CCL5</i> ; 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- κ B; 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- κ B and JNK/MAPK8; mediates type I IFN induction
TRAF3	TNF receptor-associated factor 3	Regulates activation of NF- κ B and MAP kinases; mediates activation of IRF3; mediates type I IFN induction; mediates cytokine production
TRAF6	TNF receptor-associated factor 6	Activates NF- κ B, 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 IKK ϵ -dependent

Gene	Encoded Protein	Activity/Function
		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
DHX9/DDX9	DExH/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- κ B-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- κ B 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- κ B 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- κ B, 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- κ B, 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- κ B 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 antiviral 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 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- κ B 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).

4. 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. Antibody fragments such as this 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 potential antigens to target, include, but are not limited to, the following listed below.

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- β 1, TGF- β 2, and TGF- β 3; TGF- β 1 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 and remove TGF- β from the tumor microenvironment, thereby breaking tumor immune tolerance and stimulating anti-tumor immunity, include anti-TGF-beta antibodies or antibody fragments, anti-TGF-beta receptor antibodies or antibody fragments, and soluble TGF-beta antagonist polypeptides.

Provided herein are immunostimulatory bacteria, that accumulate in the tumor microenvironment, in tumors, and in particular, in tumor-resident immune cells, 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

5 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 (bi-specific, tri-specific, etc.), joined together by a long linker. Bi-specific T-cell engager (sold 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
10 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. 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 BiTEs® have been approved by the FDA, including
15 catumaxomab, which is directed against the tumor antigen EpCAM, and CD3, and is used in the treatment of malignant ascites, and blinatumomab, a BiTE® antibody against CD19 and CD3, which is used for the treatment of relapsed, refractory acute lymphoblastic leukemia (ALL) (see, *e.g.*, Ahmadi-Fesharaki *et al.* (2019) *Mol. Ther. Oncolytics* 14:38-56). Other BiTEs® target other antigens, including
20 carcinoembryonic antigen (CEA), prostate-specific membrane antigen (PSMA), EGFR, EphA2, Her2, ADAM17/TACE, prostate stem cell antigen (PSCA), 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.

25 **c. 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
30 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 or scFv-Fc, and others known in the art or described herein, against PD-1 or against PD-L1, will synergize with activation of immune cells to potentiate anti-tumor immunity.

d. 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
 5 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
 10 thereof, such as, but not limited to, anti-CTLA-4 scFvs (see, *e.g.*, SEQ ID NO:403 for an exemplary human anti-CTLA-4 scFv fragment), and anti-CTLA-4 scFv-Fcs (see, *e.g.*, SEQ ID NO:402, for an exemplary human anti-CTLA-4 scFv-Fc fragment; see, also, Example 20).

e. Additional Exemplary Checkpoint Targets

15 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

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

Cytokines are powerful modulators of the anti-tumor immune response.

- 5 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
- 10 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 +
- 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 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
- 20 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
- 25 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
5 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
10 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
15 thereof), and/or a co-stimulatory molecule (*e.g.*, 4-1BBL, 4-1BBL Δ cyt, and other variants of 4-1BBL discussed herein); 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
20 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
25 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
30 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

5 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

10 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,

15 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

20 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

25 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

30 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

5 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*,

10 *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 encoding on a plasmid in the bacteria, nucleic acid encoding the therapeutic product(s) under control of an RNA polymerase promoter recognized by

15 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

20 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.

25 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

30 bacteria that accumulate in phagocytic cells, particularly tumor-resident immune cells, such as, for example, myeloid-derived suppressor 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
5 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.

10 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
15 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)
20 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
25 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
30 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

5 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
10 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
15 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 to improve expression in eukaryotic cells, and replacement of TATA box
20 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
25 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
30 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 polypeptides provided herein, which include those with mutations that result in constitutive expression of the type I interferons so that the interferons are expressed in the absence of induction, 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- κ B signaling activity than the NF- κ B signaling activity of human STING, and the TRAF6 binding site in the CTT optionally is deleted.

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.

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 all of *asd*⁻, flagellin⁻ (*fliC*⁻/*fliB*⁻), *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
5 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.

10 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-
15 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
20 encoding the therapeutic products are under the control of RNA polymerase II promoters. For example, promoters include, but are not limited to EF-1 α , 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
25 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.

30 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.

10 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-15R α 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, 15 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 (4-1BBL Δ cyt), ICOS, CD27, members of the B7-CD28 family, and members of the tumor necrosis factor receptor 20 (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 full-length cytoplasmic domain, or with a truncated, or partial, or partial with modifications to ensure proper orientation, cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC), where the truncated gene product 25 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 30 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 4-1BBL Δ cyt); and IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

IL-15/IL-15R α and a STING GOF variant; IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); IL-15/IL-15R α and IL-12p70; IL-15/IL-15R α and IL-21; IL-15/IL-15R α , IL-12p70, and a STING GOF variant; IL-15/IL-15R α , IL-21, and a STING GOF variant; IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); and IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

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 4-1BBL Δ cyt); IL-12p70 and a STING GOF variant; IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); IL-12p70 and IL-18; IL-12p70, IL-18, and a STING GOF variant; and
 5 IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

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

a TGF- β decoy receptor, IL-15/IL-15R α , and a STING GOF variant; a TGF- β decoy receptor, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); a TGF- β decoy receptor, IL-15/IL-15R α , and IL-12p70; a TGF- β decoy receptor, IL-15/IL-15R α , and IL-21; a TGF- β decoy receptor, IL-15/IL-15R α , IL-12p70, and a STING GOF variant; a TGF- β decoy receptor, IL-15/IL-15R α , IL-21, and a STING GOF variant; a TGF- β decoy receptor, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); and a TGF- β decoy receptor, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 20 1BBL Δ cyt);

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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); and a TGF- β decoy receptor and a STING GOF variant;

30 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 4-1BBL Δ cyt); and an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

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

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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); and a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

a CD40 agonist, IL-15/IL-15R α , and a STING GOF variant; a CD40 agonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); a CD40 agonist, IL-15/IL-15R α , and IL-12p70; a CD40 agonist, IL-15/IL-15R α , and IL-21; a CD40 agonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant; a CD40 agonist, IL-15/IL-15R α , IL-21, and a STING GOF variant; a CD40 agonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); and a CD40

agonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); 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 4-1BBL Δ cyt); and a CD40 agonist and a STING GOF variant.

In all combinations including 4-1BBL, the 4-1BBL molecule can be a full-length protein (see, *e.g.*, SEQ ID NOs:389 and 393, for human and mouse 4-1BBL, respectively); a 4-1BBL variant with the cytoplasmic domain deleted (4-1BBL Δ cyt; see *e.g.*, SEQ ID NOs:390 and 394, for human and murine 4-1BBL Δ cyt, respectively); a 4-1BBL variant with a truncated (*i.e.*, not fully deleted) cytoplasmic domain (4-1BBLcyt trunc; see, *e.g.*, SEQ ID NOs:391-392 and SEQ ID NOs:395-396, for exemplary human and mouse 4-1BBLcyt trunc variants); or a 4-1BBL molecule with a modified cytoplasmic domain, in which one or more Ser residues, which act as phosphorylation sites, are replaced at an appropriate locus or loci, such as, for human 4-1BBL, with reference to SEQ ID NO:389, Ser5 and Ser8, with a residue that reduces or eliminates reverse signaling. Additionally, all combinations including an anti-CTLA-4 antibody, can include an anti-CTLA-4 antibody fragment, such as an anti-CTLA-4 scFv (see, *e.g.*, SEQ ID NOs:403 and 404, for exemplary human and mouse anti-CTLA-4 scFv fragments, respectively), or an anti-CTLA-4 scFv-Fc (see, *e.g.*, SEQ ID NOs:402 and 405, for exemplary human and mouse anti-CTLA-4 scFv-Fc fragments, respectively). Additionally, a TGF- β receptor decoy can be replaced by other TGF-beta polypeptide antagonists, that can bind and remove TGF- β from the tumor microenvironment, including, for example, anti-TGF-beta antibodies or antibody fragments, anti-TGF-beta receptor antibodies or antibody fragments, and soluble TGF-beta antagonist polypeptides.

The following table lists exemplary products that can be encoded in plasmids in the immunostimulatory bacteria, and some effects/characteristics of such products.

Encoded Product	Effects/Characteristics
Engineered STING - to increase Type I IFN expression	Chemokine gradients recruit T-cells. Induction of Type I IFN, T-cell activation, APC tumor antigen cross-presentation. Constitutive STING variants validated in SAVI patients with Type I Interferonopathies.
IL-12	Strong Th1 immune response driver. Produced by activated APCs, particularly M1 Macrophages and DCs* Data indicate that it is a strong driver of IFN- γ
IL-15	Stimulates T-cell and NK cell proliferation. Lacks T _{reg} stimulation seen with IL-2.
IL-21	Pleiotropic cytokine that stimulates T-cells and NK cells. Converts M2 macrophages to M1 macrophages. Produced upon TLR3 activation (RNA viral sensing).
IL-36 γ	Promotes Th1 APCs and IFN- γ production by T-cells, NK cells and $\gamma\delta$ T-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

5 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

10 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.

The plasmids and immunostimulatory bacteria provided herein encode
5 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
10 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 a strong secretion of CXCL10 by bone marrow dendritic cells (BMDCs). Combining IL-36 γ with IL-12p70 and STING R284G tazCTT led to higher secretion of CXCL10 and IFN- γ by BMDCs (see, Example 26). Many of the combinations
15 induce the activation of CD8⁺ T-cell responses (*e.g.*, 4-1BB expression), and the secretion of IFN- γ . The results in the working Examples (see, Example 26) show that particular cytokine combinations can activate T-cells. For example, the combinations of IL-12p70 + IL-15; IL-12p70 + IL-15 + IFN- α 2; IL-12p70 + IL-15 + anti-4-1BB agonistic antibody; IL-12p70 + IL-15 + IL-36 γ ; IL-12p70 + IL-15 + IL-21; IL-12p70
20 + IL-21 + IL-36 γ ; IL-12p70 + IL-36 γ + IFN- α 2; IL-12p70 + IL-36 γ + anti-4-1BB agonistic antibody; IL-15 + IL-36 γ + IFN- α 2; and IL-15 + IL-36 γ + 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.
25 Additionally, several combinations of cytokines (IL-12p70, IL-15, IL-21, and IL-36 γ) and 4-1BB engagement, activate T-cells to secrete high levels of IFN- γ , with and without TCR stimulation by an anti-CD3 ϵ 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 (see, Example 24) also showed, in a
30 mouse (*mu*) model of colorectal carcinoma, that immunostimulatory bacterial strains, expressing IL-15, or the combination of 4-1BBL Δ cyt + IL-12 more potently inhibit tumor growth inhibition that the same strains expressing 4-1BBL(Δ cyt) 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 20 and SEQ ID NOs:402 and 403, respectively); one or more cytokines/chemokines, such as IL-12, IL-15, IL-18, IL-21, IL-23, IL-36 γ , IFN- β , IFN- α 2, and CXCL10; TGF- β binding decoy receptors and other TGF-beta polypeptide antagonists, such as, for example, a human soluble TGF β receptor II fused with a human IgG1 Fc (hu sTGF β RII-Fc; SEQ ID NO:407), 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.

As discussed herein, these 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.

Exemplary Combinations of Products and Exemplary Order on the Plasmids

1 st Encoded Product	1 st 2A Peptide	2 nd Encoded Product	2 nd 2A Peptide	3 rd Encoded Product	3 rd 2A Peptide	4 th Encoded Product
4-1BBL*	T2A	IL-12p70				
4-1BBL*	T2A	Chimeric STING**				
4-1BBL*	T2A	IL-12p70	P2A	Chimeric STING**		
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-36 γ				
IL-36 γ	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-36 γ		
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-36 γ	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-36 γ	P2A	Chimeric STING**		
IL-36 γ	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-36 γ	T2A	
4-1BBL*	T2A	sTGF β RIIFc [#]			T2A	
sTGF β RIIFc [#]	T2A	IL-12p70	P2A	Chimeric STING**		
4-1BBL*	T2A	sTGF β RIIFc [#]	P2A	IL-12p70	T2A	
4-1BBL*	T2A	IL-12p70	P2A	sTGF β RIIFc [#]	T2A	
IL-36 γ	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.

5 sTGF β RIIFc[#] = type II receptor betaglycan.

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;
- 5 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 ;
- 10 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
15 of immunosuppressive myeloid cells;
- 6) Cancer cell recognition by T-cells – Type I IFN, and IFN γ , and upregulation of MHC; and
- 7) Killing of cancers cells – the combination of encoded cytokines/chemokines, such as IL-12, IL-15, IL-21, and/or IL-36 γ , which induce T-
20 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
25 suppressing effects, to enhance the anti-tumor activities of the immunostimulatory bacteria provided herein.

E. CONSTRUCTING EXEMPLARY PLASMIDS ENCODING THERAPEUTIC PRODUCTS FOR BACTERIAL DELIVERY

The immunostimulatory bacteria herein can be modified to encode one or
30 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

5 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,

10 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-15R α chain complex, IL-18, IL-21, IL-23, IL-36 γ , 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

15 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 of the cytoplasmic domain (4-1BBL Δ cyt), 4-1BBL with a truncated cytoplasmic domain or otherwise modified truncated cytoplasmic domain, ICOS, ICOS ligand,

20 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.

25 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. For example, the constitutively active STING variants include those with the mutations V147L, N154S, V155M,

30 C206Y, R281Q, and/or R284G, 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), SIRP α , VISTA, and TREX-1, and others known in the art or described herein, or 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 and express 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-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 and express 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 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

5 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-1 α) 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

10 (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.*

15 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

20 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

25 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

30 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. As shown in the examples below (see, Example 30), 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.

10 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 and 2A peptides use different mechanisms for co-expression of multiple genes in one transcript. For example, when using an IRES 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 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
5 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 (SEQ ID NO:327) from *Thosea asigna* virus, P2A (SEQ ID NO:328) from
10 porcine teschovirus-1, E2A (SEQ ID NO:329) from equine rhinitis A virus, and F2A (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
15 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
20 preceding the 2A peptide (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
25 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- α 2-WPRE, where codon optimized murine IL-2 is co-expressed with murine IFN- α 2, using a CMV promoter, and a T2A peptide. Additionally, a
30 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-1 α . Exemplary of such a construct is CMV-muIL-15R α /IL-15sc_T2A_muSTING-R283G + EF-1 α -muIL-18-WPRE, where murine 15R α /IL-15sc and murine STING with the replacement R283G
5 are co-expressed under control of a CMV promoter, using T2A, and murine IL-18 is expressed separately under control of an EF-1 α promoter. This exemplary construct also includes a WPRE for enhanced expression.

b. Dual/Multiple Promoter Constructs

Alternatively, the genetic payloads/therapeutic products can be expressed
10 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
15 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

20 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
25 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.

30 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
5 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
10 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
15 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
20 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.
30 They can be adjacent to, or far from the promoter they influence, and include CMV, EF-1 α , 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

5 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
10 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
15 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
20 cells, particularly to tumor-resident myeloid cells, in cancer subjects, where the payloads are expressed, processed and trafficked. As described in the Examples (see, Example 30), 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
25 viability, and reduced growth rate in broth culture.

As shown in the Examples (see, Example 30), to minimize the negative impacts to bacterial fitness, while maintaining high ectopic expression in mammalian cells, the delivery plasmid was systematically modified to improve bacterial fitness, and to maintain or improve eukaryotic expression. The possible exemplary negative
30 impacts and solutions include, for example, the following:

1) cryptic bacterial promoter sequences encoded within the CMV promoter enhancer region were identified using PromoterHunter (available online at phisite.org/promoterhunter/; see, *e.g.*, Klucar *et al.* (2010) *Nucleic Acids Res.* 38(Database issue):D366-D370), and these putative promoter sequences were
5 replaced with CREB-binding sites and partial CREB-binding sites to promote efficient plasmid delivery;

2) to inhibit transcriptional leakiness from the CMV promoter, a number of bacterial terminators were inserted in the 5' UTR of ORF 1, to inhibit expression in bacteria (see table in Example 30, below);

10 3) to reduce the level of readthrough transcription from the origin of replication, the orientation of the expression cassette, from upstream of the CMV promoter to the end of the polyadenylation signal, was reversed, with and without a transcription terminator inserted between the expression cassette and the origin of replication; and

15 4) modifications from among 1)-3) above, that resulted in increased injection stock viability, with enhanced *in vitro* genetic payload expression, were combined for further improvement.

The results, detailed in the Examples (see, Example 30), show that, when the expression cassette was reversed on the plasmid and the BBa_B0015 bacterial
20 terminator was inserted after the coding region, and the T4 bacterial terminator was inserted downstream of the CMV promoter (see, *e.g.*, Figure 14), there was an increase in the bacterial cell viability, a reduced doubling time, growth to a higher stationary OD₆₀₀, and increased expression of the encoded payload *in vitro*, compared to the plasmid without the modifications.

25 The BBa_B0015 terminator is a composite terminator, in which a terminator derived from *E. coli* (BBa_B0010), and a terminator derived from the T7 phage (BBa_B0012), are joined.

Thus, provided herein are plasmids in which the eukaryotic promoter, such as a viral promoter, is in the opposite orientation from the bacterial promoter, such as the
30 bacterial promoter controlling expression of the exogenous *asd* gene on the plasmid. For example, the exogenous *asd* cassette (includes the regulatory sequences for expression and the exogenous *asd* gene, encoded on the plasmid in the *asd^r* bacteria)

is in the opposite orientation from the eukaryotic regulatory sequences and operatively linked payload-encoding nucleic acid. This reduces readthrough (leakiness) of the eukaryotic promoter. These constructs also include bacterial terminators flanking the payload expression cassette that includes the eukaryotic promoter, which reduces readthrough from bacterial promoters. Figure 14, for example, provides an exemplary construct configuration.

Thus, bacterial fitness, if desired, can be improved by one or more of several strategies, including orienting expression cassettes including eukaryotic promoters in the opposite direction from those under the control of bacterial promoters, and the inclusion of bacterially-recognized terminators to terminate bacterial expression at strategic loci. Other such modifications can be included to enhance bacterial growth *in vitro*, and to favor expression, or to not interfere with expression, from eukaryotic promoters *in vivo* in the eukaryotic host, such as a human. Thus, the constructs were improved by the inclusion of bacterial promoters; and by reversing the orientation of the nucleic acid encoding the payload, relative to the exogenous *asd*-encoding cassette.

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
5 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
10 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 copy numbers 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
20 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 μ g DNA per 1 ml LB culture; a low-copy plasmid will yield between 0.2-1 μ g DNA per ml of LB culture.
30 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.

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 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, induces IRF7-dependent type I interferon signaling, 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, and induce type I IFN-mediated innate and adaptive immunity. 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 (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.

5 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
10 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.

15 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
20 *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
25 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
30 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).

5 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
10 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
15 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.

20 **F. 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
25 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
30 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

5 **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
10 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
15 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
20 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.

25 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 (trehalose, sucrose, and others), cyclodextrins, and polysaccharides (*e.g.*, raffinose, maltodextrins, dextrans, *etc.*);
30 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
5 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
10 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).
15 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
20 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)
25 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
30 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.

- 5 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.

- 10 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.

- 15 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.

- 20 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, 25 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
- 30

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
5 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
10 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
15 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
20 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,
25 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
30 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, polyvinylpyrrolidone, 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.

- 5 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.

10 **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
15 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
20 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
25 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 1×10^5 to 1×10^{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
30 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 (polyoxyethylene 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.

5 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
10 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
15 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
20 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
25 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
30 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; 5 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
10 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
15 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 determined empirically, can depend on the age, weight, body surface area, and condition of the
20 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
25 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
30 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. An exemplary dose can be about 1×10^9 CFU/m². 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 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	$\Delta purI + \Delta msbB$	4.4E6	~44,000	-	6.33E+08
ΔFLG	$\Delta purI + \Delta msbB + \Delta asdA + \Delta FLG$	2.0E7	200,000	4.5	2.88E+09
$\Delta pagP$	$\Delta purI + \Delta msbB + \Delta asdA + \Delta pagP$	1.4E7	139,000	3.2	2.00E+09
$\Delta FLG/\Delta pagP$	$\Delta purI + \Delta msbB + \Delta asdA + \Delta FLG + \Delta pagP$	>6.2E7	>620,000	>14	>8.91E+09

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
5 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
10 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
15 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,
20 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;
25 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
30 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.

G. METHODS OF TREATMENT AND USES

5 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. Additionally, methods of combination therapies with one or more additional agents
10 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
15 solved. Formulations suitable for each route of administration are provided. The skilled person can establish suitable regimens and doses, and can select routes of administration.

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

20 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
25 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.

30 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

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 211

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 211

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED:

1. A nucleic acid construct, comprising nucleic acid encoding a plurality of anti-cancer products as a polycistronic sequence under control of a single promoter.
2. The construct of claim 1, wherein the polycistronic sequence comprises
5 a 2A peptide between each open reading frame (ORF) encoding each product.
3. The construct of claim 1 or claim 2, wherein the anti-cancer products are proteins.
4. The construct of claim 2 or claim 3, wherein the 2A peptide is one or more of T2A, P2A, E2A, or F2A.
- 10 5. The construct of any of claims 1-4, wherein the encoded products are one or more immunostimulatory protein(s) that confer(s) or contributes to an anti-tumor immune response in a tumor microenvironment.
6. The construct of claim 5, wherein an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor
15 microenvironment 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-18, IL-21, IL-23, IL-36 γ , IL-2 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
20 recruitment and/or persistence of T-cells, CD40, CD40 ligand (CD40L), CD28, OX40, OX40 ligand (OX40L), 4-1BB, 4-1BB ligand (4-1BBL), 4-1BBL that has a cytoplasmic domain deletion or truncation to eliminate immunosuppressive reverse signaling, members of the B7-CD28 family, CD47 antagonists, an anti-IL6 antibody or IL-6 binding decoy receptor, TGF-beta polypeptide antagonists, and members of
25 the tumor necrosis factor receptor (TNFR) superfamily.
7. The construct of claim 5, wherein an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment is selected from among one or more of: 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-
30 15/IL-15R alpha chain complex, 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 (4-1BBL Δ cyt) or with a partially deleted cytoplasmic domain, which the cytoplasmic domain is deleted or truncated to eliminate the immunosuppressive reverse signaling, members of the B7-CD28 family, and members of the tumor necrosis factor receptor (TNFR) superfamily.

8. The construct of any of claims 1-7, comprising nucleic acid encoding 4-1BBL with a deleted, or partially deleted, cytoplasmic domain, or a partially deleted cytoplasmic domain and optionally including amino acid modifications, whereby the resulting 4-1BBL assumes the proper orientation when expressed in a cell.

9. The construct of any of claims 1-8, comprising nucleic acid encoding a 4-1BBL variant with a deleted or partially deleted cytoplasmic domain, or a modified 4-1BBL with a truncated and modified cytoplasmic domain, wherein the sequence of the 4-1BBL is set forth in SEQ ID NO:390, SEQ ID NO:391, and SEQ ID NO:392.

10. The construct of any of claims 1-9, comprising nucleic acid encoding any of the following products and combinations of products:

one or more of IL-12, or IL-15, or IL12p70, or IL-15/IL-15R alpha chain complex;

a cytokine and a Stimulator of Interferon Genes (STING) pathway agonist;

a cytokine, a STING pathway agonist, and either a costimulatory receptor ligand or an immune checkpoint inhibitor;

a cytokine, a STING pathway agonist, and a TGF-beta polypeptide antagonist;

a cytokine, a STING pathway agonist, a TGF-beta polypeptide antagonist, and either a co-stimulatory receptor ligand or an immune checkpoint inhibitor,

wherein a STING pathway agonist is any product that increases type I interferon expression via activation of the STING pathway.

11. The construct of any of claims 1-10, comprising nucleic acid encoding a Stimulator of Interferon Genes (STING) polypeptide.

12. The construct of any of claims 1-11, comprising nucleic acid encoding a combination of therapeutic products selected from among the following combinations:

an anti-CTLA-4 antibody and a STING polypeptide,

- IL-15 and a STING polypeptide,
4-1BBL and a STING polypeptide,
A TGF-beta receptor decoy or antagonist polypeptide, and a STING
polypeptide,
- 5 IL-12 and a STING polypeptide,
an anti-CTLA-4 antibody, IL-15, and a STING polypeptide,
4-1BBL, IL-15, and a STING polypeptide,
A TGF-beta receptor decoy or antagonist polypeptide, and IL-15, and a
STING polypeptide,
- 10 an anti-CTLA-4 antibody, and IL-12, and a STING polypeptide,
4-1BBL, IL-12, and a STING polypeptide,
a TGF-beta receptor decoy or polypeptide antagonist, IL-12, and a STING
polypeptide,
an anti-CTLA-4 antibody, IL-15, a TGF-beta receptor decoy or polypeptide
15 antagonist, and a STING polypeptide,
4-1BBL, IL-15, a TGF-beta receptor decoy or polypeptide antagonist, and a
STING polypeptide,
an anti-CTLA-4 antibody, IL-12, a TGF-beta receptor decoy or polypeptide
antagonist, and a STING polypeptide,
- 20 4-1BBL, IL-12, a TGF-beta receptor decoy or polypeptide antagonist, and a
STING polypeptide,
an anti-CTLA-4 antibody, IL-12, IL-15, and a STING polypeptide,
4-1BBL, IL-12, IL-15, and a STING polypeptide,
a TGF-beta receptor decoy or polypeptide antagonist, IL-12, IL-15, and a
25 STING polypeptide,
a TGF-beta receptor decoy or polypeptide antagonist, IL-12, IL-15, and a
STING polypeptide,
an anti-CTLA-4 antibody, IL-12, IL-15, a TGF-beta receptor decoy or
polypeptide antagonist, and a STING polypeptide,
- 30 4-1BBL, IL-12, IL-21, a TGF-beta receptor decoy or polypeptide antagonist,
and a STING polypeptide,

an anti-CTLA-4 antibody, IL-12, IL-15, and a TGF-beta receptor decoy or polypeptide antagonist,

4-1BBL, IL-12, IL-21, and a TGF-beta receptor decoy or polypeptide antagonist,

5 IL-12, IL-15, and a STING polypeptide,

IL-15, IL-21, and a STING polypeptide,

IL-12, IL-21, and a STING polypeptide,

an anti-CTLA-4 antibody, IL-15, IL-21, and a STING polypeptide,

an anti-CTLA-4 antibody, IL-12, IL-21, and a STING polypeptide,

10 4-1BBL, IL-15, IL-21, and a STING polypeptide,

4-1BBL, IL-12, IL-21, and a STING polypeptide,

an anti-CTLA-4 antibody, and IL-15,

an anti-CTLA-4 antibody, IL-15, and a TGF-beta receptor decoy or polypeptide antagonist,

15 4-1BBL and IL-15,

4-1BBL, IL-15, and a TGF-beta receptor decoy or polypeptide antagonist,

an anti-CTLA-4 antibody, and IL-12,

an anti-CTLA-4 antibody, IL-12, and a TGF-beta receptor decoy or polypeptide antagonist,

20 4-1BBL, and IL-12,

4-1BBL, IL-12, and a TGF-beta receptor decoy or polypeptide antagonist,

an anti-CTLA-4 antibody, and a TGF-beta receptor decoy or polypeptide antagonist,

4-1BBL, and a TGF-beta receptor decoy or polypeptide antagonist,

25 IL-15, and a TGF-beta receptor decoy or polypeptide antagonist,

IL-12, and a TGF-beta receptor decoy or polypeptide antagonist,

IL-12, IL-15, and a TGF-beta receptor decoy or polypeptide antagonist, and

IL-15, IL-21, and a TGF-beta receptor decoy or polypeptide antagonist,

wherein:

30 4-1BBL is 4-1BBL with a deleted cytoplasmic domain, 4-1BBL with a modified cytoplasmic domain, 4-1BBL with a truncated cytoplasmic domain, or 4-1BBL with a truncated and modified cytoplasmic domain;

an anti-CTLA-4 antibody is an scFv or an scFv-Fc; and

a STING polypeptide is a wild-type STING, or a variant STING polypeptide, or a chimeric STING polypeptide, or a chimeric STING polypeptide with amino acid replacements.

- 5 13. The construct of any of claims 1-11 that encodes a combination of therapeutic products selected from among:
- IL-2 and IL-12p70;
- IL-2 and IL-21;
- IL-2, IL-12p70, and a STING gain-of-function (GOF) variant;
- 10 IL-2, IL-21, and a STING GOF variant;
- IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt), where Δ cyt is a deleted cytoplasmic domain;
- IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-15/IL-15R α , and a STING GOF variant;
- 15 IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-15/IL-15R α and IL-12p70;
- IL-15/IL-15R α and IL-21;
- IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- IL-15/IL-15R α , IL-21, and a STING GOF variant;
- 20 IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-12p70 and IL-21;
- 25 IL-12p70, IL-21, and a STING GOF variant;
- IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-12p70 and IL-18;
- 30 IL-12p70, IL-18, and a STING GOF variant;

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

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-2, and IL-12p70;

5 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-2, and IL-21;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-2, IL-12p70, and a STING GOF variant;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-2, IL-21, and a STING GOF variant;

10 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

15 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , and a STING GOF variant;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , and IL-12p70;

20 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , and IL-21;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;

25 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , IL-21, and a STING GOF variant;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

30 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, and IL-21;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, IL-21, and a STING GOF variant;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

5 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, and IL-12p70;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, and a STING GOF variant;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

10 a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, and IL-18;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, IL-18, and a STING GOF variant;

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, IL-12p70, IL-18, 15 a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

a TGF- β decoy receptor or a TGF- β polypeptide antagonist, 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;

20 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 4-1BBL Δ cyt);

an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL 25 (including 4-1BBL Δ cyt);

an anti-CTLA-4 antibody, IL-15/IL-15R α , and a STING GOF variant;

an anti-CTLA-4 antibody, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-12p70;

30 an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-21;

an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;

- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, a STING GOF variant,
- and 4-1BBL (including 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, a STING GOF variant, and
- 5 4-1BBL (including 4-1BBL Δ cyt);
- 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 4-1BBL Δ cyt);
- 10 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 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody, IL-12p70, and IL-18;
- 15 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 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody and a STING GOF variant;
- a CD40 agonist, IL-2, and IL-12p70;
- 20 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 4-1BBL Δ cyt);
- 25 a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-
- 1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and a STING GOF variant;
- a CD40 agonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL
- (including 4-1BBL Δ cyt);
- 30 a CD40 agonist, IL-15/IL-15R α , and IL-12p70;
- a CD40 agonist, IL-15/IL-15R α , and IL-21;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;

- a CD40 agonist, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL
- 5 (including 4-1BBL Δ cyt);
- 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 4-1BBL Δ cyt);
- 10 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 4-1BBL Δ cyt);
- a CD40 agonist, IL-12p70, and IL-18;
- 15 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 4-1BBL Δ cyt); and
- a CD40 agonist and a STING GOF variant, wherein:
 - 4-1BBL is 4-1BBL with a deleted cytoplasmic domain (4-1BBL Δ cyt),
 - 20 4-1BBL with a modified cytoplasmic domain, 4-1BBL with a truncated cytoplasmic domain, or 4-1BBL with a truncated and modified cytoplasmic domain; and
 - an anti-CTLA-4 antibody is an scFv or an scFv-Fc.
- 14. The construct of any of claims 1-13, wherein:
- 25 the STING polypeptide is modified to result in increased or constitutive expression of a type I interferon (IFN), or is a chimeric polypeptide comprising a human STING polypeptide with a C-terminal tail (CTT) from a different species that has lower NF- κ B signaling activity than the NF- κ B signaling activity of human STING, and wherein:
- 30 the TRAF6 binding site in the CTT optionally is deleted; and
- the human STING protein has the sequence set forth in any of SEQ ID NOs:305-309.

15. The construct of any of claims 1-14, wherein the encoded therapeutic proteins comprise IL-12p70, and a chimeric human STING polypeptide with a CTT from Tasmanian devil and an amino acid replacement that results in increased or constitutive expression of type I interferon, or is a STING polypeptide with an amino acid replacement that results in increased or constitutive expression of type I interferon, where a mutation that results in increased or constitutive expression of type I interferon is a gain-of-function (GOF) mutation.
16. The construct of claim 15, wherein the amino replacement in the STING polypeptide corresponds to R284G with reference, for alignment, to any of SEQ ID NOs: 305-309.
17. The construct of any of claims 11-16, wherein the STING polypeptide further comprises the replacement N154S.
18. The construct of any of claims 1-17, comprising nucleic acid encoding IL-36 γ .
19. The construct of any of claims 1-18 that encodes an immune checkpoint inhibitor antibody, or an antigen-binding portion thereof.
20. The construct of claim 19, wherein the immune checkpoint is CTLA-4, or PD-1, or PD-L1.
21. The construct of any of claims 1-20, wherein the encoded product is an antibody that is scFv, or that is an scFv-Fc two-chain polypeptide.
22. A plasmid, comprising the construct of any of claims 1-21.
23. The plasmid of claim 22 that is a bacterial plasmid, wherein the construct is operatively linked to eukaryotic transcriptional regulatory sequences.
24. The plasmid of claim 23, wherein the transcriptional regulatory sequences comprise a eukaryotic promoter.
25. A composition, comprising the mixture of anti-cancer protein products encoded by the construct or plasmid of any of claims 1-24.
26. An immunostimulatory bacterium, comprising the construct or plasmid of any of claims 1-24.
27. The immunostimulatory bacterium of claim 26, wherein the genome of the bacterium is modified so that the resulting bacterium is *msbB*⁻/*purF*⁻.

28. The immunostimulatory bacterium of claim 26 or claim 27, wherein the bacterium is *msbB*⁻ and *purI*⁻, whereby the full length of at least the coding portion of the *msbB*⁻ and *purI*⁻ genes is deleted.

29. The immunostimulatory bacterium of any of claims 26-28, wherein the
5 genome of the bacterium is modified, whereby the bacterium lacks flagella.

30. The immunostimulatory bacterium of any of claims 26-29 that comprises genome modifications, whereby the bacterium is *msbB*⁻/*pagP*⁻.

31. The immunostimulatory bacterium of any of claims 26-30 that comprises genome modifications, whereby the bacterium does not express L-
10 asparaginase II, so that the bacterium is *ansB*⁻.

32. An immunostimulatory bacterium, comprising a plasmid encoding a plurality of therapeutic products, wherein the genome of the bacterium is modified so that the bacterium is *msbB*⁻ and *purI*⁻, whereby the full length of at least the coding portion of the *msbB* and *purI* genes is deleted.

15 33. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, 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 does not activate the synthesis of secreted asparaginase.

20 34. The immunostimulatory bacterium of claim 33, wherein the asparaginase is L-asparaginase II, encoded by the gene *asnB*.

35. The immunostimulatory bacterium of claim 34 that has genome modifications, whereby the bacterium lacks flagella, and is *pagP*⁻, *ansB*⁻, and *csgD*⁻.

25 36. The immunostimulatory bacterium of claim 35 that is *Δasd/ΔFLG/ΔpagP/ΔansB/ΔcsgD*.

37. The immunostimulatory bacterium of any of claims 33-36 that has genome modifications, whereby the bacterium is *msbB*⁻.

38. The immunostimulatory bacterium of any of claims claim 33-37 that has genome modifications, whereby the bacterium is an adenosine auxotroph, or is an
30 adenosine and adenine auxotroph.

39. The immunostimulatory bacterium of any of claims claim 33-38 that has genome modifications, whereby the bacterium is *purI*⁻.

40. The immunostimulatory bacterium of any of claims 33-39, wherein the plasmid encodes aspartate-semialdehyde dehydrogenase (*asd*).

41. The immunostimulatory bacterium of any of claims 33-40 that is *purF*.

42. An immunostimulatory bacterium, comprising a plasmid encoding a
5 plurality of therapeutic products under control of a eukaryotic promoter, 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 (LPS);
hexa-acylated lipopolysaccharide is substantially reduced, by at least 10-fold,
10 compared to the wild-type bacterium, or is absent; and
the plasmid encodes a plurality of complementary anti-cancer therapeutic
products under control of a single promoter, wherein each of the complementary
therapeutic products treats a different aspect of a cancer, or acts via a different
mechanism, so that the combined effect is at least additive of the effect of each
15 product separately.

43. An immunostimulatory bacterium, comprising a plasmid encoding a
therapeutic product under control of a eukaryotic promoter, 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
20 has attenuated recognition by TLR2, TLR4, and TLR5; and
the plasmid encodes a plurality of complementary therapeutic products under
control of a single promoter.

44. An immunostimulatory bacterium, comprising a plasmid encoding a
therapeutic product under control of a eukaryotic promoter, wherein:
25 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
does not activate the synthesis of curli fimbriae and/or cellulose; and
the plasmid encodes a plurality of complementary therapeutic products under
control of a single promoter.

30 45. An immunostimulatory bacterium, comprising a plasmid encoding a
therapeutic product under control of a eukaryotic promoter, 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 is auxotrophic for purines, adenosine, or ATP, and is modified by deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium lacks

5 flagella; and

the plasmid encodes a plurality of therapeutic products under control of a single eukaryotic promoter.

46. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, wherein the genome of
10 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; and

the plasmid encodes a plurality of therapeutic products under control of a single promoter.

47. An immunostimulatory bacterium, comprising a plasmid encoding a
15 therapeutic product under control of a eukaryotic promoter, 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 specifically infect tumor-resident myeloid cells; and

the plasmid encodes a plurality of therapeutic products under control of a
20 single promoter.

48. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, 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
25 specifically infect tumor-resident myeloid cells, and is unable to replicate in tumor-resident myeloid cells.

49. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, wherein the genome of the immunostimulatory bacterium comprises two or more modifications selected from
30 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, wherein:

the genome of the immunostimulatory bacterium is modified by
5 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;

10 b) deletion or disruption of all or of a sufficient portion of a gene or genes, whereby the bacterium has attenuated recognition by one or more of TLR2, TLR4, and 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
15 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, adenosine, or ATP;

20 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;

25 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
30 therapeutic protein is increased in the tumor microenvironment and/or in tumor-resident immune cells.

50. The immunostimulatory bacterium of claim 49 comprising modifications a), d) and f), or comprising modifications c) and d).

51. The immunostimulatory bacterium of claim 49, comprising modifications a), c), d), e) and f).

5 52. The immunostimulatory bacterium of claim 49, comprising modifications a), c), d), e), f) and i), or comprising modifications a), d) f), and i), or comprising modifications c), d) and i), or comprising modifications f) and i), or comprising modifications a)-i).

53. The immunostimulatory bacterium of claim 49, comprising
10 modifications a), b), d) and f), or comprising modifications a), b), c) and d).

54. The immunostimulatory bacterium of any of claims 26-53, wherein *lppA* and *lppB* are deleted.

55. The immunostimulatory bacterium of any of claims 26-54 that lacks flagella and is *msbB*⁻/*pagP*⁻, wherein the plasmid encodes a combination of two or
15 more therapeutic proteins selected from among a co-stimulatory molecule, a cytokine, a STING pathway agonist, and an immune checkpoint inhibitor antibody.

56. 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 phenotype macrophage.

20 57. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product, wherein:

the immunostimulatory bacterium comprises modifications in its genome, whereby the bacterium infects tumor-resident macrophages, and does not infect epithelial cells; and

25 expression of the therapeutic product in a macrophage converts human M2 macrophages to an M1 or M1-like phenotype.

58. The immunostimulatory bacterium of any of claims 26-57, comprising a plasmid encoding a therapeutic product, where expression of the therapeutic product in a macrophage converts human M2 macrophages to an M1 or M1-like phenotype.

30 59. The immunostimulatory bacterium of any of claims 26-58 wherein the therapeutic product is part of a cytosolic DNA/RNA sensor pathway that leads to expression of type I interferon (IFN).

60. The immunostimulatory bacterium of claim 59, wherein the expression of the type I IFN is constitutive.

61. The immunostimulatory bacterium of claim 59 or claim 60, wherein the therapeutic product is a gain-of-function (GOF) variant of the therapeutic product
5 that is part of the cytosolic DNA/RNA sensor pathway, wherein the GOF variant product does not require cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides to result in expression of type I IFN.

62. The immunostimulatory bacterium of any of claims 26-61, wherein the therapeutic product is a variant STING protein.

10 63. The immunostimulatory bacterium of any of claims 26-62, wherein infection by the bacterium converts human M2 macrophages into M1-like, type I IFN producing cells.

64. The immunostimulatory bacterium of any of claims 26-63 that lacks flagella, wherein the wild-type bacterium has flagella, and the immunostimulatory
15 bacterium is *pagP*⁻/*msbB*⁻.

65. The immunostimulatory bacterium of any of claims 26-64, wherein the immunostimulatory bacterium contains genome modifications, whereby the bacterium lacks flagella and does not produce L-asparaginase II (*ansB*).

66. The immunostimulatory bacterium of claim 65, wherein the
20 immunostimulatory bacterium also has genome modifications whereby the bacterium is *msbB*⁻ and *pagP*⁻.

67. The immunostimulatory bacterium of any of claims 26-66, wherein immunostimulatory bacterium has modifications of the genome, whereby the bacterial phenotype conferred by the genome is $\Delta asd/\Delta FLG/\Delta pagP/\Delta ansB/\Delta csgD$ or
25 $\Delta asd/\Delta FLG/\Delta pagP/\Delta ansB/\Delta csgD/\Delta msbB/\Delta purI$, wherein the plasmid optionally encodes aspartate-semialdehyde dehydrogenase (*asd*).

68. The immunostimulatory bacterium of any of claims 26-67, comprising a plasmid that encodes a STING polypeptide.

69. The immunostimulatory bacterium of claim 68, wherein the STING
30 polypeptide is a variant STING polypeptide that results in increased or constitutive expression of type I interferon.

70. The immunostimulatory bacterium of claim 69, wherein the STING polypeptide is a human chimeric STING polypeptide that comprises a C-terminal tail (CTT) from Tasmanian devil STING.

71. The immunostimulatory bacterium of claim 69 or claim 70, wherein
5 the STING polypeptide comprises the replacement corresponding to R284, with reference, for alignment, to any of SEQ ID NOs:305-309.

72. The immunostimulatory bacterium of claim 71, further comprising a replacement corresponding to N154S, with reference to any of SEQ ID NOs:305-309.

73. The immunostimulatory bacterium of any of claims 26-72, comprising
10 a plasmid encoding one or more of IL-12, IL-15, and IL-21.

74. The immunostimulatory bacterium of any of claims 26-73 that is a *Salmonella* strain.

75. The immunostimulatory bacterium of any of claims 26-74, wherein the therapeutic product is an anti-cancer therapeutic.

15 76. The immunostimulatory bacterium of any of claims 26-75, wherein the bacterium encodes a therapeutic product that is an immunostimulatory protein.

77. The immunostimulatory bacterium of claim 76, 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
20 ligand.

78. The immunostimulatory bacterium of any of claims 26-77, wherein the bacterium comprises a genomic modification whereby it lacks flagella.

79. The immunostimulatory bacterium of any of claims 26-78, wherein the bacterium comprises a genomic modification whereby it is *pagP*⁻ or *msbB*/*pagP*⁻.

25 80. The immunostimulatory bacterium of any of claims 26-79, wherein: the bacterium comprises genomic modifications 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.
30

81. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, wherein the genome of

the immunostimulatory bacterium is modified by deletion or disruption of all or of a sufficient portion of *csgD*, whereby the bacterium is *csgD*⁻, and does not activate the synthesis of curli fimbriae.

82. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, 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.

83. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, 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.

84. The immunostimulatory bacterium of any of claims 26-83, further comprising deletion of or disruption of all or of a sufficient portion of the genes encoding the flagella, whereby the bacterium is flagellin⁻ (*fliC*⁻/*fljB*⁻) and does not produce flagella, wherein the wild-type bacterium has flagella.

85. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, wherein the genome of the immunostimulatory bacterium is modified by deletion of or disruption of all or of a sufficient portion of the genes *lppA* and *lppB*, encoding the bacterial lipoprotein, whereby the lipoprotein is not produced, and wherein the therapeutic product encoded on the plasmid is an anti-cancer therapeutic.

86. The immunostimulatory bacterium of claim 85, 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.

87. The immunostimulatory bacterium of any of claims 82-86, 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*⁻.

88. The immunostimulatory bacterium of any of claims 82, 83, and 85-87, wherein the bacterium comprises modifications of the genome, whereby the bacterium lacks flagella.

89. An immunostimulatory bacterium, comprising genome modifications
5 whereby the bacterium lacks flagella, and is *lppA*⁻/*lppB*⁻, and optionally is *csgD*⁻, or lacks flagella, is *ansB*⁻, and optionally is *csgD*⁻.

90. The immunostimulatory bacterium of any of claims 26-89, wherein the bacterium is auxotrophic for purines.

91. The immunostimulatory bacterium of any of claims 26-89, wherein the
10 bacterium is auxotrophic for adenosine.

92. The immunostimulatory bacterium of any of claims 26-89 that is auxotrophic for adenosine, adenine, and ATP.

93. The immunostimulatory bacterium of any of claims 26-92, wherein the bacterium is *purI*⁻.

94. The immunostimulatory bacterium of any of claims 26-93, wherein the
15 bacterium is *pagP*⁻.

95. The immunostimulatory bacterium of any of claims 26-94, wherein the bacterium is *asd*⁻.

96. The immunostimulatory bacterium of any of claims 26-95 that is
20 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.

97. The immunostimulatory bacterium of any of claims 26-96 that encodes
25 aspartate-semialdehyde dehydrogenase (*asd*) on the plasmid under control of a bacterial promoter.

98. The immunostimulatory bacterium of any of claims 26-97, wherein the bacterium is *msbB*⁻.

99. The immunostimulatory bacterium of any of claims 26-98 that
30 comprises genome modifications, whereby the bacterium is *asd*⁻, *purI*⁻, *msbB*⁻, flagellin⁻, and *pagP*⁻, wherein the wild-type bacterium has flagella.

100. The immunostimulatory bacterium of any of claims 26-98 that is *asd⁻*, *csgD⁻*, *purI⁻*, *msbB⁻*, flagellin⁻ and *pagP⁻*, wherein the wild-type bacterium has flagella.

101. The immunostimulatory bacterium of any of claims 26-98 that is *ansB⁻*, *asd⁻*, *csgD⁻*, *purI⁻*, *msbB⁻*, flagellin⁻ (*fliC⁻/fljB⁻*), and *pagP⁻*, wherein the wild-type bacterium has flagella.

102. The immunostimulatory bacterium of any of claims 96-100, wherein the bacterium is flagellin⁻ (*fliC⁻/fljB⁻*), wherein the wild-type bacterium has flagella.

103. The immunostimulatory bacterium of any of claims 26-102, wherein the genome of the bacterium is modified by deletion or disruption of all or of a sufficient portion of the gene *lppA* and/or *lppB*, whereby the bacterium is *lppA⁻* and/or *lppB⁻*, whereby expression in the tumor microenvironment and/or tumor-resident macrophages of the therapeutic protein encoded on the plasmid is increased, compared to the same immunostimulatory bacterium except with intact *lppA* and/or *lppB*.

104. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, 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 is *ansB⁻*, *asd⁻*, *csgD⁻*, *purI⁻*, *msbB⁻*, flagellin⁻, and *pagP⁻*, wherein the wild-type bacterium has flagella.

105. An immunostimulatory bacterium, comprising a plasmid encoding a therapeutic product under control of a eukaryotic promoter, 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 is *ansB⁻*, *asd⁻*, *csgD⁻*, *purI⁻*, *msbB⁻*, flagellin⁻, wherein the wild-type bacterium has flagella, and *pagP⁻*.

106. The immunostimulatory bacterium of claim 104 or claim 105, wherein the bacterium is flagellin⁻ (*fliC⁻/fljB⁻*).

107. The immunostimulatory bacterium of claim 105 or 106, wherein the bacterial genome comprises further modifications, whereby the bacterium is *ansB⁻*, *asd⁻*, *csgD⁻*, *purI⁻*, *msbB⁻*, flagellin⁻ (*fliC⁻/fljB⁻*), *pagP⁻*, *lppA⁻*, and *lppB⁻*.

108. The immunostimulatory bacterium of any of claims 26-107, wherein the therapeutic product is an anti-cancer therapeutic.

109. The immunostimulatory bacterium of any of claims 26-108, 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.

5 110. The immunostimulatory bacterium of any of claims 26-109, wherein one or more genes or operons involved in SPI-1 invasion are deleted or inactivated, whereby the immunostimulatory bacterium does not invade or infect epithelial cells.

 111. The immunostimulatory bacterium of claim 110, wherein one or more of *avrA*, *hilA*, *hilD*, *invA*, *invB*, *invC*, *invE*, *invF*, *invG*, *invH*, *invI*, *invJ*, *iapC*, *iagB*,
10 *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* is deleted or inactivated.

 112. The immunostimulatory bacterium of any of claims 26-111, wherein the plasmid is present in low copy number, or in medium copy number.

15 113. The immunostimulatory bacterium of any of claims 26-111, wherein the plasmid comprises a medium-to-low copy number origin of replication.

 114. The immunostimulatory bacterium of any of claims 26-111, wherein the plasmid comprises a low copy number origin of replication.

 115. The immunostimulatory bacterium of any of claims 26-111 and 114,
20 wherein the plasmid is present in low copy number.

 116. The immunostimulatory bacterium of any of claims 26-111, wherein the plasmid is present in a copy number of 150 or less.

 117. The immunostimulatory bacterium of any of claims 26-111, where in the plasmid is present in a copy number greater than 150.

25 118. The immunostimulatory bacterium of any of claims 26-111, wherein the plasmid is present in high copy number.

 119. The immunostimulatory bacterium of any of claims 26-111, wherein the plasmid is present in medium copy number, which is between 20 and 150, inclusive, is less than 150 or less than about 150 and more than 20 or about 20, or is
30 between 20 and 150 copies.

 120. The immunostimulatory bacterium of any of claims 26-111, wherein the number of copies of the plasmid is greater than 150.

121. The immunostimulatory bacterium of claim 116, wherein the number of copies of the plasmid is 150 copies or fewer, or is less than or equal to 150.

122. The immunostimulatory bacterium of any of claims 26-115, wherein the plasmid is present in low copy number, and low copy number is less than 25, or
5 less than 20, or less than about 25, or less than about 20 copies.

123. The immunostimulatory bacterium of any of claims 26-122, wherein the therapeutic product is a nucleic acid or a protein.

124. The immunostimulatory bacterium of claim 123, wherein the therapeutic product is a protein.

10 125. The immunostimulatory bacterium of any of claims 26-124, wherein the plasmid encodes two or more therapeutic products.

126. The immunostimulatory bacterium of any of claims 26-125, wherein the encoded therapeutic product(s) on the plasmid is/are anti-cancer treatment(s).

127. The immunostimulatory bacterium of any of claims 26-126, wherein
15 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.

128. The immunostimulatory bacterium of claim 127, wherein the co-stimulatory molecule lacks a cytoplasmic domain, or has a modified truncated
20 cytoplasmic domain to ensure that the encoded co-stimulatory molecule is expressed in the correct orientation in a cell, or has a truncated cytoplasmic domain whereby immunosuppressive reverse signaling is eliminated or reduced.

129. The immunostimulatory bacterium of any of claims 26-128, wherein the nucleic acid encoding one or more of the therapeutic product or products
25 comprises nucleic acid encoding a signal for secretion of the therapeutic product(s) from a cell comprising the bacterium or the plasmid.

130. The immunostimulatory bacterium of any of claims 26-129, wherein the nucleic acid encoding the product on the plasmid is operatively linked to regulatory sequences recognized by a eukaryotic host.

30 131. The immunostimulatory bacterium of any of claims 26-130, 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.

132. The immunostimulatory bacterium of claim 131, wherein the nucleic acid encodes a T2A, F2A, E2A, or P2A peptide to effect separate expression of
5 therapeutic products expressed under control of a single promoter.

133. The immunostimulatory bacterium of any of claims 26-132, wherein the eukaryotic promoter is an RNA polymerase II promoter, or an RNA polymerase III promoter.

134. The immunostimulatory bacterium of claim 133, wherein the promoter
10 is an RNA polymerase II promoter that is a viral promoter or a mammalian RNA polymerase II promoter.

135. The immunostimulatory bacterium of claim 134, 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
15 an adenovirus promoter.

136. The immunostimulatory bacterium of any of claims 26-134, 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
20 CAG promoter.

137. The immunostimulatory bacterium of any of claims 26-134, 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.

25 138. The immunostimulatory bacterium of any of claims 26-134, 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.

139. The immunostimulatory bacterium of any of claims 26-138, wherein
30 the plasmid comprises regulatory sequences that comprise a terminator and/or promoters selected from among SV40, hGH, BGH, MND, chicken beta-globulin, and rbGlob (rabbit globulin) genes, to control expression of the therapeutic product(s).

140. The immunostimulatory bacterium of any of claims 26-139, wherein the encoded therapeutic product(s) is/are operatively linked to a signal sequence for secretion from a cell containing the plasmid.

141. The immunostimulatory bacterium of any of claims 26-140, wherein
5 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.

142. The immunostimulatory bacterium of any of claims 26-141, wherein
10 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.

143. The immunostimulatory bacterium of any of claims 26-142, wherein
15 the plasmid comprises a construct that includes an enhancer, a promoter, an IRES, a localization sequence, the open reading frame encoding the therapeutic product, and a polyA tail.

144. The immunostimulatory bacterium of any of claims 26-143, wherein the plasmid comprises a construct that includes a bacterial terminator positioned to decrease read-through from a bacterial promoter on the plasmid.

145. The immunostimulatory bacterium of any of claims 26-144, wherein
20 the eukaryotic promoter on the plasmid is oriented in the opposite direction from a bacterial promoter on the plasmid.

146. The immunostimulatory bacterium of claim 145, wherein the bacterial promoter controls expression of the *asd* gene.

147. The immunostimulatory bacterium of any of claims 26-146 wherein
25 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).

148. The immunostimulatory bacterium of any of claims 26-147, wherein
30 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 is a variant thereof.

149. The immunostimulatory bacterium of claim 148, 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
5 IFN in the absence of the sensing or interacting with the cytosolic nucleic acids, nucleotides, dinucleotides, or cyclic dinucleotides.

150. The immunostimulatory bacterium of claim 148, wherein the therapeutic product is a variant that, when expressed in a subject, leads to constitutive expression of type I IFN.

10 151. The immunostimulatory bacterium of claim 148, 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.

152. The immunostimulatory bacterium of any of claims 26-151, wherein
15 the therapeutic product is selected from among STING, RIG-I, MDA-5, IRF-3, IRF-7, TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200, and variants thereof that increase activity or increase activity so that type I interferon activity is increased or constitutive.

20 153. The immunostimulatory bacterium of any of claims 26-152, 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, and variants thereof that increase activity or increase activity so that type I interferon activity is increased or constitutive.

25 154. The immunostimulatory bacterium of any of claims 26-153, wherein the therapeutic product is a variant protein that has increased activity that results in increased expression of type I interferon (IFN), or results in constitutive expression of type I IFN.

30 155. The immunostimulatory bacterium of any of claims 26-154, wherein the plasmid encodes a gain-of-function, constitutively active variant of a protein that, in humans, promotes or causes interferonopathies.

156. The immunostimulatory bacterium of any of claims 62-80 and 148-155, wherein the therapeutic product is a variant that comprises a mutation that eliminates a phosphorylation site in a STING protein to thereby reduce nuclear factor kappa-light-chain-enhancer of activated B-cell (NF- κ B) signaling.

5 157. The immunostimulatory bacterium of any of claims 148-156, wherein the therapeutic product that induces type I IFN is STING, RIG-I, IRF-3, or MDA5, or a variant thereof.

158. The immunostimulatory bacterium of any of claims 148-157, wherein:
the therapeutic product that induces expression of type I IFN is a variant
10 thereof that has increased activity or constitutive activity; and
the therapeutic product is STING, RIG-I, IRF-3, or MDA5, or a variant thereof.

159. The immunostimulatory bacterium of any of claims 148-158, wherein the therapeutic product is a variant of STING, RIG-I, IRF-3, or MDA5 that comprises
15 a gain-of-function mutation resulting in increased expression of type I IFN.

160. The immunostimulatory bacterium of any of claims 148-159, wherein the therapeutic product is 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), whereby the
20 resulting variant is a phosphomimetic that constitutively induces type I IFN.

161. The immunostimulatory bacterium of any of claims 148-160, 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, for alignment to
SEQ ID NO:312; and
25 the residues are replaced with aspartic acid residues.

162. The immunostimulatory bacterium of claim 161, wherein IRF-3 comprises the replacement S396D with reference, for alignment, to SEQ ID NO:312.

163. The immunostimulatory bacterium of claim 161, wherein IRF-3 comprises the replacements S396D/S398D/S402D/T404D/S405D with reference, for
30 alignment, to SEQ ID NO:312.

164. The immunostimulatory bacterium of any of claims 148-163, wherein the therapeutic product is selected from among STING, RIG-I, MDA-5, IRF-3, IRF-7,

TRIM56, RIP1, Sec5, TRAF3, TRAF2, TRAF6, STAT1, LGP2, DDX3, DHX9, DDX1, DDX9, DDX21, DHX15, DHX33, DHX36, DDX60, and SNRNP200, and variants thereof that increase activity or increase activity so that type I interferon activity is increased or constitutive.

5 165. The immunostimulatory bacterium of any of claims 148-164, 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
10 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.

166. The immunostimulatory bacterium of any of claims 148-165, 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,
15 or RIG-I constitutively active, whereby expression of type I IFN is constitutive.

167. The immunostimulatory bacterium of claim 165 or claim 166, wherein
the mutations are selected as follows:

a) in STING, with reference, for alignment, to SEQ ID NOs: 305-309, one or
more selected from among: S102P, V147L, V147M, N154S, V155M, G166E,
20 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,
25 N154S/R284G, and S324A/S326A;

b) in MDA5, with reference, for alignment, 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, for alignment, to SEQ ID NO:311, one or both of
30 E373A and C268F; and

d) in IRF-3, with reference, for alignment, to SEQ ID NO:312, S396D.

168. The immunostimulatory bacterium of any of claims 148-167, wherein the therapeutic product is a variant STING that contains one or more amino replacement(s) selected, with reference, for alignment, to any of SEQ ID NOs: 305-309, from among: S102P, V147L, V147M, N154S, V155M, G166E, C206Y, G207E,
5 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, S324A/S326A, and N154S/R284G, and
10 conservative replacements thereof.

169. The immunostimulatory bacterium of any of claims 26-168, wherein: the therapeutic protein increases or induces expression of a type I IFN; and the type I IFN is an interferon- α or interferon- β .

170. The immunostimulatory bacterium of any of claims 26-169, wherein
15 one or more genes or operons involved in SPI-1 invasion or SPI-1 independent invasion are deleted, disrupted, or inactivated, whereby the immunostimulatory bacterium does not invade or infect epithelial cells, or has a reduced ability to invade or infect epithelial cells.

171. The immunostimulatory bacterium of claim 170, wherein one or more
20 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* is deleted, disrupted, or inactivated.

172. The immunostimulatory bacterium of claim 171, wherein the one or
25 more genes is/are selected from among *pagN*, *hlyE*, *pefI*, *srgD*, *srgA*, *srgB*, and *srgC*.

173. The immunostimulatory bacterium of any of claims 26-172 that has a deletion or disruption of a gene encoding a protein in the SPI-2 complex.

174. The immunostimulatory bacterium of any of claims 26-173, wherein the plasmid encodes an immunostimulatory protein that confers or contributes to an
30 anti-tumor immune response in the tumor microenvironment.

175. The immunostimulatory bacterium of claim 174, wherein the immunostimulatory protein that confers or contributes to an anti-tumor immune

response in the tumor microenvironment 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-18, IL-21, IL-23, IL-36 γ , IL-2 modified so that it does not bind to IL-2Ra, CXCL9, CXCL10, CXCL11, interferon- α ,
5 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), 4-1BBL that has a deleted or truncated or otherwise modified cytoplasmic domain to eliminate the immunosuppressive reverse signaling, members of the B7-CD28 family,
10 CD47 antagonists, an anti-IL6 antibody or IL-6 binding decoy receptor, TGF-beta polypeptide antagonists, and members of the tumor necrosis factor receptor (TNFR) superfamily.

176. The immunostimulatory bacterium of claim 174 or claim 175, wherein the plasmid encodes a modified 4-1BBL, whose sequence is set forth in SEQ ID
15 NO:390, SEQ ID NO:391, or SEQ ID NO:392.

177. The immunostimulatory bacterium of claim 175 or claim 176, further comprising a tag linked to the co-stimulatory protein to facilitate purification or expression thereof.

178. The immunostimulatory bacterium of claim 177, wherein the tag is a c-
20 myc tag comprising the sequence MEQKLISEEDL, set forth as residues 1-11 of SEQ ID NO:392.

179. The immunostimulatory bacterium of any of claims 174-178, wherein the plasmid encodes a 4-1BBL polypeptide and at least one additional therapeutic protein.

25 180. The immunostimulatory bacterium of claim 179, wherein the additional therapeutic protein(s) is/are IL-15R α -IL-15sc, or IL-12, or IL-15R α -IL-15sc and IL-12.

181. The immunostimulatory bacterium of any of claims 174-180, comprising a 4-1BBL polypeptide and one or more additional therapeutic
30 polypeptides encoded in a polycistronic nucleic acid under control of a single promoter, wherein the 4-1BBL polypeptide is the first polypeptide in the polycistron.

182. The immunostimulatory bacterium of any of claims 174-181, 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 in the cytoplasmic domain or that lacks a cytoplasmic domain, for expression on an antigen-presenting cell
5 (APC), to eliminate the immunosuppressive reverse signaling; 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 truncated or deleted cytoplasmic domain.

10 183. The immunostimulatory bacterium of any of claims 26-182, wherein the encoded product comprises one or more of a STING protein, a modified STING protein, a chimeric STING protein, an anti-CTLA-4 antibody, IL-15, 4-1BBL and modified or truncated forms thereof, a TGF-beta receptor decoy or polypeptide antagonist, IL-12, and IL-21, and/or any of the following:

15 one or more of IL-12, or IL-15, or IL12p70, or IL-15/IL-15R alpha chain complex;

a cytokine, and a STING pathway agonist;

a cytokine, a STING pathway agonist, and either a co-stimulatory molecule or an immune checkpoint inhibitor;

20 a cytokine, a STING pathway agonist, and a TGF-beta receptor decoy or polypeptide antagonist; and/or

a cytokine, a STING pathway agonist, a TGF-beta receptor decoy or polypeptide antagonist, and either a co-stimulatory molecule or an immune checkpoint inhibitor.

25 184. The immunostimulatory bacterium of any of claims 26-183, wherein the plasmid includes nucleic acid encoding a product that induces tumor cell apoptosis or is cytotoxic to tumor cells.

185. The immunostimulatory bacterium of claim 184, wherein:

the product is a nucleic acid; and

30 the nucleic acid encoding the product includes nucleic acid encoding a secretion signal whereby the product is secreted.

186. The immunostimulatory bacterium of claim 183 or claim 184, wherein the product induces apoptosis.

187. The immunostimulatory bacterium of claim 186, wherein the product that induces apoptosis is azurin.

5 188. The immunostimulatory bacterium of any of claims 174-187, wherein the plasmid encodes an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment, and the immunostimulatory protein is a cytokine or a chemokine.

189. The immunostimulatory bacterium of any of claims 174-188, wherein
10 the plasmid encodes an immunostimulatory protein that confers or contributes to an anti-tumor immune response in the tumor microenvironment that is a co-stimulatory molecule or cytoplasmic domain-deleted or truncated or otherwise modified form thereof.

190. The immunostimulatory bacterium of claim 189, wherein the
15 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, B7RP1, and OX40L, and cytoplasmic domain deleted or truncated or truncated and modified forms thereof, where the modifications promote correct orientation when the protein is expressed in a cell, and the cytoplasmic deletions,
20 truncations and/or modifications eliminate or reduce immunosuppressive reverse signaling.

191. The immunostimulatory bacterium of any of claims 26-190, wherein the plasmid encodes a therapeutic product that is a TGF-beta polypeptide antagonist.

192. The immunostimulatory bacterium of claim 191, wherein the TGF-beta
25 polypeptide antagonist is selected from among 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, and a TGF-beta binding decoy receptor.

193. The immunostimulatory bacterium of claim 191 or claim 192, wherein
30 nucleic acid encoding the TGF-beta polypeptide antagonist comprises nucleic acid encoding a signal sequence for secretion of the encoded polypeptide.

194. The immunostimulatory bacterium of any of claims 26-193, wherein the therapeutic product is an antibody or antigen-binding fragment thereof.

195. The immunostimulatory bacterium of claim 194, 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, a single-chain antibody, and an scFv-Fc two-chain antibody.

5 196. The immunostimulatory bacterium of claim 195, wherein the antibody is an scFV.

197. The immunostimulatory bacterium of claim 195 or claim 196, wherein the antibody comprises an Fc, whereby the resulting antibody comprises two chains.

10 198. The immunostimulatory bacterium of any of claims 195-197, wherein the antibody is encoded by nucleic acid comprising nucleic acid encoding the variable light chain, a linker, the variable heavy chain, and an IgG Fc, whereby the encoded antibody is an scFv-Fc.

199. The immunostimulatory bacterium of any of claims 195-198, wherein the antibody is an anti-CTLA-4 antibody.

15 200. The immunostimulatory bacterium of any of claims 194-199, wherein the antibody or antigen-binding fragment thereof is humanized or is human.

201. The immunostimulatory bacterium of any of claims 194-200, wherein the antibody or antigen-binding fragment thereof is an antagonist of PD-1, PD-L1, CTLA-4, VEGF, VEGFR2, or IL-6.

20 202. The immunostimulatory bacterium of any of claims 26-201, 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;

25 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.

30 203. The immunostimulatory bacterium of claim 202, 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).

204. An immunostimulatory bacterium, comprising a plasmid that encodes two or more therapeutic products under control of a single promoter, wherein:

5 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
10 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
15 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.

205. The immunostimulatory bacterium of claim 204, wherein the immunostimulatory protein is a co-stimulatory molecule that lacks a cytoplasmic
20 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).

206. The immunostimulatory bacterium of any of claims 26-205, wherein
25 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.

207. The immunostimulatory bacterium of any of claims 26-206, wherein:
30 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 peptides, whereby, upon translation, each product is separately expressed.

208. The immunostimulatory bacterium of any of claims 202-207, wherein nucleic acid encoding one or more of the therapeutic products is operatively linked to
5 nucleic acid encoding a sequence that directs secretion of the expressed product(s).

209. The immunostimulatory bacterium of any of claims 26-208, wherein:
the therapeutic product is a co-stimulatory molecule with a cytoplasmic domain deletion or truncation or other modification for expression on an antigen-presenting cell (APC); and
10 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, truncation or other modification.

210. The immunostimulatory bacterium of claim 209, wherein the co-stimulatory molecule with the deleted, truncated or otherwise modified cytoplasmic domain is 4-1BBL, CD80, CD86, CD27L, B7RP1, or OX40L, and variants that
15 increase correct orientation of the protein when expressed in a cells.

211. The immunostimulatory bacterium of any of claims 26-210 that encodes two or more therapeutic products, wherein at least one product is selected
20 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-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
25 effect or potentiate recruitment/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,
30 DHX15, DHX33, DHX36, DDX60, or SNRNP200.

212. The immunostimulatory bacterium of any of claims 26-211 that encodes or further encodes one or more of a TGF-beta inhibitory antibody or antigen-binding fragment thereof, a TGF-beta binding decoy receptor, an anti-IL6 antibody or antigen-binding fragment thereof, and an IL-6 binding decoy receptor.

5 213. The immunostimulatory bacterium of any of claims 26-212 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 gain-of-function (GOF) variant;

10 IL-2, IL-21, and a STING GOF variant;

IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt), where Δ cyt is a deleted cytoplasmic domain;

IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

IL-15/IL-15R α , and a STING GOF variant;

15 IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

IL-15/IL-15R α and IL-12p70;

IL-15/IL-15R α and IL-21;

IL-15/IL-15R α , IL-12p70, and a STING GOF variant;

IL-15/IL-15R α , IL-21, and a STING GOF variant;

20 IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

IL-12p70 and IL-21;

25 IL-12p70, IL-21, and a STING GOF variant;

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

IL-12p70 and a STING GOF variant;

IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

30 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 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-2, and IL-12p70;
- a TGF- β decoy receptor or polypeptide antagonist, IL-2, and IL-21;
- 5 a TGF- β decoy receptor or polypeptide antagonist, IL-2, IL-12p70, and a STING GOF variant;
- a TGF- β decoy receptor or polypeptide antagonist, IL-2, IL-21, and a STING GOF variant;
- a TGF- β decoy receptor or polypeptide antagonist, IL-2, IL-12p70, a STING
- 10 GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , and a STING GOF variant;
- 15 a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , and IL-12p70;
- a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , and IL-21;
- 20 a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- 25 a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, and IL-21;
- 30 a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, IL-21, and a STING GOF variant;

- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, and IL-12p70;
- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, and a STING
- 5 GOF variant;
- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, and IL-18;
- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, IL-18, and a
- 10 STING GOF variant;
- a TGF- β decoy receptor or polypeptide antagonist, IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor or polypeptide antagonist, and a STING GOF variant;
- an anti-CTLA-4 antibody, IL-2, and IL-12p70;
- 15 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 4-1BBL Δ cyt);
- 20 an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody, IL-15/IL-15R α , and a STING GOF variant;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 25 an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-12p70;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-21;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- 30 an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody, IL-12p70, and IL-21;
- an anti-CTLA-4 antibody, IL-12p70, IL-21, and a STING GOF variant;
- 5 an anti-CTLA-4 antibody, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 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
- 10 (including 4-1BBL Δ cyt);
- 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 4-1BBL Δ cyt);
- 15 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;
- 20 a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and a STING GOF variant;
- 25 a CD40 agonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and IL-12p70;
- a CD40 agonist, IL-15/IL-15R α , and IL-21;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- 30 a CD40 agonist, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

- a CD40 agonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-12p70, and IL-21;
- a CD40 agonist, IL-12p70, IL-21, and a STING GOF variant;
- 5 a CD40 agonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 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 4-1BBL Δ cyt);
- 10 1BBL Δ cyt);
- 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 4-1BBL Δ cyt); and
- 15 a CD40 agonist and a STING GOF variant, wherein:
- 4-1BBL is 4-1BBL with a deleted cytoplasmic domain, 4-1BBL with a modified cytoplasmic domain, 4-1BBL with a truncated cytoplasmic domain, or 4-1BBL with a truncated and modified cytoplasmic domain; and
- an anti-CTLA-4 antibody is an scFv or an scFv-Fc; and
- 20 214. The immunostimulatory bacterium of any of claims 26-213, the comprises nucleic acid encoding a combination of therapeutic products selected from among the following combinations:
- an anti-CTLA-4 antibody and STING,
- IL-15 and STING,
- 25 4-1BBL and STING,
- TGF-beta decoy receptor or polypeptide antagonist, and STING,
- IL-12 and STING,
- an anti-CTLA-4 antibody, IL-15, and STING,
- 4-1BBL, IL-15, and STING,
- 30 TGF-beta decoy receptor or polypeptide antagonist, and IL-15, and STING,
- an anti-CTLA-4 antibody, and IL-12, and STING,
- 4-1BBL, IL-12, and STING,

TGF-beta decoy receptor or antagonist polypeptide, IL-12, and STING,
an anti-CTLA-4 antibody, IL-15, a TGF-beta decoy receptor or polypeptide
antagonist, and STING,
4-1BBL, and IL-15, a TGF-beta decoy receptor or polypeptide antagonist, and
5 STING,
an anti-CTLA-4 antibody, and IL-12, and a TGF-beta decoy receptor or
polypeptide antagonist, and STING,
4-1BBL, and IL-12, a TGF-beta decoy receptor or polypeptide antagonist, and
STING,
10 an anti-CTLA-4 antibody, IL-12, IL-15, and STING,
4-1BBL, IL-12, IL-15, and STING,
a TGF-beta decoy receptor or polypeptide antagonist, IL-12, IL-15, and
STING,
a TGF-beta decoy receptor or polypeptide antagonist, IL-12, IL-15, and
15 STING,
an anti-CTLA-4 antibody, IL-12, IL-15, a TGF-beta decoy receptor or
polypeptide antagonist, and STING,
4-1BBL, IL-12, IL-21, a TGF-beta decoy receptor or polypeptide antagonist,
and STING,
20 an anti-CTLA-4 antibody, IL-12, IL-15, and a TGF-beta decoy receptor or
polypeptide antagonist,
4-1BBL, IL-12, IL-21, and a TGF-beta decoy receptor or polypeptide
antagonist,
IL-12, IL-15, and STING,
25 IL-15, IL-21, and STING,
IL-12, IL-21, and STING,
an anti-CTLA-4 antibody, IL-15, IL-21, and STING,
an anti-CTLA-4 antibody, IL-12, IL-21, and STING,
4-1BBL, IL-15, IL-21, and STING,
30 4-1BBL, IL-12, IL-21, and STING,
an anti-CTLA-4 antibody, and IL-15,

- an anti-CTLA-4 antibody, IL-15, and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL and IL-15,
- 4-1BBL, IL-15, and TGF-beta decoy receptor or polypeptide antagonist,
- 5 an anti-CTLA-4 antibody and IL-12,
- an anti-CTLA-4 antibody, and IL-12, and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL and IL-12,
- 4-1BBL, IL-12, and a TGF-beta decoy receptor or polypeptide antagonist,
- 10 an anti-CTLA-4 antibody, and a TGF-beta decoy receptor or polypeptide antagonist,
- 4-1BBL and a TGF-beta decoy receptor or polypeptide antagonist,
- IL-15 and a TGF-beta decoy receptor or polypeptide antagonist,
- IL-12 and a TGF-beta decoy receptor or polypeptide antagonist,
- 15 IL-12, IL-15, and a TGF-beta decoy receptor or polypeptide antagonist, and
- IL-15, and IL-21, and a TGF-beta decoy receptor or polypeptide antagonist,
- wherein:
- IL-15 is IL-15 or IL-15/IL-15R alpha chain complex;
- STING is a STING polypeptide, or a variant STING polypeptide, or a
- 20 chimeric STING polypeptide or a chimeric STING with amino acid replacements;
- TGF-beta is a soluble TGF-beta decoy or TGF-beta antagonist;
- 4-1BBL is 4-1BBL with a deleted cytoplasmic domain, 4-1BBL with a modified cytoplasmic domain, 4-1BBL with a truncated cytoplasmic domain,
- 25 or 4-1BBL with a truncated and modified cytoplasmic domain; and
- an anti-CTLA-4 antibody is an scFv or an scFv-Fc.
215. The immunostimulatory bacterium of claim 214, wherein the TGF-beta decoy comprises an Fc fusion or is an anti-TGF antibody or an antigen-binding fragment thereof.
- 30 216. The immunostimulatory bacterium of claim 214 or claim 215, wherein the 4-1BBL is full-length, or is full-length with amino acid replacements at Ser5, and/or Ser8, whereby immunosuppressive reverse signaling is reduced or eliminated,

or is 1-4BBL with a deleted cytoplasmic domain, or a truncated cytoplasmic domain that eliminates or reduces immunosuppressive reverse signaling, or is 4-1BBL that has amino acid replacements in the truncated cytoplasmic domain, whereby the 4-1BBL is in the correct orientation when expressed in a cell.

5 217. The immunostimulatory bacterium of any of claims 214-215, wherein the STING polypeptide comprises the replacement R284G, or comprises the replacements N154S/R284, or is a chimeric STING polypeptide that is a chimeric human STING polypeptide with a CTT from Tasmanian Devil, or is a chimeric human STING polypeptide with a CTT from Tasmanian Devil and the replacement
10 corresponding to R284G or the replacements corresponding to N154S/R284G, all with reference, for alignment, to SEQ ID NOs:305-309.

 218. The immunostimulatory bacterium of any of claims 213-215, wherein the STING gain-of-function (GOF) variant is/are selected from among any set forth in claim 167 or claim 168.

15 219. 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;
20 IL-2, IL-21, and a STING GOF variant;
 IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt), where Δ cyt is a deleted cytoplasmic domain;
 IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 IL-15/IL-15R α , and a STING GOF variant;
25 IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
 IL-15/IL-15R α and IL-12p70;
 IL-15/IL-15R α and IL-21;
 IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
 IL-15/IL-15R α , IL-21, and a STING GOF variant;
30 IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

- IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-12p70 and IL-21;
- IL-12p70, IL-21, and a STING GOF variant;
- 5 IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-12p70 and a STING GOF variant;
- IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- IL-12p70 and IL-18;
- 10 IL-12p70, IL-18, and a STING GOF variant;
- IL-12p70, IL-18, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor, IL-2, and IL-12p70;
- a TGF- β decoy receptor, IL-2, and IL-21;
- 15 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 4-1BBL Δ cyt);
- a TGF- β decoy receptor, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 20 a TGF- β decoy receptor, IL-15/IL-15R α , and a STING GOF variant;
- a TGF- β decoy receptor, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a TGF- β decoy receptor, IL-15/IL-15R α , and IL-12p70;
- 25 a TGF- β decoy receptor, IL-15/IL-15R α , and IL-21;
- a TGF- β decoy receptor, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- a TGF- β decoy receptor, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- a TGF- β decoy receptor, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 30 a TGF- β decoy receptor, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

- 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 4-1BBL Δ cyt);
- 5 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 4-1BBL Δ cyt);
- a TGF- β decoy receptor, IL-12p70, and IL-18;
- 10 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 4-1BBL Δ cyt);
- a TGF- β decoy receptor and a STING GOF variant;
- an anti-CTLA-4 antibody, IL-2, and IL-12p70;
- 15 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 4-1BBL Δ cyt);
- 20 an anti-CTLA-4 antibody, IL-2, IL-21, a STING GOF variant, and 4-1BBL
(including 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody, IL-15/IL-15R α , and a STING GOF variant;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , a STING GOF variant, and 4-
1BBL (including 4-1BBL Δ cyt);
- 25 an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-12p70;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , and IL-21;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, and a STING GOF
variant;
- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- 30 an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-12p70, a STING GOF variant,
and 4-1BBL (including 4-1BBL Δ cyt);

- an anti-CTLA-4 antibody, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- an anti-CTLA-4 antibody, IL-12p70, and IL-21;
- an anti-CTLA-4 antibody, IL-12p70, IL-21, and a STING GOF variant;
- 5 an anti-CTLA-4 antibody, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 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
- 10 (including 4-1BBL Δ cyt);
- 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 4-1BBL Δ cyt);
- 15 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;
- 20 a CD40 agonist, IL-2, IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-2, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and a STING GOF variant;
- 25 a CD40 agonist, IL-15/IL-15R α , a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-15/IL-15R α , and IL-12p70;
- a CD40 agonist, IL-15/IL-15R α , and IL-21;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, and a STING GOF variant;
- 30 a CD40 agonist, IL-15/IL-15R α , IL-21, and a STING GOF variant;
- a CD40 agonist, IL-15/IL-15R α , IL-12p70, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);

- a CD40 agonist, IL-15/IL-15R α , IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- a CD40 agonist, IL-12p70, and IL-21;
- a CD40 agonist, IL-12p70, IL-21, and a STING GOF variant;
- 5 a CD40 agonist, IL-12p70, IL-21, a STING GOF variant, and 4-1BBL (including 4-1BBL Δ cyt);
- 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 4-1BBL Δ cyt);
- 10 1BBL Δ cyt);
- 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 4-1BBL Δ cyt); and
- 15 a CD40 agonist and a STING GOF variant.
220. The immunostimulatory bacterium or cell of claim 219, wherein the STING GOF variant is selected from among any set forth in claim 167 or claim 168.
221. The immunostimulatory bacterium any of claims 26-220, wherein an encoded therapeutic product comprises an Fc domain.
- 20 222. The immunostimulatory bacterium of any of claims 26-221, wherein an encoded therapeutic product comprises the B7 protein transmembrane domain.
223. The immunostimulatory bacterium of any of claims 26-222, wherein an encoded therapeutic product is GPI-anchored.
224. The immunostimulatory bacterium of any of claims 26-223, wherein
- 25 an encoded therapeutic product comprises human serum albumin or its derivatives thereof that increase serum half-life of the encoded product.
225. The immunostimulatory bacterium of any of claims 26-224, wherein an encoded therapeutic product comprises a fusion to collagen.
226. The immunostimulatory bacterium of any of claims 26-225, wherein
- 30 the bacterium is a Gram-negative bacterium.
227. The immunostimulatory bacterium of any of claims 26-226,

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*,
5 *Pseudomonas*, *Helicobacter*, *Bacillus*, or *Erysipelothrix*, or an attenuated strain
thereof or a modified strain thereof of any of the preceding list of bacterial strains.

228. The immunostimulatory bacterium of any of claims 26-226, wherein
the bacterium is *Rickettsia rickettsiae*, *Rickettsia prowazekii*, *Rickettsia*
tsutsugamuchi, *Rickettsia mooseri*, *Rickettsia sibirica*, *Bordetella bronchiseptica*,
10 *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*,
15 *Erysipelothrix rhusiopathiae*, *Yersinia enterocolitica*, *Rochalimaea quintana*, or
Agrobacterium tumefaciens.

229. The immunostimulatory bacterium of any of claims 26-228 that is an
attenuated bacterium or is a Gram-negative bacterium.

230. The immunostimulatory bacterium of any of claims 26-229 that is a
20 strain of *Salmonella*.

231. The immunostimulatory bacterium of claim 230 that is a *Salmonella*
typhimurium strain.

232. The immunostimulatory bacterium of claim 230 or claim 231, wherein
the unmodified *Salmonella* is a wild-type strain.

25 233. The immunostimulatory bacterium of claim 230 or claim 231, wherein
the unmodified *Salmonella* strain is attenuated.

234. The immunostimulatory bacterium of any of claims 26-233, wherein
the immunostimulatory bacterium is derived from strain AST-100 (VNP20009 or
YS1646), or strain ATCC 14028, or a strain having all of the identifying
30 characteristics of strain ATCC 14028.

235. The immunostimulatory bacterium of any of claims 227-233 that is
ansB⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*fliC*⁻/*fljB*⁻), and *pagP*⁻.

236. The immunostimulatory bacterium of any of claims 26-235, wherein the bacterium encodes and expresses the gene resistance to complement killing (*rck*).

237. The immunostimulatory bacterium of claim 236, wherein the *rck* gene is a *Salmonella rck* gene.

5 238. The immunostimulatory bacterium of any of claims 26-237 that, when intravenously administered at a therapeutic dose, induces less than 150 pg/ml of each of serum IL-6, serum TNF-alpha, and serum IL-10, when measures at 7 days post-treatment.

239. An immunostimulatory bacterium that, when intravenously
10 administered at a therapeutic dose, induces less than 150 pg/ml of each of serum IL-6, serum TNF-alpha, and serum IL-10, when measured at 7 days post treatment.

240. The immunostimulatory bacterium of claim 238 or claim 239, wherein the dose is 1×10^8 CFUs (colony forming units).

241. The immunostimulatory bacterium of claim 238 or claim 239, wherein
15 the dose is 1×10^8 CFUs – 1×10^9 CFUs.

242. An immunostimulatory bacterium, comprising a plasmid that encodes a therapeutic product, and that is modified to encode and express the gene resistance to complement killing (*rck*), wherein the wild-type bacterium does not encode *rck*.

243. The immunostimulatory bacterium of claim 242 that is an *E. coli*
20 strain.

244. A delivery vehicle encoding a truncated co-stimulatory molecule with a full or partial cytoplasmic domain deletion, for expression on an antigen-presenting cell (APC), wherein the truncated gene product is capable of constitutive immunostimulatory signaling to a T-cell through co-stimulatory receptor engagement,
25 and is unable to counter-regulatory signal to the APC due to the deleted cytoplasmic domain.

245. The delivery vehicle of claim 244, wherein the co-stimulatory molecule is 4-1BBL, CD80, CD86, CD27L, B7RP1, or OX40L, or a variant thereof with a deleted or truncated cytoplasmic domain and/or mutations that confer proper
30 orientation of the protein when expressed in a cell.

246. The delivery vehicle of claim 244 or claim 245 that is an immunostimulatory bacterium, an exosome, a nanoparticle, an oncolytic virus, or a cell.
247. The delivery vehicle of any of claims 244-246 that is a cell.
- 5 248. The delivery vehicle of claim 247, wherein the cell is a stem cell.
249. The delivery vehicle of claim 248, wherein the stem cell is a mesenchymal stem cell (MSC).
250. The delivery vehicle of claim 249, wherein the MSC is genetically modified to express a combination of immunomodulatory cytokines.
- 10 251. The delivery vehicle of claim 250, wherein the cytokines are Interleukin 12 (IL-12) and Interleukin 21 (IL-21).
252. An isolated cell, comprising the delivery vehicle of any of claims 244-251.
253. An isolated cell, comprising the immunostimulatory bacterium of any
15 of claims 26-243.
254. The cell of claim 252 or claim 253 that is an immune cell, a stem cell, a tumor cell, or a primary cell line.
255. The cell of claim 254 that is a hematopoietic cell.
256. The cell of claim 255 that is a T-cell.
- 20 257. The cell of any of claims 252-256 that is produced *ex vivo* by infecting the cell with the delivery vehicle or immunostimulatory bacterium.
258. The cell of any of claims 252-254 that is a stem cell that is not of embryonic origin.
259. The cell of any of claims 252-254, wherein the cell is a stem cell.
- 25 260. The cell of claim 259, wherein the stem cell is a mesenchymal stem cell (MSC).
261. The cell of claim 260, wherein the MSC is genetically modified to express a combination of immunomodulatory cytokines.
262. The cell of claim 261, wherein the cytokines are Interleukin 12 (IL-12)
30 and Interleukin 21 (IL-21).

263. A pharmaceutical composition, comprising the immunostimulatory bacterium of any of claims 25-243, or the delivery vehicle of any of claims 244-251, or the cell of any of claims 252-262, in a pharmaceutically acceptable vehicle.

264. A pharmaceutical composition, comprising the immunostimulatory bacterium of claim 84 in a pharmaceutically acceptable vehicle.

265. The pharmaceutical composition of claim 263 or claim 264 that is formulated for administration without dilution.

266. The pharmaceutical composition of any of claims 263-265 that is formulated for systemic administration.

267. The pharmaceutical composition of any of claims 263-266 that is formulated for parenteral administration.

268. The pharmaceutical composition of claim 267 that is formulated for intravenous administration.

269. The pharmaceutical composition of claim 267 that is formulated for intratumoral administration.

270. The pharmaceutical composition of claim 267 that is formulated for intraperitoneal administration.

271. The pharmaceutical composition of claim 266 that is formulated for oral administration.

272. A method of treatment of cancer that comprises a solid tumor or a hematological malignancy in a subject, comprising administering the immunostimulatory bacterium of any of claims 26-243, or the delivery vehicle of any of claims 244-251, or the cell of any of claims 252-262, or the pharmaceutical composition of any of claims 263-271.

273. Use of the immunostimulatory bacterium of any of claims 26-244, or the delivery vehicle of any of claims 244-251, or the cell of any of claims 252-262, or the pharmaceutical composition of any of claims 263-271, for the treatment of a cancer that comprises a solid tumor or a hematological malignancy in a subject.

274. The immunostimulatory bacterium of any of claims 26-244, or the delivery vehicle of any of claims 244-251, or the cell of any of claims 252-262, or the pharmaceutical composition of any of claims 263-271, for use for the treatment of a cancer that comprises a solid tumor or a hematological malignancy in a subject.

275. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-274, wherein the subject is a human.

276. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-275, wherein the cancer comprises a solid tumor.

277. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-275, wherein the cancer comprises a hematological malignancy.

278. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-277, wherein the treatment comprises combination therapy in which a second anti-cancer agent or treatment is administered.

279. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of claim 278, wherein the second anti-cancer agent or treatment is administered before, concomitantly with, after, or intermittently with, the immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition.

280. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of claim 278 or claim 279, wherein the second anti-cancer agent or treatment is an immunotherapy.

281. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-280, wherein administration of the immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition is parenteral.

282. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-280, wherein administration of the immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition is oral, or rectal, or by aerosol into the lung, or intratumoral.

283. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-280, wherein administration of the immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition is intravenously, intramuscularly, or subcutaneously.

284. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-283, 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.

285. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of claim 280, wherein the immunotherapy comprises administration of an anti-PD-1, or anti-PD-L1, or anti-CTLA-4 antibody, or antigen-binding portions or forms thereof.

286. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-285, wherein the immunostimulatory bacterium is a *Salmonella*, *Shigella*, *Listeria*, or *E. coli* species.

287. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-286, wherein the immunostimulatory bacterium is a *Salmonella* species.

288. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-287, wherein the immunostimulatory bacterium is a *Salmonella typhimurium* strain.

289. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-288, wherein administration of the immunostimulatory bacterium is by intraperitoneal or intratumoral administration.

290. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-289, wherein the subject has metastatic cancer.

291. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-290, 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, or antigen-binding portions or forms thereof.

292. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-291, 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.

293. The method, use, immunostimulatory bacterium, delivery vehicle, cell, or pharmaceutical composition of any of claims 272-292, wherein the immunostimulatory bacterium is *ansB*⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*fliC*⁻/*fljB*⁻), and *pagP*⁻.

294. A method of selecting a subject for treatment with the immunostimulatory bacterium of any of claims 26-244, or the delivery vehicle of any of claims 244-251, or the cell of any of claims 252-262, or the pharmaceutical composition of any of claims 263-271, comprising:

obtaining a biological sample from the subject; and
detecting one or more biomarkers that indicate an immune-excluded or immune-desert tumor phenotype.

295. The method of claim 294, wherein the biomarker is selected from among:
a test indicative of T-cell or tumor-infiltrating lymphocyte (TIL) infiltration into the tumor microenvironment;
a biomarker that measures the restriction of T-cells or TILs to the invasive margin of the tumor and tumor core;
the level of TILs;
an adenosine signature (Nanostring) indicative of CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, THBS1, IL-6, CSF-3, IL-1beta, CCL2, CCL3, or CCL7;
a myeloid signature (Nanostring) indicative of CXXCL1, CXCL2, CXCL3, CXCL8, IL-6, or PTGS2; and
levels of one or more of CD3, CD8, CD73, CD39, TNAP (tissue-nonspecific alkaline phosphatase), CD38, CD45, CD68, PD-L1, and FoxP3.

296. A method of monitoring therapy with the immunostimulatory bacterium of any of claims 26-244, or the delivery vehicle of any of claims 244-251,

or the cell of any of claims 252-262, or the pharmaceutical composition of any of claims 263-271, comprising:

obtaining a biological sample from a subject, and detecting a change in the level of a biomarker, wherein:

- 5 an increase in a biomarker indicative of an anti-cancer phenotype indicates that treatment is effective; and
- the biomarkers are any that indicate a cytokine response, activation of Type I Interferon, or activation of Type II Interferon.
297. The method of claim 296, wherein:
- 10 the biomarker is selected from among the level of one or more of CXCL10/IP-10, CXCL9, Interferon-alpha, Interferon-beta, the pro-inflammatory serum cytokines IL-6, TNF- α , MCP-1, or CCL2, and IL-18 binding protein; and
- an increase in the level of one or more of the biomarkers indicates that treatment is effective.
- 15 298. The method of any of claims 294-297, wherein the biological sample is a tumor biopsy, or a sample of a body fluid.
299. The method of any of claims 294-298, further comprising administering the immunostimulatory bacterium of any of claims 26-244, or the delivery vehicle of any of claims 244-251, or the cell of any of claims 252-262, or the
- 20 pharmaceutical composition of any of claims 263-271.
300. A modified Stimulator of Interferon Genes (STING) protein from a non-human species, where the non-human STING is one that has lower NF- κ B signaling activity compared to human STING, and, optionally, higher type I interferon (IFN) pathway signaling activity compared to human STING, wherein:
- 25 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
- 30 the STING protein optionally has a deletion or disruption of the TRAF6 binding site.

301. 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
5 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- κ B) signaling activity,
10 compared to human STING; and

the mutation or mutations result in increased STING activity or constitutive activity in inducing IFN-beta production.

302. The modified STING protein of claim 300 or claim 301, wherein the human STING protein comprises the sequence set forth in any of SEQ ID NOs:305-
15 309, 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.

303. The modified STING protein of any of claims 300-302, 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
20 protein from a second species;

the STING protein of the second species has lower NF- κ B signaling activity than the NF- κ B signaling activity of human STING; and

the TRAF6 binding site in the CTT optionally is deleted.

304. The modified STING protein of any of claims 300-303, wherein the
25 mutation or mutations is/are any that correspond to those associated with the auto-inflammatory disease STING-associated vasculopathy (SAVI).

305. 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,
30 wherein:

the STING protein of the second species has lower NF- κ B signaling activity than the NF- κ B signaling activity of human STING; and

the TRAF6 binding site in the CTT optionally is deleted.

306. The modified STING protein of any of claims 303-305, 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.

307. The modified STING protein of any of claims 303-306, 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.

308. The modified STING protein of any of claims 300-307, wherein the type I IFN signaling activity is at least or at least about 30% that of a wild type human STING protein.

309. The modified STING protein of any of claims 300-308, wherein the NF- κ B 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- κ B signaling activity.

310. The modified STING protein of any of claims 300-309, 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.

311. The modified STING protein of any of claims 300-310, 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.

312. The modified STING protein of any of claims 300-311 that comprises replacement of the C-terminal tail (CTT) with the CTT from a STING protein that has reduced NF- κ B signaling activity compared to the NF- κ B signaling activity of human STING.

313. The modified STING protein of claim 312, 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.

314. The modified STING protein of claim 312, 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.

- 5 315. The modified STING protein of claim 313 or claim 314, wherein the replacing CTT is selected from among the following species, and has a sequence:
Tasmanian devil RQEEFAIGPKRAMTVTTSSTLSQEPQLLISGMEQPLSLRTDGF
SEQ ID NO:371,
Marmoset EEEEVTVGSLKTSEVPSTSTMSQEPPELLISGMEKPLPLRSDLF SEQ
10 ID NO:372,
Cow EREVTMGSTETSVMPGSSVLSQEPPELLISGLEKPLPLRSDVF SEQ ID
NO:373,
Cat EREVTVGSVGTSMVRNPSVLSQEPNLLISGMEQPLPLRTDVF SEQ ID
NO:374,
15 Ostrich RQEEYTVCDGTLCSLDLSLQISESDLPQPLRSDCL SEQ ID NO:375,
Boar EREVTMGSAETSVVPTSSTLSQEPPELLISGMEQPLPLRSDIF SEQ ID
NO:376,
Bat EKEEVTVGTVGTYEAPGSSTLHQEPPELLISGMDQPLPLRTDIF SEQ ID
NO:377,
20 Manatee EREEVTVGSVGTSSVPSPPSTSSLSQEPKLLISGMEQPLPLRTDVF
SEQ ID NO:378,
Crested ibis CHEEYTVYEGNQPHNPSTTLHSTELNLQISESDLPQPLRSDCF SEQ
ID NO:379,
Coelacanth
25 (variant 1) QKEEYFMSEQTQPNSSSTSCSTEPQLMISDTPAPHTLKRQVC SEQ
ID NO:380,
Coelacanth
(variant 2) QKEEYFMSEQTQPNSSSTSCSTEPQLMISDTPAPHTLKSFG SEQ ID
NO:381,
30 Ghost shark LTEYPVAEPSNANETDCMSSEPHLMISDDPKPLRSYCP SEQ ID
NO:383, and

Mouse EKEEVTMNAPMTSVAPPPSVLSQEPRLISGMDQPLPLRTDLI SEQ ID NO:384,

or allelic variants of each of these sequences, having at least 98% sequence identity thereto.

5 316. The modified STING protein of claim 314 or claim 315, wherein the human CTT comprises the sequence EKEEVTVGSLKTSVPSTSTMSQEPPELLISGMEKPLPLRTDFS (SEQ ID NO:370), or is an allelic variant having at least 98% sequence identity thereto.

10 317. The modified STING protein of any of claims 305-316, wherein the modified STING protein is a chimera in which the human STING CTT is replaced with a CTT from the Tasmanian devil STING.

 318. The modified STING protein of claim 317, 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.

 319. The modified STING protein of any of claims 300-318, comprising a deletion or disruption of the TRAF6 binding site.

20 320. The modified STING protein of claim 319, wherein the STING protein is a human STING protein, and the TRAF6 binding site comprises the amino acid residues DFS at the C-terminus.

 321. The modified STING protein of any of claims 300-320, comprising a modification that increases type I interferon signaling activity, or that renders the activity constitutive in the absence of cytosolic nucleic acids.

25 322. The modified STING protein of claim 321, 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.

30 323. The modified STING protein of any of claims 300-322, 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, for alignment, to the sequence of human STING, as set forth in any of SEQ ID NOs:305-309.

324. The modified STING protein of claim 323 that comprises a replacement corresponding to C206Y or R284G, with reference, for alignment, to the sequence of human STING, as set forth in any of SEQ ID NOs:305-309.

325. The modified STING protein of claim 324 that comprises replacements corresponding to N154S/R284G

326. The modified STING protein of claim 300 that is a Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, or ghost shark STING protein.

327. A delivery vehicle, comprising nucleic acids encoding the modified STING protein of any of claims 300-326.

328. A delivery vehicle, comprising nucleic acids encoding a STING protein from a non-human species, wherein the STING protein has type I IFN signaling activity, and attenuated NF- κ B signaling activity compared to the NF- κ B signaling activity of human STING.

329. The delivery vehicle of claim 328, 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.

330. The delivery vehicle of claim 328 or claim 329, wherein the type I IFN signaling activity is at least or at least about 30% of the type I IFN signaling activity of wild-type human STING.

331. The delivery vehicle of any of claims 327-330, wherein the NF- κ B signaling activity of the non-human STING protein is less than 30%, less than 20%, less than 15%, less than 10%, or less than 5% of the NF- κ B signaling activity of human STING.

332. The delivery vehicle of any of claims 327-331, wherein the non-human species is Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, or ghost shark.

333. The delivery vehicle of any of claims 327-332 that is a cell, an
5 exosome, an oncolytic virus or viral vector, a liposome or other lipid-based vehicle, or an immunostimulatory bacterium.

334. The delivery vehicle of claim 333, wherein the delivery vehicle is a cell that is a stem cell or an immune cell.

335. The delivery vehicle of claim 333, wherein the delivery vehicle is the
10 immunostimulatory bacterium of any of claims 26-243.

336. The delivery vehicle of claim 335, wherein the immunostimulatory bacterium is *ansB*⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*fliC*⁻/*fljB*⁻), and *pagP*⁻.

337. The delivery vehicle of claim 335, wherein the immunostimulatory bacterium is *ansB*⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*fliC*⁻/*fljB*⁻), *pagP*⁻, *lppA*⁻ and
15 *lppB*⁻.

338. An immunostimulatory bacterium, comprising a plasmid encoding the modified STING protein of any of claims 300-326, or encoding a STING protein from a non-human species, wherein the encoded STING protein has type I IFN signaling activity, and attenuated NF-κB signaling activity compared to the NF-κB signaling
20 activity of human STING.

339. The immunostimulatory bacterium of claim 338, that is *ansB*⁻, *asd*⁻, *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻, wherein the wild-type bacterium has flagella, and *pagP*⁻.

340. The immunostimulatory bacterium of claim 338, that is *ansB*⁻, *asd*⁻,
25 *csgD*⁻, *purI*⁻, *msbB*⁻, flagellin⁻ (*fliC*⁻/*fljB*⁻), wherein the wild-type bacterium has flagella, *pagP*⁻, *lppA*⁻, and *lppB*⁻.

341. The immunostimulatory bacterium of any of claims 338-340, wherein the non-human species is selected from among Tasmanian devil, marmoset, cattle, cat, ostrich, boar, bat, manatee, crested ibis, coelacanth, mouse, and ghost shark.

30 342. The immunostimulatory bacterium of any of claims 338-341, or the delivery vehicle of any of claims 295-297, wherein the immunostimulatory bacterium is a Gram-negative bacterium.

343. The immunostimulatory bacterium of any of claims 338-342, or the delivery vehicle of any of claims 295-297, wherein the immunostimulatory bacterium is a strain of *Salmonella*, *Shigella*, *E. coli*, *Bifidobacteriae*, *Rickettsia*, *Vibrio*, *Listeria*, *Klebsiella*, *Bordetella*, *Neisseria*, *Aeromonas*, *Francisella*, *Cholera*,
5 *Corynebacterium*, *Citrobacter*, *Chlamydia*, *Haemophilus*, *Brucella*, *Mycobacterium*, *Mycoplasma*, *Legionella*, *Rhodococcus*, *Pseudomonas*, *Helicobacter*, *Bacillus*, or *Erysipelothrix*, or an attenuated strain thereof, or a modified strain thereof of any of the preceding list of bacterial strains.

344. The immunostimulatory bacterium of any of claims 338-342, or the
10 delivery vehicle of any of claims 327-337, wherein the immunostimulatory 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*,
15 *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*, or *Agrobacterium tumefaciens*, or an attenuated strain thereof, or a modified strain thereof of any of the
20 preceding list of bacterial strains.

345. The immunostimulatory bacterium of any of claims 338-342, or the delivery vehicle of any of claims 327-337 wherein the immunostimulatory bacterium is an attenuated bacterium.

346. The immunostimulatory bacterium of any of claims 338-342, or the
25 delivery vehicle of any of claims 327-337, wherein the bacterium is a strain of *Salmonella*.

347. The immunostimulatory bacterium or delivery vehicle of claim 346, wherein the bacterium is a *Salmonella typhimurium* strain.

348. The immunostimulatory bacterium or delivery vehicle of claim 346 or
30 claim 347, wherein the unmodified *Salmonella* is a wild-type strain.

349. The immunostimulatory bacterium or delivery vehicle of claim 346 or claim 347, wherein the unmodified *Salmonella* strain is attenuated.

350. The immunostimulatory bacterium or delivery vehicle of any of claims 327-349, wherein the immunostimulatory bacterium is derived from strain AST-100 (VNP20009 or YS1646), or strain ATCC 14028, or a strain having all of the identifying characteristics of strain ATCC 14028.

5 351. A pharmaceutical composition, comprising the modified STING protein of any of claims 300-326, or the delivery vehicle of any of claims 327-337, or the immunostimulatory bacterium of any of claims 338-350, in a pharmaceutically acceptable vehicle.

10 352. An isolated cell, comprising the modified STING protein of any of claims 300-326, or the delivery vehicle of any of claims 327-337, or the immunostimulatory bacterium of any of claims 338-350, wherein the cell is not a zygote of a fertilized human egg.

353. The cell of claim 352 that is an immune cell, a stem cell, a tumor cell, or a primary cell line.

15 354. An isolated cell or cultured cells, comprising the immunostimulatory bacterium of any of claims 338-350.

355. The cell of claim 353 or claim 354 that is a hematopoietic cell.

356. The cell of claim 355 that is a T-cell.

20 357. The cell of any of claims 352-356 that is produced *ex vivo* by infecting the cell with the immunostimulatory bacterium or delivery vehicle.

358. The cell of claim 357 that is a T-cell, or a hematopoietic cell.

25 359. A method of treatment of cancer, comprising administering the modified STING protein of any of claims 300-326, or the delivery vehicle of any of claims 327-337, or the immunostimulatory bacterium of any of claims 348-350, or the pharmaceutical composition of claim 351, or the cell of any of claims 352-358, to a subject with a cancer that comprises a solid tumor or is a hematological malignancy.

360. The method of claim 359, wherein the cancer comprises a solid tumor.

361. The method of claim 359 or claim 360, wherein the cancer is metastatic.

30 362. The method of any of claims 359-361, wherein the cancer is selected from among lymphoma; leukemia; 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.

363. Use of the modified STING protein of any of claims 300-326, or the delivery vehicle of any of claims 327-337, or the immunostimulatory bacterium of
5 any of claims 338-350, or the pharmaceutical composition of claim 351, or the cell of any of claims 352-358, for the treatment of cancer.

364. The use of claim 363, wherein the cancer comprises a solid tumor or a hematological malignancy.

365. The use of claim 363 or claim 364, wherein the cancer is metastatic.

10 366. The use of any of claims 363-365, wherein the cancer is selected from among lymphoma; leukemia; 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.

15 367. The modified STING protein, delivery vehicle, immunostimulatory bacterium, pharmaceutical composition, cell, method, or use of any of claims 300-366, wherein the non-human STING protein has the sequence of amino acids set forth in any of SEQ ID NOs: 349, 356, or 359-369, or is an allelic variant of the STING protein of each species, having at least 98% sequence identity to the sequence of
20 amino acids set forth in any of SEQ ID NOs: 349, 356, or 359-369.

368. The modified STING protein, delivery vehicle, immunostimulatory bacterium, pharmaceutical composition, cell, method, or use of any of claims 300-366, wherein the non-human STING protein or chimera has the sequence of amino acids set forth in any of SEQ ID NOs: 350-355, 357, or 358.

25 369. A method of converting an M2 macrophage to an M1 or M1-like phenotype, comprising administering the immunostimulatory bacterium of any of claims 26-343 and 338-350 to a subject with a condition, disease, or disorder treated by enhancing an anti-tumor immune response.

370. Use of an immunostimulatory bacterium of any of claims 25-343 and
30 338-350 or the immunostimulatory bacterium of any of claims 25-343 and 338-350 for use to convert an M2 macrophage to an M1 or M1-like phenotype macrophage in

a subject with cancer, whereby an anti-tumor immune response is induced or enhanced.

371. The method of claim 369 or use of bacterium of claim 370, wherein the disease, disorder, or condition is a cancer that comprises a solid tumor.

5 372. An isolated cell that comprises an immunostimulatory bacterium of any of claims 26-243 and 338-350.

373. The cell of claim 372 that is an immune cell or a stem cell.

374. The cell of claim 373, wherein the cell is a stem cell is not an embryonic stem cell.

10 375. The cell of claim 373 that is a T-cell.

376. The cell of claim 375 that is a CAR-T cell.

377. A method of treatment of cancer, comprising administering the cell of any of claims 372-376.

378. The cell of any of claims 372-376 for use for the treatment of cancer.

15 379. Use of the cell of any of claims 372-376 for the treatment of cancer.

1/14

Human and Tasmanian devil STING:

Human	1	MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTIWGLGEPPEHTLRYLVLH	50
		. .	
Tasmanian	1	MPRSHLHPSIPQPRGWGAQKAACVLLSLCMAAVYILGESAAFTLQWLLFY	50
Human	51	LASLQLGLLLVGCSLAEELRHIHSRYRGSYWRTVRACIGCPLRRGALLL	100
		..: .	
Tasmanian	51	FTTVQVGLLLKGAYCLAEELYHIQSRHQGSYWQAIQACIHYPFQRISLLL	100
Human	101	LSIYFYYSLPNAVGPPTWMLALLGLSQALNILLGLKGLAPAEISAVCEK	150
		. .	
Tasmanian	101	LSGYFYVTLSNKPNSLTWTWFALLGLSHALSFILGLQNLTSAEISEVCEK	150
Human	151	GNFNVAHGLAWSYYIGYLRILPELQARIRTYNQHYNNLLRGAVSQRLYI	200
		.: .	
Tasmanian	151	RHLNVAHGLAWSYYIGYLRILPGLQTRIQIYNQFNKDVLRSPEGHRLHI	200
Human	201	LLPLDCGVDPNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN	250
		: .	
Tasmanian	201	LIPLDCSVDPNLSQADPNIQFLQELPQHKLDRAKIGRIYTNISIYKISED	250
Human	251	GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLAQAKLFCRTLEDILA	300
		. .	
Tasmanian	251	GQPAEVCVLEFATPLQTLFAMSQDARAGFSREDRLAQAKLFCRTLEDILE	300
Human	301	DAPESQNNCRLLIAYQEPADD--SSFSLSQEVLRLRQEEKEEVTVGSLKT	348
		: .	
Tasmanian	301	DAPEARDCCLVVYQEPEDKAVSNFSLSKEILRLRQERQEEFAIGPKRA	350
Human	349	SAVPSTSTMSQEPPELLISGMEKPLPLRTD-FS	379
		.. .: .	
Tasmanian	351	MTVTTSSSTLSQEPQLLISGMEQPLSLRTDGF-	381

FIGURE 1

2/14

Human and Marmoset STING:

Human	1	MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH	50
		. : 	
Marmoset	1	MPHSSLHPSIPHPRGHGAQEAAALVLLSVCLVTLWWLREAPEDILRFLVLH	50
Human	51	LASLQLGLLLVGVCSLAEELRHHSRYRGSYWRTVRACLGCPLRGALLL	100
		. . : : : : . : . .	
Marmoset	51	LASLQLGLLLVNRLCSLAEELRHVHTRYQGSYWRAVRACLGCPIRLGAQLL	100
Human	101	LSIYFYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK	150
			
Marmoset	101	LSIYFYCFLPN--GRPFTWMLALLGFSQALNILLGLKGLAPAEISAVCEK	148
Human	151	GNFNVAHGLAWSYYIGYLRILPELQARIRTYNQHYNNLLRGAVSQRLYI	200
		 : : .	
Marmoset	149	RNFNVAHGLAWSYYIGYLRILPGFQARIRTYNQHNNVLRGPASQRLYI	198
Human	201	LLPLDCGVDPNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN	250
		. . . : . . . :	
Marmoset	199	LFPLDCGVDPNLSTADPNIRFLDKLPQETIDRAGIKGRVYTNSIYELLEN	248
Human	251	GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLAQAKLFCRTLEDILA	300
		. : 	
Marmoset	249	GQRAGACVLEYASPLQTLFAMSQYQAGFSREDRLAQAKLFCRTLEDILA	298
Human	301	DAPESQNNCRLIAYQEPADSSFSLSQEVLRHLRQEEKEEVTGSLKTS	350
		. : : . . : : .	
Marmoset	299	DAPESQNNCRLIVYEEPADGSSFLLSQEVLQHLRQEEEEVTGSLKTSE	348
Human	351	VPSTSTMSQEPELLISGMEKPLPLRTDFS	379
		. . : ..	
Marmoset	349	VPSTSTMSQEPELLISGMEKPLPLRSDLF	377

FIGURE 2

3/14

Human and cattle STING:

Human	1	MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTWGLGEPPEHTLRYLVLH	50
		: : : : : : : :	
Cattle	1	MPHSSLHPSIPQPRGLRAQKAALVLLSACLVALWGLGEPDDYTLKWLVH	50
		: : : : : : :	
Human	51	LASLQLGLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL	100
		: : : : : : :	
Cattle	51	LASQQMGLLIKGICSLAEELCHVHSRYHGSYWRAVRACLCSSMRCGALLL	100
		: : : : : : :	
Human	101	LSIYFYYSLPNAVGPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK	150
		: : : : : : :	
Cattle	101	LSCYFYCSLPNMADLPFTWMLALLGLSQALNILLGLQGLAPAEVSAICEK	150
		: : : : : : :	
Human	151	GNFNVAHGLAWSYYIGYLRLLPELQARIRTYNQHYNNLLRGAVSQRLYI	200
		. : : : : : : :	
Cattle	151	RNFNVAHGLAWSYYIGYLRLLPGLPARIQIYNQFHNNTLQGAGSHRLHI	200
		: : : : : : :	
Human	201	LLPLDCGVPDNLMSADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN	250
		: : : : : : :	
Cattle	201	LFPLDCGVPDDLNVDPNIRFLHELPQQSADRAGIKGRVYTNSIYELLEN	250
		: : : : : : :	
Human	251	GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA	300
		: : : : : : :	
Cattle	251	GQRAGVCVLEYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRTLEDILA	300
		: : : : : : :	
Human	301	DAPESQNNCRLLIAYQEPADDSSFSLSQEVLRLRQEEKEEVTVGSLK TSA	350
		: : : : : : : :	
Cattle	301	NAPESQNNCRLLIVYQEPAGSSFSLSQEILQHLRQEER-EVTMGSTETSV	349
		: : : : : : : :	
Human	351	VPSTSTMSQEPPELLISGMEKPLPLRTDFS	379
		: .:.: : : : : : :	
Cattle	350	MPGSSVLSQEPPELLISGLEKPLPLRSDVF	378

FIGURE 3

Species	Position	Sequence	Position
Human	1	MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH	50
Cat	1	MPRTGLHPSIPRPRMGGAQKAALVLLAVCLAALWRLGESPDHTLRWLVLH	50
Human	51	LASLQLGLLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRRGALLL	100
Cat	51	LASLQLGLLFTGVCHLTEELCHLSRYQGSYW RAMKACLGSPVRSGALLL	100
Human	101	LSIYFYYSLPNAVGPPTWMLALLGLSQALNILLGLKGLAPAEISAVCEK	150
Cat	101	LSCYFYSTLP-STDLPPTWMLALLGLSQALNILLDLQGLAPAEVSAVCEK	149
Human	151	GNFNVAHGLAWSYYIGYLRLLPELQARIRTYNQHYNNLLRGAVSQRLYI	200
Cat	150	KNFNVAHGLAWSYYIGYLRLLPGLPARVLTQNLHNNILRGTGSHRLHI	199
Human	201	LLPLDCGVPDNLMSADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYELLEN	250
Cat	200	LFPLDCGVPDDMSVADPNIRFLYELPQQSADRAGIKGRVYTNSVYALLEN	249
Human	251	GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLF CRTLEDILA	300
Cat	250	GOQAGICVLEYATPLQTLFAMSQDGRAGFSREDRLEQAKLF CRILEDILA	299
Human	301	DAPESQNNCRLLIAYQEPADDSSFSLSQEVLRHLRQEKEEEVTVGSLKTS A	350
Cat	300	DTPECQNNCRLLIVYQEP EEGSNFSLSQEILRHLRQEER-EVTVGSGVTSM	348
Human	351	VPSTSTMSQEP ELLISGMEKPLPLRTDFS	379
Cat	349	VRNPSVLSQEPNLLISGMEQPLPLRTDVF	377

FIGURE 4

5/14

Human and ostrich STING:

Human	1	MPHSSLHPS-----IPCPRGHGAQKAALVLLSACLVTWGLGEPPEHTL	44
			
Ostrich	1	MAHESGTPSNPATPLIPKAREGRAQHAAYVLLALCAAALYLAGEPVVHIA	50
Human	45	RYLVHLHLASLQLGLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPRLR	94
		 : . . . : . . .: .: : : : : : : : : : : : .	
Ostrich	51	RSFTSHFVALQIGALLKGICYLVEEIIHFHLETRHRGSFRRALSACL--HLR	98
Human	95	-RGALLLSIYFYSLPNAVGPPFTWMLALLGLSQALNILLGLKGLAPAE	143
		... :	
Ostrich	99	WHVTLLLVCGSAYVALLDGDEQPLGLHLGLACLCQLLILALGLHKPSAVE	148
Human	144	ISAVCEKGNFNVAHGLAWSYIIGYLRILPELQARITYNQHYNNLLRGA	193
		: :	
Ostrich	149	ISEMSERSKQNVAGHLAWSYVGYLKIVLPRLKESMENIGRTNPNMLAHK	198
Human	194	VSQRLYILLPLDCGVPDNLMSADPNIRFLDKLPQQTGDHAGIKDRVYSNS	243
		:.: :	
Ostrich	199	ETWKLHILVPLSCNICDDLEKADSNIQYAMDLPETTLTRAGTKKRVYRNT	248
Human	244	IYELLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLAQAKLFCR	293
		: : :.: :	
Ostrich	249	LYK-IKDEKFRFCVVEYATPLQSLYAMSQDECAAFSREDRIEQAKLFYR	297
Human	294	TLEDILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRLRLRQEEKEEVTV	343
		: :	
Ostrich	298	TLEEILQSAKECAGTYRLIVYEESGEAETHFLSREILRHLQQQRQEYTV	347
Human	344	--GSLKTSAPVSTSTMSQEPPELLISGMEKPLPLRTDFS	379
		: :. :	
Ostrich	348	CDGTL-----CSTDLSLQISESDLPLRSDCL	375

FIGURE 5

Human	1	---MPHSSLHPS---IPCPRGHGAQKAALVLLSACLVTLWGLGEPPEHTL	44
Crested	1	MSQEPQWLSHPTALLIPKARGGRAQHAYVLLLLALCAAVLYLAGEPLVPSPA	50
Human	45	RYLVLHLASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPPLR	94
Crested	51	RSLISHFIMALQIGVLLKGTCYLAEEIFHLQSRHHGSFWRALSACF--PLR	98
Human	95	RGALLLSIYFYSLPNAVGPFFTWMALLGLSQALNILLGLKGLAPAEI	144
Crested	99	WHGLMLLVCGSAYVALLEDGQPLGLHLGLASLCQLLILALGLHKPSAVEM	148
Human	145	SAVCEKGNFNVAHGLAWSYYIGYLRILIPELQARIRTYNQHYNNLLRGAV	194
Crested	149	SEMSERSKQNVAHGLAWSYYVGYLKIVLPRVKKSMEEFSTRANPNVLARRE	198
Human	195	SQRLYILLPLDCGVDPDNLMSADPNIRFLDKLPQQTGDHAGIKDRVYSNSI	244
Crested	199	TWKHLHILVPLSCDVYDDLEKADSNIQYLMDLPETTLTRAGTKKRVIKHSL	248
Human	245	YELLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLAQAKLFCRT	294
Crested	249	YTIRDEGNKLWHCAVEYATPLQSLYAMSQDEYAASFREDRLAQAKLFYRT	298
Human	295	LEDILADAPESQNNCRLIAQEPADDSSFSLSQEVLRLHRQEEKEEVTVG	344
Crested	299	LEEILKGSKECAGTYRLIVYEESGEAETHSLSRDILWHLRQQCHEEYTVY	348
Human	345	SLKTSAVPSTSTMSQEPPELLISGMKPLPLRTDFS	379
Crested	349	EGNOPHNPPSTTLHSTELNLQISESDLPOPLRSDC-	382

FIGURE 6

7/14

Human and coelacanth (variant with SEQ ID NO:345) STING:

Human	1	MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTWGLGEPPEHTLRYLVLH	50
			
Coelacanth	1	FGLQNMSAII PQPRGNRANQMAYFIITVILMLLWIFRNIMDNVLEVIALH	50
Human	51	LASLQLGLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRGALLL	100
		.. .	
Coelacanth	51	ILLLQGAAIMKGICNFAEEVNHVQPRYGGSYWKALEACLNLSKYDVMKIV	100
Human	101	LS--IYFYSLPNAVGPPFTWMLALL--GLSQALNILLGLKGLAPAEISA	146
		.: : .	
Coelacanth	101	FAGVLWWHFSSSLFV---VWFLHLLVSCICHLLNNVLGVLKPSPEVSE	146
Human	147	VCEKGNFNVAGHLAWSYYIGYLRILPELQARIRTYNQHYNNLLRGAVSQ	196
		:. .	
Coelacanth	147	IYERNRIGVAHGLAWSYYLGYLKLVLPELEDRIKSYNVSHGNLLKHKETW	196
Human	197	RLYILLPLDCGVPDNLSMADPNIRFLDKLPQQTGDHAGIKDRVYSNSIYE	246
		: . .:.	
Coelacanth	197	RLHILLPLSCSIFDNLADVDSNIEFLDNLSELQINRAGIKGRSYKHSLYQ	246
Human	247	LENGQ RAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLQAKLFCRTLE	296
		: :	
Coelacanth	247	VLDEDKRPHYCILEYATPLKSLEMSKEASAEFTKEDRLQTKLFYRTLK	296
Human	297	DILADAPESQNNCRLIAY--QEPADDSSFSLSQEVLRLHLRQEEKEEVTVG	344
		: :	
Coelacanth	297	DILDNSQECRGRFRLVIYDGREDSGKSHF-LSKELLRHLNQQQKEEYFMS	345
Human	345	SLKTSAVPSTSTMSQEPPELLISGMEKPLPLRTDFS	379
			
Coelacanth	346	EQTQPNSSSTSCLSTEPQLMISDTPAPHTLKRQVC	380

FIGURE 7

Human	1	-----MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTLWGLGEPPE	41
Zebrafish	1	MSVMGEDALVPRARSRLPVMCAAGLG----FLTLAVAWLLSDDKFSE---	43
Human	42	HTLRYLVLHLASLQLGLLLNG---VCSLAEELR-HIHSRYRGSYWRTVR	86
Zebrafish	44	-----RAGIIAFGLMLERFIYCICLLAEELLFHRSQRYPHGMRMSEIFR	85
Human	87	ACLGCP LRRGA--LLLLSIYFYYSLPNAVGPFFTWMALLGLSQAL----	130
Zebrafish	86	ACF-----RSGSIGLMCAIFL-----MLMLGGVSFSVKQWS	116
Human	131	--NIL-----LGLKGLAPAEISAVCEKGNFNVAHGLAWSYYIGYLR	169
Zebrafish	117	HFNLMCAGYMLLSNLGVLPAPVEISEICEAKMNVAHGLAWSFYIGYLYK	166
Human	170	LILPELQARIRTYNQHYNNLLRGAVSQRLYIILLPLDCGVPDNLMSADPNI	219
Zebrafish	167	FLLPALEVN NVREYSRR----ERLSSPRLHILLPLNARVPSKPPEEDTNV	211
Human	220	RFLDKLPQQTGDHAGIKDRVYSNSIYEELLENGQRAGTCVLEYATPLQTLF	269
Zebrafish	212	VFHENLPDLKLDRAGVRKRSTNSVYKITHNNE-TFSCILEYATPLLTLTY	260
Human	270	AMSQYSQAGFSREDRLAQAKLFRCRTLEDILADAPESQNNCRLIAYQEPAD	319
Zebrafish	261	QMSQESSAGFGGERERKQQVLLFYRTLSQLDNSLECRNRRLILLNDEHT	310
Human	320	DSSFSLSQEVLRLHLRQEE-----KEEVTVGSLKTSAVPST-	354
Zebrafish	311	GDPHYLSREL FQNLIKQDG EIFMDPTNEVHPVPEEGPVGN C-NGALQATF	359
Human	355	--STM SQEPellisGM EKPLPLR-----TD FS-	379
Zebrafish	360	HEEPSM SDEPTLMFS---RPQSLRSEP VETTDYFN PSSAMKQN	398

FIGURE 8

9/14

Human and boar STING:

Human	1	MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTWGLGEPPEHTLRYLVLH	50
		: :	
Boar	1	MPYSSLHPSIPQPRGLRAQVAALVLLGACLVALWGLGELPEYTLRWLVLH	50
Human	51	LASLQLGLLLVGCSLAEELRHIHSRYRGSYWRTVRACLGCPLRGALLL	100
		. : . : . : . . :	
Boar	51	LASQQIGLLVKGLCSLAEELCHVHSRYQSSYWRAARACLGCPIRC GALLL	100
Human	101	LSIYFYYSLPNAVGPFTWMLALLGLSQALNILLGLKGLAPAEISAVCEK	150
		. : : . . . : . : :	
Boar	101	LSCYFYFSIRDKAGLPLPWLALLGLSQALNILLGLQHLAPAEVSAICEK	150
Human	151	GNFNVAHGLAWSYYIGYLRILPELQARIRTYNQHYNNLLRGAVSQRLYI	200
		. : : . : : . . : . . :	
Boar	151	RNFNVAHGLAWSYYIGYLRILPGLRARIQAYNQRHKNVLGGIGNHRLHI	200
Human	201	LLPLDCGVDPNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYELLEN	250
		. : : : . : : : . . : : :	
Boar	201	LFPLDCGVDDLSVADPNIRFLHELPPQSADRAGIKGRVYTNSIYELLEN	250
Human	251	GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDILA	300
		. . . : : . : : . : : . : :	
Boar	251	GQPAGVCVLGYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRTLEDILA	300
Human	301	DAPESQNNCRLIAYQEPADSSFSLSQEVLRLRQEEKEEVTGSLKTS A	350
		: . . : : . : : . : : . :	
Boar	301	DAPEAQNNCRLIVYQEPTEGGSFSLSQEILRLRQEEER-EVTMGSAETSV	349
Human	351	VPSTSTMSQEPPELLISGMEKPLPLRTDFS	379
		: : : : : : : : : : : : : : :	
Boar	350	VPTSSTLSQEPPELLISGMEQPLPLRSDIF	378

FIGURE 9

10/14

Human and bat STING:

Human	1	MPHSSLHPSIPCPRGHGAQK-AALVLLSACLVTWGLGEPPEHTLRYLVL	49
		. : . .:. :. .: . .	
Bat	1	MSHSSLHPSVPRPRGRARRNIAAFVLLIVCLAALWLSGKPSEYTLRCLVL	50
Human	50	HLASLQLGLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRGALL	99
		 : : . . . : . .:	
Bat	51	HLASQQLGLLSNRVCYLAEELSHIHSRYQGNWRAVRACLSCPIRFGVVL	100
Human	100	LLSIYFYYSLPNAVGPFFTWMALLGLSQALNILLGLKGLAPAEISAVCE	149
		: .:. . . .:. . . . : : . . . : . .:	
Bat	101	LVSFFFYTSLPNIDDLPLTWMLAHLGLSEALNILLGLRGLTPAEVSTVCE	150
Human	150	KGNFNVAGHLAWSYYIGYLRILPELQARIRTYNQHYNNLLRGAVSQRLY	199
		:.:. . . : ::. . .:. . .:	
Bat	151	QRHFNVAGHLAWSYYIGYLRILPGLRARIHTYNQLHGNTLQGVGSHRLY	200
Human	200	ILLPLDCGVDPNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYELLE	249
		. . : . : : .: . : : .: : : : : :	
Bat	201	ILFPLDCGVLDLSDAADPNIRFLRELPRQSSDRAGIKNRVYTNSVYELLE	250
Human	250	NGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLEDIL	299
		. :.. . . : : .: . : : .: : : : : : : :	
Bat	251	KGKPVGTVCVLEYATPLQTLFAMSQDARAGFSQEDRLEQAKLFCRTLADIL	300
Human	300	ADAPESQNNCRLIAYQEPADDSSFSLSQEVLRLRHLRQEEKEEVTVGSLKTS	349
		. .: : . . .: . .: : : : : : : : : :	
Bat	301	ADDPESQKSCRLIVYQEPTEESDFSLSQAILKHLRQEEKEEVTVGTVGTY	350
Human	350	AVPSTSTMSQEPPELLISGMEKPLPLRTDFS	379
		.. .:. : . : : : : : : : : : :	
Bat	351	EAPGSSTLHQEPPELLISGMDQPLPLRTDIF	380

FIGURE 10

11/14

Human and manatee STING:

Human	1	---MPHSSLHPSIPCPRGHGAQKAALVLLSACLVTWGLGEPPEHTLRYL	47
Manatee	1	MVEMPHSSLHPSIPRHRHGIGQKAAFVLLVACLVALWELGEPGHTLQWL	50
Human	48	VLHLASLQLGLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCP LRGA	97
		..	
Manatee	51	VCQLASLQLGLLLKVICSLAEELCHVHSRYQSSYWRAVRACMGCP IRRGA	100
Human	98	LLLLSIYFYSLPNAVGPPTWMLALLGLSQALNILLGLKGLAPAEISAV	147
Manatee	101	LLLLSCYFYFCFPMADLPLTWTLALLGLLQAMNILLSLQGLTPAEISAI	150
Human	148	CEKGNFNVAHGLAWSYYIGYLRILPELQARITYNQHYNNLLRGAVSQR	197
		.	
Manatee	151	CEKRNLNVAHGLAWSYYIGYLRILPGLQDRIRIYNLQHNMLRGTSQR	200
Human	198	LYILLPLDCGVDPNLSMADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYEL	247
		: ..	
Manatee	201	LHILFPLDCGVDPNLSVADANIRFLQKLPQQSADCAIKGRVYTNSVYQL	250
Human	248	LENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLED	297
		: ..	
Manatee	251	LEHGQPAGICVLEYATPLQTLFAMSQDGRAGFSREDRLEQAKLFCRTLED	300
Human	298	ILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRLRLRQEEKEEVTVGSLK	347
Manatee	301	ILADAPEYQNNCR LIVYQESAEGSSFSLSQEILRLRLRQEERE ETVGSVG	350
Human	348	TSAV-----PSTSTMSQEP ELLISGMEKPLPLRTDFS	379
		.	
Manatee	351	TSVVPSPPSPSTSSLSQEPKLLISGMEQPLPLRTDVF	387

FIGURE 11

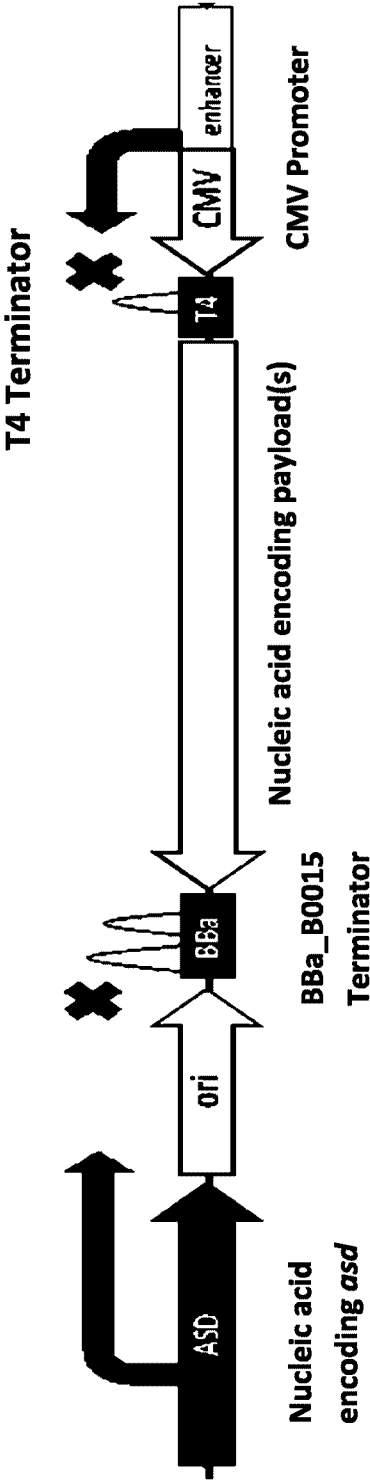
Human and ghost shark STING:

Human	1	MPHSSLHPSPICPRGHGAQKAALVLLS--ACLVTLWGLGPEPHTLRYLV	48
GhostShark	1	-----MSDSIPRPRGKVATYVGLILMSGLACLYEML----TPDYHIKFIV	41
Human	49	LHLASLQL-----GLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPL	93
GhostShark	42	QQVAQ-QLIVSFFALCLRLCEFVEELGHKQTRYQNSWVRALNASLDWK-	89
Human	94	RRGALLLSIYFYFYSLPNAVGPFTWMLALLGLSQALNI-----LL	134
GhostShark	90	KYGFCLLISLLFNWVLPYEKHSY---AVPTVGQTISLTCFCVFLFKVF	135
Human	135	GLKGLAPAEISAVCEKGNFNAHGLAWSYYIGYLRLLPELQARIRTYNQ	184
GhostShark	136	QLDVLSPAQISEISETNKCNAHGLAWSYYLGYLKIVLPKLEEKINQYHR	185
Human	185	HYNNLLRGAVSQRLYILLPLDCGVDPNLSMADPNIRFLDKLPQQTGDRAG	234
GhostShark	186	EYGNVLQ-QKGKKLYILIPFSCKVLDKLEKVDTNIVFYQNLPELLVDRAG	234
Human	235	IKDRVYSNSIYELL-ENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSRED	283
GhostShark	235	IKRSRYKHSIYLIYDQKKQQPHHCILEYATPLRSLFEMTNDNSAAAFSKEQ	284
Human	284	RLEQAKLFCRTLEDILADAPESQNNCRLLIAYQEPADSSFS---LSQEVL	330
GhostShark	285	RLDQAKLFYRTLKSLNNVPEVTGSYRLIPYDDLEGAELGPHFVSEIIL	334
Human	331	RHLRQEEKEEVTGSLKTSAVPSTSTMSQEPELLISGMEKPLPLRTDFS	379
GhostShark	335	KHMRQELTEYPVA---EPSNANETDCMSSEPHLMIS--DDPKPLRSYCP	378

FIGURE 12

Human	1	MPHSSLHPSPICPRGHGAQKAALVLLSACLVTLWGLGEPPEHTLRYLVLH	50
Mouse	1	MPYSNLHPAIPRPRGHRSKYVALIFLVASLMILWVAKDPPNHTLKYLAH	50
Human	51	LASLQLGLLLNGVCSLAEELRHIHSRYRGSYWRTVRACLGCPLRGALLL	100
Mouse	51	LASHELGLLLLKNLCCLAEEELCHVQSRYSYQGSYWKAVRACLGCPHMCAMIL	100
Human	101	LSIYFYSLPNAVGPFFTWMALLGLSQALNILLGLKGLAPAEISAVCEK	150
Mouse	101	LSSYFYF-LQNTADIYLSWMFGLLVLYKSLSMLLGLQSLTPAEVSAVCEE	149
Human	151	GNFNVAHGLAWSYIYGRLRILPELQARIRTYNQHYNNLLRGAVSQRLYI	200
Mouse	150	KKLNVAHGLAWSYIYGRLRILPGLQARIRMFNLHNNMLSGAGSRRLYI	199
Human	201	LLPLDCGVPDNLMSADPNIRFLDKLPQQTGDRAGIKDRVYSNSIYELLEN	250
Mouse	200	LFPLDCGVPDNLVVDPNIRFRDMLPQQNIDRAGIKNRVYSNSVYEILEN	249
Human	251	GQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLQAKLFCRTLEDILA	300
Mouse	250	GQPAGVCILEYATPLQTLFAMSQDAKAGFSREDRLQAKLFCRTLEEILE	299
Human	301	DAPESQNNCRLIAYQEPADSSFSLSQEVLRHLRQEEKEEVTVGSLKTS	350
Mouse	300	DVPESRNNCR LIVQEPTDGNFSFSLSQEVLRHLRQEEKEEVTMNAPMTSV	349
Human	351	VPSTSTMSQPELLISGMEKPLPLRTDFS	379
Mouse	350	APPPSVLSQEPRLISGMDQPLPLRTDLI	378

FIGURE 13



Plasmid construct in which the expression cassette is reversed on the plasmid and is followed by the BBa_B0015 terminator, and the T4 terminator is inserted downstream of the CMV promoter.

FIGURE 14