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**Declarations under Rule 4.17:**
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

(54) Title: ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF

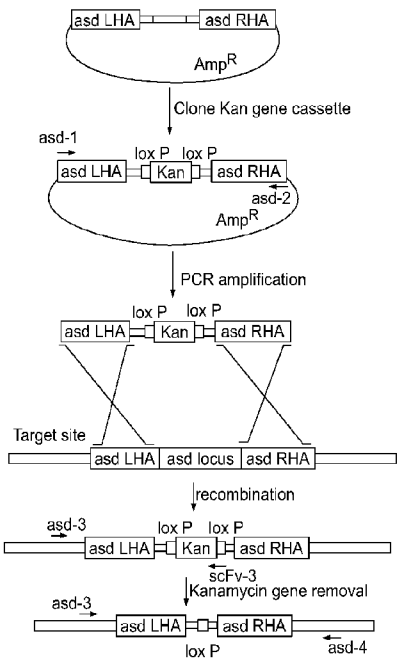



Figure 1

(57) Abstract: Provided are immunostimulatory bacteria and pharmaceutical compositions containing the bacteria. The immunostimulatory bacteria provided herein contain one or more modalities that enhance the anti-tumor activity of the immunostimulatory bacteria. Among the immunostimulatory bacteria provided are bacteria, such as Salmonella species, which are modified to be auxotrophic or are auxotrophic for adenosine and/or contain plasmids encoding RNAi, such as shRNA and micro RNA, that mediate gene disruption and/or expression of immune checkpoints, such as TREX1, VISTA, PD-L1 and, genes that influence the immune system. The bacteria contain additional modifications to enhance their anti -tumor activity. Also provided are methods of inhibiting the growth or reducing the volume of a solid tumor by administering the pharmaceutical compositions.

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[Continued on next page]

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# ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF

## RELATED APPLICATIONS

Benefit of priority is claimed to U.S. Provisional Application Serial No. 62/531,327, filed July 11, 2017, to Christopher D. Thanos and Laura Hix Glickman, and entitled "ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF." Benefit of priority also is claimed to U.S. Provisional Application Serial No. 62/648,380, filed March 26, 2018, to Christopher D. Thanos, Laura Hix Glickman, and Justin Skoble, entitled "ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF."

This application is related to U.S. Application. Serial No. (Attorney Docket No.: 62131.01701.US03/1701), filed the same day herewith, entitled "ENGINEERED IMMUNOSTIMULATORY BACTERIAL STRAINS AND USES THEREOF," which claims priority to U.S. Provisional Application Serial Nos. 62/531,327 and 62/648,380.

Where permitted, the subject matter of each of these applications is incorporated by reference in its entirety.

## INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED ELECTRONICALLY

An electronic version of the Sequence Listing is filed herewith, the contents of which are incorporated by reference in their entirety. The electronic file was created on July 11, 2018, is 410 kilobytes in size, and is titled 1701SEQPC001.txt.

## BACKGROUND

The field of cancer immunotherapy has made great strides, as evidenced by clinical successes of anti-CTLA4, 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.* (2015) *J. Clin. Invest.* 125:3392-4000; and Chen *et al.* (2015) *J. Clin. Invest.* 125:3384-3391). Tumors have evolved a profoundly immunosuppressive environment. They initiate multiple mechanisms to evade immune surveillance, reprogram anti-tumor immune cells to suppress immunity, and continually mutate resistance to the latest cancer therapies (see, *e.g.*, Mahoney *et al.* (2015) *Nat. Rev. Drug Discov.* 14(8):561-584). Designing immunotherapies that overcome immune

tolerance and escape, while limiting the autoimmune-related toxicities of current immunotherapies, challenges the field of immuno-oncology. Hence additional and innovative immunotherapies and other therapies are needed.

**SUMMARY**

Provided are bacteria modified to be immunostimulatory for anti-cancer therapy. Immunostimulatory bacteria, as provided herein, provide a multi-faceted approach to anti-tumor therapy. As provided herein, bacteria, such as species of *Salmonella*, can be fine-tuned to have potent anti-tumor activity. Bacteria provide a platform in which there are numerous avenues for eliciting anti-tumor immunostimulatory activity. The bacteria contain plasmids that encode anti-cancer therapeutics, such as RNA, including microRNA, shRNA, and siRNA, that are designed to suppress, inhibit, disrupt or otherwise silence immune checkpoint genes and products, and other targets that play a role in pathways that are immunosuppressive and pathways that are immunostimulatory and improve an anti-tumor response, such as Stimulator of Interferon Genes (STING) and cGAS. Bacteria by their nature stimulate the immune system; bacterial infection induces immune and inflammatory pathways and responses, some of which are desirable for anti-tumor treatment, and others, are undesirable. Modification of the bacteria by deleting or modifying genes and products that result in undesirable inflammatory response, and genes that induce desirable immunostimulatory anti-tumor responses can improve the anti-tumor activity of the bacteria. Bacteria also accumulate in tumor cells and tissues, and by replicating therein can lyse cells. Bacteria migrate from the sites of administration and can accumulate other tumors and tumor cells to provide an abscopal effect. Herein, all of these properties of bacteria are exploited to produce demonstrably immunostimulatory bacteria with a plurality anti-tumor activities and properties that can act synergistically.

Provided are compositions, uses thereof and methods that modulate immune responses for treatment of diseases, including for treatment of cancer. The compositions contain immunostimulatory bacteria provided herein. Methods of treatment and uses of the bacteria for treatment also are provided. The subjects for treatment include humans and other primates, pets, such as dogs and cats, and other animals, such as horses.

Provided are pharmaceutical compositions containing the immunostimulatory bacteria, and methods and uses thereof for treatment of diseases and disorders, particularly proliferative disorders, such as tumors, including solid tumors.

Also provided are methods of inhibiting the growth or reducing the volume of a solid tumor by administering the immunostimulatory bacteria or pharmaceutical compositions or using the compositions for treatment. For example, provided are methods of administering or using a composition that contains, for a single dosage, an effective amount of an attenuated *Salmonella* sp. to a subject, such a human patient, having a solid tumor cancer.

It is understood that all of the RNAis and modifications of the bacteria and the plasmids described can be combined in any desired combination. So reference to immunostimulatory bacteria refers to bacteria that include RNAi against at least one target and that can have any or all of the modifications described herein.

Provided are immunostimulatory bacteria that contain a sequence of nucleotides encoding RNA (RNAi) that inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increases the anti-tumor immune response in a subject; the RNA is encoded on a plasmid in the bacterium; and the immunostimulatory bacterium is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$).

For purposes herein RNAi includes all forms of double stranded RNA that can be used to silence expression of targeted nucleic acids. RNAi includes shRNA, siRNA and micro RNA. Any of these forms can be interchanged in the embodiments disclosed and described herein. In general, the RNAi is encoded on a plasmid in the bacterium. The plasmids can include other heterologous nucleic acids that encode products of interest that modulate or add activities or products to the bacterium, or other such products that can modulate the immune system of a subject to be treated with the bacterium. Bacterial genes also can be added, deleted or disrupted. These genes can encode products for growth and replication of the bacteria, or products that also modulate the immune response of the host to the bacterium.

Also provided are immunostimulatory bacteria that contain a sequence of nucleotides encoding RNA (RNAi) that inhibits, suppresses or disrupts expression of three prime repair exonuclease 1 (TREX1), and is auxotrophic for adenosine. Also

provided are immunostimulatory bacterium that contain a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of VISTA (the gene encoding V-domain Ig suppressor of T cell activation), and is auxotrophic for adenosine. Also provided are immunostimulatory bacteria that comprise a sequence of nucleotides encoding RNA that inhibits, suppresses, disrupts expression of programmed death-ligand 1(PD-L1).

Among these immunostimulatory bacteria are those of *Salmonella* species. These include *Salmonella* that contain nucleic acid that encodes an RNA that inhibits or suppresses, disrupts or silences expression of three prime repair exonuclease 1 (TREX1) and/or VISTA.

Also provided are immunostimulatory bacteria that contain a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of three prime repair exonuclease 1 (TREX1), and a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of PD-L1.

Also provided are immunostimulatory bacteria that contain a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of VISTA, and a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of PD-L1.

Provided are immunostimulatory bacteria, such as *S. typhimurium*, carrying plasmids encoding RNAi, such as miRNA or shRNA, that mediate gene disruption of one or more of TREX1, VISTA and PD-L1 and other such targets known to those of skill in the art and/or enumerated or exemplified herein. Bacterial species that carry such plasmids, 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*.

Species include, for example, strains of *Salmonella, Shigella, E. coli, Bifidobacteriae, Rickettsia, Vibrio, Listeria, Klebsiella, Bordetella, Neisseria, Aeromonas, Francisella, Cholera, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Bacillus*, and *Erysipelothrix*, or an attenuated strain thereof or modified strain thereof of any of the preceding list of bacterial strains.

Other suitable bacterial species include *Rickettsia, Klebsiella, Bordetella, Neisseria, Aeromonas, Franciesella, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Vibrio, Bacillus*, and *Erysipelothrix*. For example, *Rickettsia Rikettsiae, Rickettsia prowazekii, Rickettsia tsutsugamuchi, Rickettsia mooseri, Rickettsia sibirica, Bordetella bronchiseptica, Neisseria meningitidis, Neisseria gonorrhoeae, Aeromonas eucrenophila, Aeromonas salmonicida, Franciesella tularensis, Corynebacterium pseudotuberculosis, Citrobacter freundii, Chlamydia pneumoniae, Haemophilus sornnus, Brucella abortus, Mycobacterium intracellulare, Legionella pneumophila, Rhodococcus equi, Pseudomonas aeruginosa, Helicobacter mustelae, Vibrio cholerae, Bacillus subtilis, Erysipelothrix rhusiopathiae, Yersinia enterocolitica, Rochalimaea quintana*, and *Agrobacterium tumerfacium*.

*Salmonella* is exemplified herein, and particularly *Salmonella typhimurium* strain, such as the strain designated YS1646 (ATCC #202165) or VNP20009. Other strains include, RE88, SL7207, χ 8429, χ 8431, and χ 8468. Exemplary of modified *Salmonella* strains provided herein are immunostimulatory bacterium strains AST-104, AST-105, AST-106, AST-108, AST-110, AST-112, AST-113, AST-115, AST-117, AST-118, AST-119, AST-120, AST-121, AST-122, and AST-123. Sequences thereof and descriptions are provided in the detailed description, examples and sequence listing. The immunostimulatory bacteria can be derived from attenuated strains of bacteria or they become attenuated by virtue of the modifications described herein, such as deletion of *asd*, whereby replication is limited *in vivo*.

The immunostimulatory bacteria provided herein encode inhibitors of various genes and/or expression of genes and/or gene products that contribute to reduced anti-tumoral immune responses and/or products that stimulate the immune system, and thereby are immunostimulatory. As described herein, inhibition of TREX1 is immunostimulatory, as is inhibition of PD-L1. Adenosine auxotrophy also is immunostimulatory. Provided are inhibitory RNA (RNAi), such as shRNA or microRNA or siRNA, targeted for disruption or inhibition of expression of TREX1, PD-L1, VISTA (the gene encoding V-domain Ig suppressor of T cell activation), TGF-beta, and CTNNB1 (the gene that encodes β-catenin) among others, combinations thereof and combinations thereof with any shRNAs that inhibit or

disrupt expression of other immune suppressive genes whose expression is activated, or enhanced by tumors or the tumor microenvironment (TME). Expression of these RNA exploits two independent immunostimulatory pathways, and leads to enhanced tumor colonization in a single therapy. The effects of this combination are enhanced by the strains provided herein that are auxotrophic for adenosine, which provides preferential accumulation in or recruitment into adenosine-rich immunosuppressive tumor microenvironments. Reducing adenosine in such TMEs further enhances the immunostimulatory effects. Such combinations of traits in any of the bacterial strains known or that can be engineered for therapeutic administration provide similar immunostimulatory effects.

Among the targets is TGF-beta, which has three isoforms: 1, 2 and 3. Among the targets is TGF-beta, particularly isoform 1, and not isoforms 2 and 3. Toxicities are associated with isoforms 2 and 3. For example, cardiac valve toxicity is associated with inhibition of isoform 2. Isoform 1 is present in most cancers (see, *e.g.*,TCGA database). It is advantageous to inhibit only isoform 1. RNAi can be advantageously employed for this purpose, since it can be designed to very specifically recognize a target. For TGF-beta, specific inhibition of isoform 1 can be effected by targeting a sequence unique to isoform 1 (see, *e.g.*, the RNA against TGF-beta isoform 1 in Example 2) that is not present in isoform 2 or 3, or to select a sequence to target isoforms 1 and 3, and not 2. Also provided are immunostimulatory bacteria in which the plasmid encodes an shRNA or microRNA that specifically inhibits, suppresses or disrupts expression of TGF-beta isoform 1 but not TGF-beta isoform 2 or TGF-beta isoform 3; or the plasmid encodes an shRNA or microRNA that specifically inhibits, suppresses or disrupts expression of TGF-beta isoforms 1 and 3, but not isoform 2.

Also, RNAi, such a miRNA or shRNA-mediated gene disruption of PD-L1 provided by immunostimulatory bacteria provided herein also improves colonization. It has been shown that knockout of PD-L1 enhances *S. typhimurium* infection. For example, an at least 10-fold higher bacterial load in PD-L1 knockout mice than in wild-type mice has been observed, indicating that PD-L1 is protective against *S. typhimurium* infection (see, *e.g.*, Lee *et al.* (2010) *Immunol.* 185:2442-2449).

Engineered immunostimulatory bacteria, such as the *S. typhimurium* immunostimulatory bacteria, provided herein contain multiple synergistic modalities

to induce immune re-activation of cold tumors to promote tumor antigen-specific immune responses, while inhibiting immune checkpoint pathways that the tumor utilizes to subvert and evade durable anti-tumor immunity. Included in embodiments is adenosine auxotrophy and enhanced vascular disruption. This improvement in tumor targeting through adenosine auxotrophy and enhanced vascular disruption increases potency, while localizing the inflammation to limit systemic cytokine exposure and the autoimmune toxicities observed with other immunotherapy modalities.

Provided are immunostimulatory bacteria that are auxotrophic for adenosine and/or target the TREX1 gene, such as encoding a double-stranded RNA, such as an shRNA or miRNA that inhibits expression thereof, and optionally encodes additional RNAs, such as miRNA or shRNA, that target and inhibit expression of other checkpoint inhibitors. Among these bacteria are immunostimulatory bacteria that are auxotrophic for adenosine. Methods of treatment and uses for treatment of tumors, including solid tumors and hematologic malignancies are provided. Among the methods and uses are those in which the immunostimulatory bacteria are auxotrophic for adenosine and the uses and treatments treat tumors that are cd73+ and/or cd73+/cd39+.

The RNAs are expressed under the control of promoters that are recognized by the eukaryotic host cell transcription machinery, such as RNA polymerase II (RNAPII) and RNA polymerase III (RNAPIII) promoters. RNAP III promoters generally are constitutively expressed in a eukaryotic host; RNAP II promoters can be regulated. The RNAs, such as miRNA and shRNA, are provided on plasmids stably expressed by the bacteria. Exemplary of such bacteria are *Salmonella* strains, generally attenuated strains, either attenuated by passage or other methods or by virtue of modifications described herein, such as adenosine auxotrophy. Exemplary of the bacteria are *Salmonella* strains. Exemplary of *Salmonella* strains are modified *S. typhimurium* strains that contain an *asd* mutation for antibiotic-free selection. These strains also can contain the *asd* mutation.

The promoters can be selected for the environment of the tumor cell, such as a promoter expressed in a tumor microenvironment (TME), such as a promoter expressed in hypoxic conditions, or in conditions where the pH is less than 7.

Provided are strains of bacteria that contain miRNA or shRNA against the TREX1 or VISTA gene. The TREX1 or VISTA gene can be under control of an RNAPIII promoter, such as the H1 promoter. TREX1 knockdown induces vascular disruption, which increases colonization, and also decreases immune suppression. The strains provided herein can include miRNA or shRNA that inhibits expression of other checkpoint inhibitors, including, but not limited to PD-L1. Strains that include a plurality of RNAs, such as miRNA or shRNAs, generally include different promoters, for each RNA. For example, the bacterium can include a genetically modified *S. typhimurium* strain that contains miRNA or shRNA under control of the U6 promoter against the PD-L1 gene and also contains miRNA or shRNA against TREX1 under control of the H1 promoter. Also provided are genetically modified *S. typhimurium* strains that contain miRNA or shRNA against the SIRP-α gene under control of the H1 promoter. The exemplary bacteria, such as *S. typhimurium* strains, can contain miRNA or shRNA against the β-catenin gene under control of an RNAPIII promoter, such as the H1 promoter and/or miRNA or shRNA against the VISTA gene under control of an RNAPIII promoter, such as the H1 promoter. Various combinations of adenosine auxotrophy, miRNA or shRNA against TREX1, and/or optionally against other immune checkpoint targets, such as RNA that inhibits, suppresses or disrupts PD-L1 or one or both of TREX1 and PD-1 or VISTA, can be included in the modified immunostimulatory bacteria.

Provided are immunostimulatory bacteria that are cGAS agonists. Exemplary of such bacteria is *S. typhimurium* that is one or both of a cGAS agonist and Stimulator of Interferon Genes (STING) agonist. These can be administered, for example, in uses and methods, such as radiotherapy and chemotherapy, in which cytosolic DNA is produced or accumulates. STING activates innate immunity in response to sensing nucleic acids in the cytosol. Downstream signaling is activated through binding of cyclic dinucleotides (CDNs), which are synthesized by bacteria or by host enzyme cGAS in response to binding to cytosolic dsDNA. Bacterial and host-produced CDNs have distinct phosphate bridge structures, which differentiates their capacity to activate STING. CDNs are synthesized by bacteria or by host enzyme cGAS in response to binding cytosolic dsDNA. IFN-β is the signature cytokine of activated STING.

The plasmids in any of the bacteria described and enumerated above and herein contain plasmids that encode the RNAi and other heterologous nucleic acid. Plasmids can be present in many copies or fewer. This can be controlled by selection of elements, such as the origin of replication. Low and high and medium copy number plasmids and origins of replication are well known to those of skill in the art and can be selected. In embodiments of the immunostimulatory bacteria here, the plasmid can be present in low to medium copy number, such as about 150 or 150 and fewer copies, to low copy number which is less than about 25 or about 20 or 25 copies. Exemplary origins are those derived from pBR322, p15A, pSC101, pMB1, colE1, colE2, pPS10, R6K, R1, RK2, and pUC.

As discussed, the plasmids can include RNAi such that the RNA inhibits, suppresses or disrupts expression of an immune checkpoint or other target and additionally their products. Among these are sequences of nucleic acids encoding listeriolysin O (LLO) protein lacking the signal sequence (cytoLLO), a CpG motif, a DNA nuclear targeting sequence (DTS), a deletion of the gene encoding a flagellin subunit(s), and a retinoic acid-inducible gene-I (RIG-I) binding element.

The immunostimulatory bacteria provided herein can be aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$), which permits growth in DAP supplemented medium, but limits replication *in vivo* when administered to subjects for treatment. Such bacteria will be self-limiting, which can be advantageous for treatment. The bacterium can be *asd*$^-$ by virtue of disruption or deletion of all or a portion of the endogenous gene encoding aspartate-semialdehyde dehydrogenase (*asd*), whereby the endogenous *asd* is not expressed. In other embodiments, the gene encoding *asd* can be included on the plasmid for expression *in vivo*.

Any of the immunostimulatory bacteria provided herein can include nucleic acid, generally on the plasmid, that includes a CpG motif or a CpG island, wherein the motif is recognized by toll-like receptor 9 (TLR9). Nucleic acid encoding CpG motifs or islands are plentiful in prokaryotes, and, thus, the CpG motif can be included in or part of a bacterial gene that is encoded in the plasmid. The bacterial gene that encodes *asd* contains immunostimulatory CpGs.

The immunostimulatory bacteria provided herein can be auxotrophic for adenosine or adenosine and adenine. Any of the bacteria herein can be rendered

autotrophic for adenosine, which advantageously can increase the anti-tumor activity, since adenosine accumulates in many tumors, and is immunosuppressive.

The immunostimulatory bacteria provided herein can be flagellin deficient, where the wild-type bacterium comprises flagella. They can be rendered flagellin deficient by disrupting or deleting all or a part of the gene or genes that encode flagella. For example, provided are immunostimulatory bacteria that have deletions in the genes encoding one or both of flagellin subunits *fliC* and *fljB*, whereby the bacteria is flagella deficient.

The immunostimulatory bacteria provided herein can include a nucleic acid encoding cytoLLO, which is a listeriolysin O (LLO) protein lacking the periplasmic secretion signal sequence so that it accumulates in the cytoplasm. This mutation is advantageously combined with *asd*- bacteria. LLO is a cholesterol-dependent pore forming hemolysin from *Listeria monocytogenes* that mediates phagosomal escape of bacteria. When the autolytic strain is introduced into tumor bearing hosts, such as humans, the bacteria are taken up by phagocytic immune cells and enter the vacuole. In this environment, the lack of DAP prevents bacterial replication, and results in autolysis of the bacteria in the vacuole. Lysis then releases the plasmid and the accumulated LLO forms pores in the cholesterol-containing vacuole membrane and allows for delivery of the plasmid into the cytosol of the host cell.

The immunostimulatory bacteria can include a DNA nuclear targeting sequence (DTS), such as an SV40 DTS, encoded on the plasmid.

The immunostimulatory bacteria can have a deletion or modification in the gene encoding endonuclease-I (*endA*), whereby *endA* activity is inhibited or eliminated. Exemplary of these are immunostimulatory bacteria that contain one or more of a CpG motif, an *asd* gene selectable marker for plasmid maintenance and a DNA nuclear targeting sequence.

The immunostimulatory bacteria can contain nucleic acids on the plasmid encoding two or more different RNA molecules that inhibit, suppress or disrupt expression of an immune checkpoint or an RNA molecule that encodes an inhibitor of a metabolite that is immunosuppressive or is in an immunosuppressive pathway.

The nucleic acids encoding the RNAi, such as shRNA or miRNA or siRNA can include a transcriptional terminator following the RNA-encoding nucleic acid.

In all embodiments, the RNAi encoded on the plasmid in the immunostimulatory bacteria can be short hairpin RNA (shRNA) or micro-RNA (miRNA).

The immunostimulatory bacteria contain RNAi that inhibits, suppresses or disrupts expression or silences expression of immune checkpoints and other targets whose inhibition, disrupting or silencing is immunostimulatory. These targets include, but are not limited to, one or more of three prime repair exonuclease 1 (TREX1), PD-1, PD-L1 (B7-H1), VEGF, TGF-beta isoform 1, Beta-catenin, CTLA-4, PD-L2, PD-1, PD-2, IDO1, IDO2, SIRPα, CD47, VISTA (B7-H5), LIGHT, HVEM, CD28, LAG3, TIM3, TIGIT, Galectin-9, CEACAM1, CD155, CD112, CD226, CD244 (2B4), B7-H2, B7-H3, ICOS, GITR, B7-H4, B7-H6, CD27, CD40/CD40L, CD48, CD70, CD80, CD86, CD137( 4-1BB), CD200, CD272 (BTLA), CD160, CD39, CD73, A2a receptor, A2b receptor, HHLA2, ILT-2, ILT-4, gp49B, PIR-B, HLA-G, ILT-2/4, OX40/OX-40L, BTLA, KIR, TIM1, TIM4 and STAT3, Stabilin-1 (CLEVER-1), DNASE II and RNASE H2. For example, any of the immunostimulatory bacteria can contain RNA that inhibits, suppresses or disrupts expression of one or a combination of TREX1, PD-L1, VISTA, TGF-beta, such as TGF-beta isoform 1 or isoforms 1 and 3, beta-catenin, SIRP-alpha, VEGF, RNase H2, DNase II, and CLEVER-1/Stabilin-1.

Immunostimulatory bacteria where the plasmid comprises a sequence of nucleotides that encodes RNA that inhibits, suppresses or disrupts expression of at least two targets, and each RNA is expressed from a different promoter, are provided. Exemplary of these are where the targets for inhibition, suppression or disruption combinations are at least two that are selected from among TREX1 and PD-L1, TREX1 and PD-1, TREX1 and VISTA, TREX1 and SIRP-alpha, PD-L1 and TGF-beta isoform 1, PD-L1 and beta-catenin, PD-L1 and VISTA, TGF-beta isoform 1 and VISTA, SIRP-alpha and VISTA and TREX1 and RNASE H2.

Other combinations of RNAi, include RNAi that inhibits, suppresses or disrupts expression of one or a combination of TREX1, PD-L1, VISTA, TGF-beta isoform 1, beta-catenin, SIRP-alpha, VEGF, RNase H2, DNase II, and CLEVER-1/Stabilin-1. Other combinations include those where the target for inhibition, suppression or disruption is a combination of at least two that are selected from

among TREX1 and PD-L1, TREX1 and PD-1, TREX1 and VISTA, TREX1 and SIRP-alpha, PD-L1 and TGF-beta isoform 1, PD-L1 and beta-catenin, PD-L1 and VISTA, TGF-beta isoform 1 and VISTA, SIRP-alpha and VISTA, TREX1 and RNASE H2, VISTA and RNASE H2, and VISTA and DNASE H2, or TREX1 and SIRPα, or TREX1 and VISTA, or TREX1 and VEGF, or PD-L1 and β-catenin, or PD-L1 and TGF-beta isoform 1, or PD-L1 and VEGF, or TREX and PD-1.

The immunostimulatory bacterium can also include nucleic acids encoding RNA that inhibits, suppresses or disrupts expression of another different immune checkpoint or target to be inhibited, suppressed or disrupted, selected from among any of CTLA-4, PD-L1 (B7-H1), PD-L2, PD-1, PD-2, IDO1, IDO2, SIRPα, CD47, VISTA (B7-H5), VEGF, TGF-beta, LIGHT, HVEM, CD28, LAG3, TIM3, TIGIT, Galectin-9, CEACAM1, CD155, CD112, CD226, CD244 (2B4), B7-H2, B7-H3, ICOS, GITR, B7-H4, B7-H6, CD27, CD40/CD40L, CD48, CD70, CD80, CD86, CD137( 4-1BB), CD200, CD272 (BTLA), CD160, CD39, CD73, A2a receptor, A2b receptor, HHLA2, ILT-2, ILT-4, gp49B, PIR-B, HLA-G, ILT-2/4, OX40/OX-40L, BTLA, KIR, TIM1, TIM4, STAT3, CLEVER-1, DNASE II and RNASE-H2. Exemplary thereof are among human PD-L1 (SEQ ID NO:31), human Beta-catenin (SEQ ID NO:32), human SIRPα (SEQ ID NO:33), human TREX1 (SEQ ID NO:34), human VISTA (SEQ ID NO:35), human TGF-beta isoform 1 (SEQ ID NO:193), and human VEGF (SEQ ID NO:194). RNA can target or contain a sequence in the immune checkpoint nucleic acid set forth in any of SEQ ID NOs.: 1-30, 36-40, and 195-217.

The plasmids in any of the immunostimulatory bacteria also can encode a sequence of nucleotides that is an agonist of retinoic acid-inducible gene I (RIG-I) or a RIG-I binding element.

The immunostimulatory bacteria can include one or more of deletions in genes, such as one or more of *purI (purM), msbB*$^-$, *purD*$^-$, flagellin$^-$ *(fliC*$^-$*/fljB*$^-$*), pagP*$^-$, *adrA*$^-$, *CsgD*$^-$ and *hilA*$^-$. The immunostimulatory bacteria can be *msbB*$^-$. For example, the immunostimulatory bacteria can contain a *purI* deletion, an *msbB* deletion, an *asd* deletion, and *adrA* deletion, and optionally a *CsgD* deletion. Exemplary of bacterial gene deletions are any of the following:

one or more of a mutation in a gene that alters the biosynthesis of lipopolysaccharide selected from among one or more of *rfaL*, *rfaG*, *rfaH*, *rfaD*, *rfaP*, *rFb*, *rfa*, *msbB*, *htrB*, *firA*, *pagL*, *pagP*, *lpxR*, *arnT*, *eptA*, *and lpxT*; and/or

one or more of a mutation that introduces a suicide gene and is selected from one or more of *sacB*, *nuk*, *hok*, *gef*, *kil* or *phlA*; and/or

one or more of a mutation that introduces a bacterial lysis gene and is selected from one or both of *hly* and *cly*; and/or

a mutation in one or more virulence factor(s) selected from among *IsyA*, *pag*, *prg*, *iscA*, *virG*, *plc* and *act*; and/or

one or more mutations that modify the stress response selected from among *recA*, *htrA*, *htpR*, *hsp* and *groEL*; and/or

a mutation in *min* that disrupts the cell cycle; and/or

one or more mutations that disrupt or inactivate regulatory functions selected from among *cya*, *crp*, *phoP/phoQ*, and *ompR*.

As described, the RNAi includes shRNA and miRNA. Exemplary of an miRNA backbone into which the RNA that encodes the target or complement thereof is inserted is one based on miR-16-2 (SEQ ID NO:248), or the miRNA backbone of SEQ ID NO:249. The immunostimulatory bacteria can include miR-103 (SEQ ID NO:252), where mature miR-103 comprises the sequence: 5'-AGCAGCAUUGUACAGGGCUAUGA-3.'

The RNAi can be expressed under control of an RNA polymerase III or RNA polymerase II promoter. Generally shRNA is expressed under control of an RNAP III promoter; and miRNA is expressed under control of an RNAP II promoter. Many RNAP III and II promoters are known and available to those of skill in the art. RNAP III promoters include, for example, U3, H1, U6, 7SK and 7SL; and RNAP II promoters include viral promoters, a cytomegalovirus SV40 promoter, and adenovirus promoters. Many viral promoters, particularly later promoters, are strong constitutive promoters.

The immunostimulatory bacterium can be a strain of *Salmonella*, *Shigella*, *E. coli*, *Bifidobacteriae*, *Rickettsia*, *Vibrio*, *Listeria*, *Klebsiella*, *Bordetella*, *Neisseria*, *Aeromonas*, *Francisella*, *Cholera*, *Corynebacterium*, *Citrobacter*, *Chlamydia*, *Haemophilus*, *Brucella*, *Mycobacterium*, *Mycoplasma*, *Legionella*, *Rhodococcus*,

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

Exemplary of the immunostimulatory bacteria are those where the plasmid contains one or more of sequence of nucleic acids encoding a listeriolysin O (LLO) protein lacking the signal sequence (cytoLLO), a CpG motif, a DNA nuclear targeting sequence (DTS), a deletion of the gene encoding a flagellin subunit(s), and a retinoic acid-inducible gene-I (RIG-I) binding element.

Where the plasmid contains two or more encoding RNAs that inhibit, suppress or disrupt expression, each is separated by at least about 75 nucleotides, or at least 75 nucleotides, up to about or at least 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500 nucleotides (or base pairs), up to about 1600 or 1600 nucleotides (or base pairs), or between 75-1500 or 1600 nucleotides (or base pairs).

Other exemplary immunostimulatory bacteria include those that are auxotrophic for adenosine, and comprise: a deletion in the gene(s) encoding the flagella; a deletion in *endA*; a plasmid that encodes CytoLLO; a nuclear localization sequence; and an *asd* plasmid complementation system; and encode RNA that inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increases the anti-tumor immune response in a subject.

Such immunostimulatory bacteria include strains of *Salmonella*, such as a *Salmonella typhimurium* strain, such as for example, an attenuated *Salmonella typhimurium* strain selected from among strains designated as AST-100, VNP20009, or strains YS1646 (ATCC #202165), RE88, SL7207, χ 8429, χ 8431, and χ 8468.

The immunostimulatory bacterium can contain a plasmid encoding an shRNA encoded by the sequence of nucleotides set forth in any of SEQ ID NOs: 36-40 and 75-78, or an miRNA encoded by the sequence of nucleotides set forth in any of SEQ ID NOs: 214-217.

Any of the immunostimulatory bacteria are those that, when grown, are harvested at stationary phase. Methods of producing the immunostimulatory bacteria include those that are cultured by standard methods, and harvested at stationary phase.

Compositions containing the immunostimulatory bacteria are provided. Such compositions contain the bacteria and a pharmaceutically acceptable excipient or vehicle. A single dose is therapeutically effective for treating a disease or disorder in which immune stimulation effects treatment. Exemplary of such stimulation is an immune response, that includes, but is not limited to, one or both of a specific immune response and non-specific immune response, both specific and non-specific responses, innate response, primary immune response, adaptive immunity, secondary immune response, memory immune response, immune cell activation, immune cell proliferation, immune cell differentiation, and cytokine expression.

Pharmaceutical compositions containing any of the immunostimulatory bacteria are provided. As are uses thereof for treatment of cancers, and methods of treatment of cancer. Methods and uses include treating a subject who has cancer, comprising administering an immunostimulatory bacterium or the pharmaceutical composition to a subject, such as a human. A method of treating a subject who has cancer, comprising administering an immunostimulatory bacterium is provided. The Methods and uses include combination therapy in which a second anti-cancer agent or treatment is administered. The second anti-cancer agent is a chemotherapeutic agent that results in cytosolic DNA or radiotherapy, or an anti- immune checkpoint inhibitor, such as an anti-PD-1, or anti-PD-L1 or anti-CTLA4 antibody, or CAR-T cells or other therapeutic cells, such as stem cells, TIL cells and modified cells for cancer therapy.

As described herein, the immunostimulatory bacteria, such as the *Salmonella* strains, that encode RNAi, such as miRNA and shRNA, against TREX1 are complementary to therapies that are genotoxic or target or harm DNA to result in cytosolic DNA.

Administration can be by any suitable route, such as parenteral, and include additional agents that can facilitate or enhance delivery. Administration can be oral or rectal or by aerosol into the lung or intratumoral, intravenously, intramuscularly, or subcutaneously.

Cancers include solid tumors and hematologic malignancies, such as, but not limited to, cancer of the breast, heart, lung, small intestine, colon, spleen, kidney,

bladder, uterus, head and neck, ovary, prostate, brain, pancreas, skin, bone, liver, bone marrow, blood, thymus, uterus, testicles, cervix or liver.

The immunostimulatory bacteria can be formulated into compositions for administration, such as suspensions. They can be dried and stored as powders. Combinations of the immunostimulatory bacteria with others of the anti-cancer agents also are provided.

Also provided are shRNA and miRNA, such as the nucleic acid molecules comprising the sequence of nucleic acids set forth in any of SEQ ID NOs.: 36-40 and 75-78. Plasmids containing such DNA also are provided. The immunostimulatory bacteria, such as *Salmonella* containing the plasmids are provided.

Combination therapies for treatment of cancers and malignancies are provided. The immunostimulatory bacteria can be administered before, or concurrently with other cancer therapies, including radiotherapy, chemotherapies, particularly genotoxic chemotherapies that result in cytosolic DNA, and immunotherapies, such as anti-checkpoint inhibitor antibodies, including anti-PD-L1, anti CTLA4, and other such immunotherapies.

Also provided are methods of treatment and uses for treating a subject who has a tumor that is $cd73^+$. The immunostimulatory bacterium for such treatment is auxotrophic for adenosine; and the subject has been or is identified as having a tumor that is $cd73^+$ by testing a tumor biopsy or other body tissue or fluid sample.

Methods of increasing colonization of an immunostimulatory bacterium in a subject are provided. These methods include administering the immunostimulatory bacterium to the subject; and inhibiting or suppressing expression of TREX1 and/or the activity of the encoded product of TREX1 in the subject.

The terms and expressions that are employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions to exclude any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are contemplated.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1** depicts a schematic of the process used to delete the *asd* gene from strain YS1646. The *asd* gene from *S. typhimurium* strain YS1646 was deleted using

lambda-derived Red recombination system as described in Datsenko and Wanner (*Proc Natl Acad Sci USA 97*:6640–6645 (2000)).

**Figure 2** depicts the results of human PD-L1 shRNA screening using qPCR and Western blot. HEK 293 cells were co-transfected with a PD-L1 cDNA expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting PDL1. **Fig. 2A** depicts the results of qPCR analysis to determine the level of mRNA knockdown. **Fig. 2B** depicts the Western blot analysis of human PD-L1 shRNAs. Western blotting and densitometry were used to measure the level of PD-L1 protein expression.

**Figure 3** depicts the results of human TREX1 shRNA screening using qPCR and Western blot. HEK 293 cells were co-transfected with a TREX1 cDNA expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting TREX1. **Fig. 3A** depicts results of qPCR analysis, used to determine the level of mRNA knockdown. **Fig. 3B** depicts results of Western blot analysis of the human TREX1 shRNAs. Western blotting and densitometry were used to measure the level of PD-L1 protein expression.

**Figure 4** depicts the results of human beta-catenin shRNA screening using qPCR and Western blot. HEK 293 cells were co-transfected with a beta-catenin cDNA expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting beta-catenin. **Fig. 4A** depicts results of qPCR, used to determine the level of mRNA knockdown. **Fig. 4B** depicts the results of Western blot analysis of the human beta-catenin shRNAs. Western blotting and densitometry were used to measure the level of beta-catenin protein expression.

**Figure 5** depicts the results of human SIRP-alpha shRNA screening using qPCR and Western blot. HEK 293 cells were co-transfected with a SIRP-alpha cDNA expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting SIRP-alpha. **Fig. 5**A depicts results of qPCR, used to determine the level of mRNA knockdown. **Fig. 5**B depicts the results of Western blot analysis of human SIRP-alpha shRNAs. Western blotting and densitometry were used to measure the level of SIRP-alpha protein expression.

**Figure 6** depicts the results of human TGF-beta isoform 1 shRNA screening using qPCR. HEK 293 cells were co-transfected with a TGF-beta isoform 1 cDNA

expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting TGF-beta. qPCR was used to determine the level of mRNA knockdown.

**Figure 7** depicts the results of human VEGF shRNA screening using qPCR. HEK 293 cells were co-transfected with a VEGF cDNA expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting VEGF. qPCR was used to determine the level of mRNA knockdown.

**Figure 8** depicts the results of human VISTA shRNA screening using qPCR and Western blot. HEK 293 cells were co-transfected with a VISTA cDNA expression plasmid and various pEQU6 plasmids encoding distinct shRNAs targeting VISTA. **Fig. 8A** depicts results of qPCR, used to determine the level of mRNA knockdown. **Fig. 8B** depicts the results of Western blot analysis of human VISTA shRNAs. Western blotting and densitometry were used to measure the level of VISTA protein expression.

**Figure 9** depicts the results of qPCR assessment of combination gene knockdown with HuPD-L1 + HuTREX1 RNAi's. HEK 293 cells were co-transfected with a TREX1 cDNA expression plasmid, a PD-L1 cDNA expression plasmid, and pEQU6-H1 plasmid encoding ARI-134 shRNAs targeting PD-L1 and TREX1, or pEQU6 plasmid encoding ARI-123 shRNA targeting PD-L1 alone, or pEQU6 plasmid encoding ARI-114 shRNA targeting TREX1. **Fig. 9A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 9B** depicts results of qPCR, used to determine the level of TREX1 mRNA knockdown.

**Figure 10** depicts the results of qPCR assessment of combination gene knockdown with HuPD-L1 + HuSIRP-alpha RNAi's. HEK 293 cells were co-transfected with a PD-L1 cDNA expression plasmid, a SIRP-alpha cDNA expression plasmid, and pEQU6-H1 plasmid encoding ARI-135 containing shRNAs targeting PD-L1 and SIRP-alpha, or pEQU6 plasmid encoding ARI-123 shRNA targeting PD-L1 alone, or pEQU6 plasmid encoding ARI-175 shRNA targeting SIRPalpha. **Fig. 10A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 10B** depicts results of qPCR, used to determine the level of SIRP-alpha mRNA knockdown.

**Figure 11** depicts the results of qPCR assessment of combination gene knockdown with HuPD-L1 + Hu beta-catenin RNAi's. HEK 293 cells were co-

transfected with a PD-L1 cDNA expression plasmid, a beta-catenin cDNA expression plasmid, and pEQU6-H1 plasmid encoding ARI-136 containing shRNAs targeting PD-L1 and beta-catenin, or pEQU6 plasmid encoding ARI-123 shRNA targeting PD-L1 alone, or pEQU6 plasmid encoding ARI-169 shRNA targeting beta-catenin. **Fig. 11A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 11B** depicts results of qPCR, used to determine the level of beta-catenin mRNA knockdown.

**Figure 12** depicts the results of qPCR assessment of combination gene knockdown with HuPD-L1 + HuVISTA RNAi's. HEK 293 cells were co-transfected with a PD-L1 cDNA expression plasmid, a VISTA cDNA expression plasmid, and pEQU6-H1 plasmid encoding ARI-137 (SEQ ID NO:213) containing shRNAs targeting PD-L1 and VISTA, or pEQU6 plasmid encoding ARI-123(SEQ ID NO:2) shRNA targeting PD-L1 alone, or pEQU6 plasmid encoding ARI-195 (SEQ ID NO:25) shRNA targeting VISTA. **Fig. 12A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 12B** depicts results of qPCR, used to determine the level of VISTA mRNA knockdown.

**Figure 13** depicts the results of qPCR assessment of combination gene knockdown with mouse TREX1 + mouse PD-L1 RNAi's. HEK 293 cells were co-transfected with a mouse TREX1 cDNA expression plasmid, a mouse PD-L1 cDNA expression plasmid, and pEQU6-H1 plasmid encoding containing shRNA (designated ARI-128) targeting mouse TREX1 and mouse PD-L1, or pEQU6 plasmid encoding shRNA (designated ARI-115 targeting mouse PD-L1 alone, or pEQU6 plasmid encoding shRNA(designated ARI-108) targeting mouse TREX1. **Fig. 13A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 13B** depicts results of qPCR, used to determine the level of TREX1 mRNA knockdown.

**Figure 14** depicts the results of qPCR assessment of combination gene knockdown with mouse PD-L1 + mouse SIRP-alpha RNAi's. HEK 293 cells were co-transfected with a mouse PD-L1 cDNA expression plasmid, a mouse SIRP-alpha cDNA expression plasmid, and pEQU6-H1 plasmid encoding shRNA (designated ARI-129) targeting mouse PD-L1 and SIRP-alpha, or pEQU6 plasmid encoding shRNA (designated ARI-115) targeting PD-L1 alone, or pEQU6 plasmid encoding

shRNA (designated ARI-138) targeting SIRP-alpha. **Fig. 14A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 14B** depicts results of qPCR, used to determine the level of SIRP-alpha mRNA knockdown.

**Figure 15** depicts the results of qPCR assessment of combination gene knockdown with mouse PD-L1 + mouse VISTA RNAi's. HEK 293 cells were co-transfected with a mouse PD-L1 cDNA expression plasmid, a mouse VISTA cDNA expression plasmid, and pEQU6-H1 plasmid encoding containing shRNA (designated ARI-132) targeting PD-L1 and VISTA, or pEQU6 plasmid encoding shRNA (designated ARI-115) targeting PD-L1 alone, or pEQU6 plasmid encoding shRNA (designated ARI-157) targeting VISTA. **Fig. 15A** depicts results of qPCR, used to determine the level of PDL1 mRNA knockdown. **Fig. 15B** depicts results of qPCR, used to determine the level of beta-catenin mRNA knockdown.

**Figure 16** depicts the results of qPCR assessment of combination gene knockdown with mouse TREX1+ mouse SIRP-alpha RNAi's. HEK 293 cells were co-transfected with a mouse TREX1 cDNA expression plasmid, a mouse VISTA cDNA expression plasmid, and pEQU6-H1 plasmid encoding containing shRNA (designated ARI-131) targeting PD-L1 and VISTA, or pEQU6 plasmid encoding shRNA (designated ARI-108) targeting TREX1 alone, or pEQU6 plasmid encoding shRNA(designated ARI-138) targeting SIRP-alpha. **Fig. 16A** depicts results of qPCR, used to determine the level of TREX1 mRNA knockdown. **Fig. 16B** depicts results of qPCR, used to determine the level of SIRP-alpha mRNA knockdown.

**Figure 17** depicts the results of qPCR assessment of combination gene knockdown with mouse PD-L1 + mouse beta-catenin RNAi's. HEK 293 cells were co-transfected with a mouse PD-L1 cDNA expression plasmid, a mouse beta-catenin cDNA expression plasmid, and pEQU6-H1 plasmid encoding containing shRNA (designated ARI-133) targeting PD-L1 and VISTA, or pEQU6 plasmid encoding shRNA(designated ARI-115) targeting PD-L1 alone, or pEQU6 plasmid encoding shRNA (designated ARI-166) targeting beta catenin. **Fig. 17A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 17B** depicts results of qPCR, used to determine the level of beta-catenin mRNA knockdown.

**Figure 18** depicts the results of qPCR assessment of combination gene knockdown with mouse TREX1 + mouse VISTA RNAi's. HEK 293 cells were co-

transfected with a mouse TREX1 cDNA expression plasmid, a mouse VISTA cDNA expression plasmid, and pEQU6-H1 plasmid encoding shRNA (designated ARI-130) targeting PD-L1 and VISTA, or pEQU6 plasmid encoding shRNA (designated ARI-108) targeting TREX1 alone, or pEQU6 plasmid encoding shRNA (designated ARI-157) targeting VISTA. **Fig. 18A** depicts results of qPCR, used to determine the level of TREX1 mRNA knockdown. **Fig. 18B** depicts results of qPCR, used to determine the level of VISTA mRNA knockdown.

**Figure 19** depicts a comparison of micro-RNA and shRNA-mediated knockdown of mouse PD-L1. HEK 293 cells were co-transfected with a mouse PD-L1 cDNA expression plasmid and either pEQU6 plasmids encoding micro-RNA (ARI-201) or shRNA (designated ARI-115) targeting PD-L1. **Fig. 19A** depicts results of qPCR, used to determine the level of PD-L1 mRNA knockdown. **Fig. 19B** depicts results of Western blot analysis; Western blotting and densitometry were used to measure the level of PD-L1 protein expression.

**Figure 20** depicts a comparison of micro-RNA and shRNA-mediated knockdown of mouse TREX1. HEK 293 cells were co-transfected with a mouse TREX1 cDNA expression plasmid and pEQU6 plasmids encoding micro-RNA (designated ARI-203) or shRNA (designated ARI-108) targeting TREX1. Western blot was used to determine the level of mRNA knockdown.

**Figure 21** depicts the results of TREX1 knockdown with RNA Pol II expression of micro-RNA. HEK 293 cells were co-transfected with a mouse TREX1 cDNA expression plasmid and pEQU6 plasmid shRNA targeting mouse TREX1 (designated ARI-108) or a pEQ plasmid encoding a CMV promoter and micro-RNA targeting mouse TREX1 (designated ARI-204). **Fig. 21A** depicts results of qPCR, used to determine the level of mouse TREX1 mRNA knockdown. **Fig. 21B** depicts results of Western blot analysis; Western blotting and densitometry were used to measure the level of mouse TREX1 protein expression.

**Figure 22** depicts the results of PD-L1 knockdown with RNA Pol II expression of micro-RNA. HEK 293 cells were co-transfected with a mouse PD-L1 cDNA expression plasmid and pEQU6 plasmid shRNA targeting mouse PD-L1 (designated ARI-115) or a pEQ plasmid encoding a CMV promoter and micro-RNA targeting mouse TREX1 (designated ARI-202). **Fig. 22A** depicts results of qPCR,

used to determine the level of mouse PD-L1 mRNA knockdown. **Fig. 22B** depicts results of Western blot analysis; Western blotting and densitometry were used to measure the level of mouse PD-L1 protein expression.

**Figure 23** depicts the efficacy of systemically administered strain AST-104 in a CT26 colon tumor model. BALB/c mice were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=8 per group). Mice with established tumors were IV injected with $1\times10^7$ CFU of YS1646 strains containing either plasmid control (strain AST-102) or the TREX1 shRNA plasmid (of strain AST-104), or PBS control, on the days indicated by the arrows. Spaghetti plots depict tumor growth, each line representing an individual mouse. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. % Tumor Growth Inhibition (TGI) was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. * $p < 0.05$ vs. plasmid control, student's *t*-test.

**Figure 24** depicts the correlation of strain AST-104 mediated cytokine changes with STING signature. BALB/c were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=8 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (strain AST-102) or the TREX1 shRNA plasmid (AST-104), or PBS control. Mice were bled 6 hrs following the first dose and systemic serum cytokines tested on a Luminex 200 device (Luminex Corporation) and mouse cytometric bead array (BD bead array, FACS Fortessa, FCAP software, BD Biosciences). **Fig. 24A** depicts levels of pro-inflammatory cytokines. Fig. 24**B** depicts levels of immuno-suppressive cytokines. * $p < 0.05$, ** $p < 0.01$, student's *t*-test.

**Figure 25** depicts the efficacy of systemically administered strain AST-104 in a MC38 colon tumor model. C57Bl/6 mice (6-8 wk old) were implanted with a single MC38 ($2\times10^5$ cells) subcutaneous flank tumor (n=10 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (strain AST-102) or the TREX1 shRNA plasmid (strain AST-104), or PBS control, on the days indicated by the arrows. Spaghetti plots depict tumor growth, each line representing an individual mouse. Tumor measurements were

performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. * $p < 0.05$ vs. plasmid control, student's *t*-test.

**Figure 26** depicts the efficacy of AST-104 in a checkpoint-resistant B16.F10 melanoma model. C57Bl/6 mice (6-8 wk old) were implanted with a single B16.F10 ($5\times10^5$ cells) subcutaneous flank tumor (n=10 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (AST-102) or the TREX1 shRNA plasmid (AST-104), or PBS control, on the days indicated by the arrows. Spaghetti plots depict tumor growth, each line representing an individual mouse. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. * $p < 0.05$ vs. plasmid control, student's *t*-test.

**Figure 27** depicts the efficacy of systemically administered AST-105 (shPD-L1) in a CT26 tumor model. BALB/c (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=8 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (AST-102) or the PD-L1 shRNA plasmid (AST-105), or PBS control, on the days indicated by the arrows. A separate group was administered 100 μg anti-PD-L1 antibody (clone 10F.9G2 clone, BioXCell) by IP injection weekly, beginning with the first IV injection. Spaghetti plots depicting tumor growth, each line representing an individual mouse. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. * $p < 0.05$ vs. plasmid control, student's *t*-test.

**Figure 28** depicts results showing that AST-105 induces significant cytokine responses observed over PD-L1 mAb. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=8 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (AST-102) or the PD-L1 shRNA plasmid (AST-105), or PBS control, on the days indicated by the arrows. A separate group was administered 100 μg anti-PD-L1 antibody IP (clone 10F.9G2 clone, BioXCell) weekly, beginning with the first IV injection. Mice were bled 6 hrs following the first dose and systemic serum cytokines tested by Luminex (BD bead array and Luminex 200) and mouse cytometric bead array (FACS Fortessa, FCAP software, all BD Biosciences). * $p < 0.05$, ** $p < 0.01$, student's *t*-test.

**Figure 29** depicts the effects of intratumoral administration of strains AST-104 and AST-105 in dual flank colon tumors on tumor volume. BALB/c mice (6-8 wk old) were implanted with dual CT26 ($2\times10^5$ cells) subcutaneous flank tumors on the right and left flanks (n=10 per group). Mice with established tumors were IT injected into the right flank with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (AST-102) or the strain containing TREX1 shRNA plasmid (AST-104), or PD-L1 shRNA plasmid (AST-105), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reaches >20% of body weight or became necrotic, as per IACUC regulations. % Tumor Growth Inhibition (TGI) is calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The plots depict mean tumor growth of each group in the injected (left graph) and distal (right graph) groups, ± SEM. * $p < 0.05$, *** $p < 0.001$, student's *t*-test.

**Figure 30** depicts the curative effects of intratumoral AST-104 administration in dual flank colon tumors in mice. BALB/c mice (6-8 wk old) were implanted with dual CT26 ($2\times10^5$ cells) subcutaneous flank tumors on the right and left flanks (n=10 per group). Mice with established tumors were IT injected into the right flank with $5\times10^6$ CFU of YS1646 strains containing either plasmid control (AST-102) or the TREX1 shRNA plasmid (AST-104), or the shPD-L1 plasmid (AST-105), or PBS control on days 10 and 14 after tumor implantation. Mice were euthanized when

tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. The figure depicts the overall survival of the mice, ** $p < 0.01$, log-rank (Mantel-Cox) test.

**Figure 31** depicts the levels of tumor colonization in injected and distal tumors after IT administration of AST-104. BALB/c mice (6-8 wk old) were implanted with dual CT26 ($2\times10^5$ cells) subcutaneous flank tumors on the right and left flanks (n=10 per group). Mice with established tumors were IT injected into the right flank with $5\times10^6$ CFU of the YS1646 strain containing a TREX1 shRNA plasmid (AST-104). At 35 days post tumor implantation (12 days after the last dose of AST-104), three mice were sacrificed, and injected and distal tumors were homogenized (GentleMACs™, Miltenyi Biotec) and plated on LB plates to enumerate the number of colony forming units (CFU) per gram of tumor tissue. The figure depicts the mean CFU per gram of tissue, ± SD.

**Figure 32** depicts that CpG scrambled plasmid has immuno-stimulatory anti-tumor properties. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of the YS1646 strain (AST-100), or the YS1646 strain containing the scrambled shRNA control plasmid (AST-103), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2 (length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI is calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts mean tumor growth of each group, ± SEM. ** $p < 0.01$, student's *t*-test.

**Figure 33** depicts the efficacy of AST-106 (microRNA TREX1) vs. AST-104 (shRNA TREX1). BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of the YS1646 containing the TREX1 shRNA plasmid (AST-104) or the YS1646 strain containing a TREX1 microRNA plasmid (AST-106), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA).

Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts the mean tumor growth of each group, ± SEM. * $p < 0.05$, student's *t*-test.

**Figure 34** depicts a schematic of the process used to delete the *fliC* gene. The *flic* gene was deleted from the chromosome of *S. typhimurium* strain AST-101 (*asd* deleted strain of YS1646) using lambda-derived Red recombination system as described in Datsenko and Wanner (*Proc Natl Acad Sci USA 97*:6640–6645 (2000)).

**Figure 35** depicts that the Flagellin deletion strain grows normally in LB. The figure depicts the growth of strains AST-108 ASD (pATI-shTREX1) and AST-112 ASD/FLG (pATI-shTREX1) at 37ºC in LB broth, as measured by $OD_{600}$ using a Spectramax 96 well plate reader (Molecular devices).

**Figure 36** depicts that Flagellin knockout improves anti-tumor efficacy. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of the *asd/flgB/fliC* knockout strain containing the pATI shTREX1 plasmid (AST-113), or *asd* knockout strain containing the pATI shTREX1 plasmid (AST-110), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts the mean tumor growth of each group, ± SEM. * $p < 0.05$, student's *t*-test.

**Figure 37** depicts that Flagellin knockout shows an increased IFN-gamma signature. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of the *asd/flgB/fliC* knockout strain containing the pATI shTREX1 plasmid (AST-113), or *asd* knockout strain containing the pATI shTREX1 plasmid (AST-110), or PBS control. Mice were bled 6 hrs following the first dose and systemic serum cytokines tested by Luminex 200 device (Luminex Corporation) and

mouse cytometric bead array (BD bead array, FACS Fortessa, FCAP software, all BD Biosciences). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, student's *t*-test.

**Figure 38** depicts that Flagellin is not required for tumor colonization. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of the *asd/flgB/fliC* knockout strain containing the pATI shTREX1 plasmid (AST-113), or *asd* knockout strain containing the pATI shTREX1 plasmid (AST-110), or PBS control. At 35 days post tumor implantation (12 days after the last dose of engineered *Salmonella* therapy), three mice per group were sacrificed, and tumors were homogenized (GentleMACs™, Miltenyi Biotec) and plated on LB plates to enumerate the number of colony forming units per gram of tumor tissue. The figure depicts the mean colony forming units (CFU) per gram of tissue, ± SD.

**Figure 39** depicts that a cytoLLO expressing strain grows normally *in vitro*. The figure depicts the growth of strains AST-110 (YS1646 with *asd* deletion containing (pATI-shTREX1)) and AST-115 (YS1646 with *asd* deletion and knock-in of cytoLLO expression cassette containing (pATI-shTREX1)) at 37 °C in LB broth, as measured by $OD_{600}$ using a Spectramax 96 well plate reader (Molecular devices).

**Figure 40** depicts that AST-115 (ASD knockout + CytoLLO Knock-in strain carrying shTREX1 plasmid) demonstrates potent, single-dose efficacy in a murine CT26 tumor model. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5\times10^6$ CFU of AST-115 (YS1646 with *asd* deletion and knock-in of cytoLLO expression cassette at *asd* locus containing (pATI-shTREX1), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts the mean tumor growth of each group, ± SEM. ** $p < 0.01$, student's *t*-test.

**Figure 41** depicts that strain YS1646 requires tumor microenvironment levels of adenosine for growth. Growth of strains YS1646 (*purI-/msbB-*) and the wild-type parental strain ATCC14028 at 37 °C in LB broth are shown, as measured by $OD_{600}$ using a Spectramax 96 well plate reader (Molecular devices).

**Figure 42** depicts that ASD, FLG, and CytoLLO engineered strains require high adenosine for growth. The growth of strains AST-117 (YS1646 Δ*asd* containing a low copy shTREX-1 plasmid), AST-118 (YS1646 Δ*asd/filC/fljB* containing a low copy shTREX-1 plasmid), and AST-119 (YS1646 Δ*asd:*LLO containing a low copy shTREX-1 plasmid) at 37 °C in LB broth are shown, as measured by $OD_{600}$ using a Spectramax 96 well plate reader (Molecular devices).

**Figure 43** depicts that a strain with a low copy origin of replication *asd*-encoding plasmid has superior growth kinetics than a strain with a high copy origin of replication *asd*-encoding plasmid. The growth of strains YS1646, AST-117 (YS1646 Δ*asd* containing a low copy shTREX-1 plasmid with a functional *asd* gene), AST-104 (YS1646 containing a low copy pEQ shTREX-1 plasmid without an *asd* gene), and AST-110 (YS1646 Δ*asd* containing a high copy pATI-shTREX-1 plasmid with a functional *asd* gene) at 37 °C in LB broth are shown, as measured by $OD_{600}$ using a Spectramax 96 well plate reader (Molecular devices).

**Figure 44** depicts that a strain with a low copy *asd* plasmid is more fit than a strain with a high copy *asd* plasmid in mouse tumor cells. The intracellular growth of strains AST-117 (YS1646 Δ*asd* containing a low copy shTREX-1 plasmid with a functional *asd* gene) and AST-110 (YS1646 Δ*asd* containing a high copy pATI-shTREX-1 plasmid with a functional *asd* gene) are shown in B16F.10 mouse melanoma cells and CT26 mouse colon carcinoma cells. $5\times10^5$ cells in a 24 well dish were infected with the *S. typhimurium* strains at a MOI of 5. After 30 minutes of infection, media was replaced with media containing gentamycin to kill extracellular bacteria. At indicated time points, cell monolayers were lysed by osmotic shock the cell lysates were diluted and plated on LB agar to enumerate CFU.

**Figure 45** depicts that *in vivo*, *asd* gene complementation systems result in retention of plasmids in *S. typhimurium*-infected tumors. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2\times10^5$ cells) subcutaneous flank tumor (n=9 per

group). Mice with established tumors were IV injected with $5\times10^6$ CFU of the *asd* knockout strain containing the pATI shTREX1 plasmid (AST-110) or the YS1646 containing a pEQ shTREX-1 plasmid without an *asd* gene (AST-104). At 35 days post tumor implantation (12 days after the last dose of engineered Salmonella therapy), three mice per group were sacrificed, and tumors were homogenized using a GentleMACs™ homogenizer (Miltenyi Biotec) and plated on LB agar plates or LB agar plates with 50ug/mL of Kanamycin. The figure depicts the percentage of Kanamycin resistant CFU in tumor tissue homogenates, ± SD.

**Figure 46** depicts that the therapeutic efficacy of a strain containing a plasmid with *asd* gene complementation system and shTREX1 (AST-110) is improved. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2x10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5x10^6$ CFU of the *asd* knockout strain containing the pATI-shTREX1 plasmid (AST-110) or the *asd* knockout strain containing the pATI-scramble plasmid (AST-109), or the YS1646 strain containing a pEQ-shTREX-1 plasmid without an *asd* gene (AST-104), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reaches >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts the mean tumor growth of each group, ± SEM.

**Figure 47** depicts that a strain containing a low copy shTREX1 plasmid (AST-117) has superior anti-tumor properties compared to a strain containing a high copy plasmid (AST-110). BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2x10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5x10^6$ CFU of the *asd* knockout strain containing the pATI-shTREX1 plasmid with a high copy number origin of replication (AST-110) or the *asd* knockout strain containing the pATI-shTREX1 plasmid with a low copy number origin of replication (AST-117), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula

1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts the mean tumor growth of each group, ± SEM. * $p < 0.05$, student's *t*-test.

**Figure 48** depicts that the AST-117 low copy plasmid strain colonizes tumors better and has a higher tumor to spleen colonization ratio than the AST-110 high copy plasmid strain. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2x10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5x10^6$ CFU of the *asd* knockout strain containing the pATI-shTREX1 plasmid with a high copy number origin of replication (AST-110) or the *asd* knockout strain containing the pATI-shTREX1 plasmid with a low copy number origin of replication (AST-117). At 35 days post tumor implantation (12 days after the last dose of engineered Salmonella therapy), 3 mice per group were sacrificed, and tumors were homogenized using a GentleMACs™ homogenizer (Miltenyi Biotec) and plated on LB plates to enumerate the number of CFU per gram of tumor tissue. **Fig. 48A** depicts the mean CFU per gram of tumor tissue, ± SD. **Fig. 48B** depicts the tumor to spleen colonization ratios.

**Figure 49** depicts that a strain grown to stationary phase is equivalently potent, and less inflammatory than the same strain grown to log phase. BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2x10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5x10^6$ CFU of the YS1646 strain containing a pEQ-shTREX-1 plasmid (AST-104) harvested at log phase or stationary phase, or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × width$^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. **Fig. 49A** depicts the mean tumor growth of each group, ± SEM. * $p < 0.05$, student's *t*-test. **Fig. 49B** depicts the levels of TNF-alpha and IL-6. Mice were bled 6 hrs following the first dose and systemic serum cytokines tested by Luminex (Luminex Corp.) and mouse cytometric bead array (FACS Fortessa, FCAP software, all BD Biosciences). ** $p < 0.01$, student's *t*-test.

**Figure 50** depicts that autolytic strain (AST-120) cannot grow in the absence of DAP. The figure depicts the growth of Δ*asd*:cytoLLO strain containing a pEQU6-shTREX1 plasmid that does not contain an *asd* gene (AST-120) over time in LB broth alone, or in LB broth supplemented with 50μg/mL DAP, as measured by $OD_{600}$ using a Spectramax 96 well plate reader (Molecular devices).

**Figure 51** depicts the anti-tumor activity of the autolytic strain (AST-120). BALB/c mice (6-8 wk old) were implanted with a single CT26 ($2x10^5$ cells) subcutaneous flank tumor (n=9 per group). Mice with established tumors were IV injected with $5x10^6$ CFU of the of Δ*asd*:cytoLLO strain containing a pEQU6-shTREX1 plasmid that does not contain an *asd* gene (AST-120), or PBS control, on the days indicated by the arrows. Tumor measurements were performed using electronic calipers (Fowler, Newton, MA). Tumor volume was calculated using the modified ellipsoid formula 1/2(length × $width^2$). Mice were euthanized when tumor size reached >20% of body weight or became necrotic, as per IACUC regulations. TGI was calculated as 1-(mean test tumor volume/mean control tumor volume) x 100. The figure depicts the mean tumor growth of each group, ± SEM. * $p < 0.05$, student's *t*-test.

**Figure 52** depicts that TREX1 expression is increased in several human tumor types. Analysis of the relative gene expression of the TREX1 gene using the TCGA database was performed from a broad array of tumor types. Tumor types with a significant upregulation of TREX1 compared to normal tissue are displayed: prostate, breast, cervical, uterine and bladder (p values: BRCA - 7.7e-16; PRAD - 9.4e-12; UCEC - 2.5e-05; BLCA - 3.7e-03; CESC - 7.7e-03) and multiple forms of kidney cancer (p values: KIPAN - 8.9e-39; KIRC - 9.6e-35; KIRP - 5.8e-14; KICH - 4.9e-08).

**Figure 53** depicts that radiotherapy after administration of *S. typhimurium* strain AST-106 increases tumor colonization. BALB/c mice (6-8 wk old) were inoculated subcutaneously in the right flank with $1x10^5$ mouse TSA breast carcinoma cells. Mice bearing established tumors were administered the following: IV injection of $5x10^6$ CFUs of AST-106 (YS1646 transformed with pEQU6-miTREX1) followed 4 hours later with 0 Gy (3 mice), or 5 $x10^6$ CFUs of AST-106 followed 4 hours later with 20 Gy (3 mice); 20 Gy irradiation followed 4 hours later with $5x10^6$ CFUs of

AST-106 (3 mice), or PBS IV followed by 0 Gy radiation (1 mouse). Focal radiotherapy was administered using a small animal radiation research platform (SARRP) device (XStrahl Life Sciences). Mice were sacrificed 24 hours later, and tumors were harvested and weighed. Tumors were homogenized in 10mL sterile PBS using M tubes in a GentleMACs™ device (Miltenyi Biotec), then 10-fold serial dilutions were performed and plated on LB agar plates containing kanamycin. The following day, colony forming units (CFU) were counted and CFU per gram of tumor tissue was calculated. * $p < 0.05$, student's *t*-test.

**DETAILED DESCRIPTION**

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## A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can

change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, therapeutic bacteria are bacteria that effect therapy, such as 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, and replicate and/or express products that are immunostimulatory or that result in immunostimulation. 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, 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, at least one of an shRNA that targets, disrupts or inhibits a checkpoint gene or gene encoding such inhibitor or a metabolite that is immunosuppressive or is in an immunosuppressive pathway. These include encoding an siRNA, such as an shRNA, that targets or inhibits TREX1 expression, a modification that renders the bacterium auxotrophic for adenosine, and/or an inhibitor or disruptor of an immune checkpoint gene or product thereof, such as an shRNA that disrupts or inhibits PD-L1.

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) and YS1646 and 41.2.9 are used interchangeably and each refer to the strain deposited with the American Type Culture Collection 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 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 and assigned Accession No. 202164 (see, U.S. Patent No. 6,863,894).

As used herein, an origin of replication is a sequence of DNA at which replication is initiated on a chromosome, plasmid or 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, it is between about 25–50 copies/cell, and if derived from the high-copy-number plasmid pUC, it can be 150-200 copies/cell.

As used herein, medium copy number of a plasmid in cells is about or is 150 or less than 150, low copy number is 15-30, such as 20 or less than 20. Low to medium copy number is less than 150. High copy number is greater than 150 copies/cell.

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

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

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

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

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

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

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

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

As used herein, small interfering RNAs (siRNAs) are small pieces of double-stranded (ds) RNA, usually about 21 nucleotides long, with 3' overhangs (2 nucleotides) at each end that can be used to "interfere" with the translation of proteins by binding to and promoting the degradation of messenger RNA (mRNA) at specific sequences. In doing so, siRNAs prevent the production of specific proteins based on the nucleotide sequences of their corresponding mRNAs. The process is called RNA interference (RNAi), and also is referred to as siRNA silencing or siRNA knockdown.

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

As used herein, a tumor microenvironment (TME) is the cellular environment in which the tumor exists, including surrounding blood vessels, immune cells, fibroblasts, bone marrow-derived inflammatory cells, lymphocytes, signaling molecules and the extracellular matrix (ECM). Conditions that exist include, but are

not limited to, increased vascularization, hypoxia, low pH, increased lactate concentration, increased pyruvate concentration, increased interstitial fluid pressure and altered metabolites or metabolism, such as higher levels of adenosine, indicative of a tumor.

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. IFN-β proteins are produced by fibroblasts, and have antiviral activity that is involved mainly in innate immune response. Two types of IFN-β are IFN-β1 (IFNB1) and IFN-β3 (IFNB3).

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

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

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

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

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

residues or nucleotides of the molecule. Amino acid replacements compared to a particular polypeptide can be expressed in terms of the number of the amino acid residue along the length of the polypeptide sequence.

As used herein, "at a position corresponding to," or recitation that nucleotides or amino acid positions "correspond to" nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence Listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm, such as the GAP algorithm. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides. In general, to identify corresponding positions, the sequences of amino acids are aligned so that the highest order match is obtained (see, *e.g.*, *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carrillo *et al.* (1988) *SIAM J Applied Math* 48:1073).

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

guides to find corresponding amino acid or nucleotide residues between and among human and non-human sequences. Corresponding positions also can be based on structural alignments, for example by using computer simulated alignments of protein structure. In other instances, corresponding regions can be identified. One skilled in the art also can employ conserved amino acid residues as guides to find corresponding amino acid residues between and among human and non-human sequences.

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

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

As used herein, "bind," "bound" or grammatical variations thereof refers to the participation of a molecule in any attractive interaction with another molecule,

resulting in a stable association in which the two molecules are in close proximity to one another. Binding includes, but is not limited to, non-covalent bonds, covalent bonds (such as reversible and irreversible covalent bonds), and includes interactions between molecules such as, but not limited to, proteins, nucleic acids, carbohydrates, lipids, and small molecules, such as chemical compounds including drugs.

As used herein, "antibody" refers to immunoglobulins and immunoglobulin fragments, whether natural or partially or wholly synthetically, such as recombinantly produced, including any fragment thereof containing at least a portion of the variable heavy chain and light region of the immunoglobulin molecule that is sufficient to form an antigen binding site and, when assembled, to specifically bind an antigen. Hence, an antibody includes any protein having a binding domain that is homologous or substantially homologous to an immunoglobulin antigen-binding domain (antibody combining site). For example, an antibody refers to an antibody that contains two heavy chains (which can be denoted H and H') and two light chains (which can be denoted L and L'), where each heavy chain can be a full-length immunoglobulin heavy chain or a portion thereof sufficient to form an antigen binding site (*e.g.*, heavy chains include, but are not limited to, VH chains, VH-CH1 chains and VH-CH1-CH2-CH3 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, VL chains and VL-CL chains). Each heavy chain (H and H') pairs with one light chain (L and L', respectively). Typically, antibodies minimally include all or at least a portion of the variable heavy (VH) chain and/or the variable light (VL) chain. The antibody also can include all or a portion of the constant region.

For purposes herein, the term antibody includes full-length antibodies and portions thereof including antibody fragments, such as anti-EGFR 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 (scFv), single-chain Fabs (scFab), diabodies, anti-idiotypic (anti-Id) antibodies, or antigen-binding fragments of any of the above. Antibody also includes synthetic antibodies, recombinantly produced antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human antibodies, non-human antibodies, humanized antibodies, chimeric antibodies, and intrabodies.

Antibodies provided herein include members of any immunoglobulin class (*e.g.*, IgG, IgM, IgD, IgE, IgA and IgY), any subclass (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or sub-subclass (*e.g.*, IgG2a and IgG2b).

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

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

As used herein, "operably linked" with reference to nucleic acid sequences, regions, elements or domains means that the nucleic acid regions are functionally related to each other. 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 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 protein by the specific recognition of the charged tRNA molecule with its cognate mRNA codon in humans.

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

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

As used herein, an "amino acid" is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids contained in the antibodies 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 according to their well-known, three-letter or one-letter abbreviations (see Table below). The nucleotides, which occur in the various nucleic acid molecules and fragments, are designated with the standard single-letter designations used routinely in the art.

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

**Table of Correspondence**

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

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

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

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

**Exemplary conservative amino acid substitutions**

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

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

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

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

As used herein, a DNA construct is a single 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 means by using recombinant DNA methods means the use of the well-known methods of molecular biology for expressing proteins encoded by cloned DNA.

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

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

As used herein, a "vector" is a replicable nucleic acid from which one or more heterologous proteins, can be expressed when the vector is transformed into an appropriate host cell. Reference to a vector includes those vectors into which a nucleic acid encoding a polypeptide or fragment thereof can be introduced, typically by restriction digest and ligation. Reference to a vector also includes those vectors that contain nucleic acid encoding a polypeptide, such as a modified anti-EGFR antibody. The vector is used to introduce the nucleic acid encoding the polypeptide into the host cell for amplification of the nucleic acid or for expression/display of the polypeptide

encoded by the nucleic acid. The vectors typically remain episomal, but can be designed to effect integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well-known to those of skill in the art. A vector also includes "virus vectors" or "viral vectors." Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

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

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

As used herein, "sequence identity" refers to the number of identical or similar amino acids or nucleotide bases in a comparison between a test and a reference polypeptide or polynucleotide. Sequence identity can be determined by sequence alignment of nucleic acid or protein sequences to identify regions of similarity or identity. For purposes herein, sequence identity is generally determined by alignment to identify identical residues. The alignment can be local or global. Matches, mismatches and gaps can be identified between compared sequences. Gaps are null amino acids or nucleotides inserted between the residues of aligned sequences so that identical or similar characters are aligned. Generally, there can be internal and

terminal gaps. When using gap penalties, sequence identity can be determined with no penalty for end gaps (*e.g.*, terminal gaps are not penalized). Alternatively, sequence identity can be determined without taking into account gaps as the number of identical positions/length of the total aligned sequence x 100.

As used herein, a "global alignment" is an alignment that aligns two sequences from beginning to end, aligning each letter in each sequence only once. An alignment is produced, regardless of whether or not there is similarity or identity between the sequences. For example, 50% sequence identity based on "global alignment" means that in an alignment of the full sequence of two compared sequences each of 100 nucleotides in length, 50% of the residues are the same. It is understood that global alignment also can be used in determining sequence identity even when the length of the aligned sequences is not the same. The differences in the terminal ends of the sequences will be taken into account in determining sequence identity, unless the "no penalty for end gaps" is selected. Generally, a global alignment is used on sequences that share significant similarity over most of their length. Exemplary algorithms for performing global alignment include the Needleman-Wunsch algorithm (Needleman *et al.* (1970) *J. Mol. Biol.* 48: 443). 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 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, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Whether any two nucleic acid molecules have nucleotide sequences or any two polypeptides have amino acid sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical," or other similar variations reciting a percent identity, can be determined using known computer algorithms based on local or global alignment (*see e.g.*, wikipedia.org/wiki/Sequence_alignment_software, providing links to dozens of known and publicly available alignment databases and programs). Generally, for purposes herein sequence identity is determined using computer algorithms based on global alignment, such as the Needleman-Wunsch Global Sequence Alignment tool available from NCBI/BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&Page_TYPE=BlastHome); LAlign (William Pearson implementing the Huang and Miller algorithm (*Adv. Appl. Math.* (1991) 12:337-357)); and program from Xiaoqui Huang available at deepc2.psi.iastate.edu/aat/align/align.html. Typically, the full-length sequence of each of the compared polypeptides or nucleotides is aligned across the full-length of each sequence in a global alignment. Local alignment also can be used when the sequences being compared are substantially the same length.

Therefore, as used herein, the term "identity" represents a comparison or alignment between a test and a reference polypeptide or polynucleotide. In one non-limiting example, “at least 90% identical to” refers to percent identities from 90 to 100% relative to the reference polypeptide or polynucleotide. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide or polynucleotide length of 100 amino acids or nucleotides are compared, no more than 10% (*i.e.*, 10 out of 100) of amino acids or nucleotides in the test polypeptide or polynucleotide differ from those of the reference polypeptide. Similar comparisons can be made between a test and reference

polynucleotides. 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 difference (approximately 90% identity). Differences also can be due to deletions or truncations of amino acid residues. Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. Depending on the length of the compared sequences, at the level of homologies or identities above about 85-90%, the result can be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from cause or condition including, but not limited to, infections, acquired conditions, genetic conditions, and characterized by identifiable symptoms.

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

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

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

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

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

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

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

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

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

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

As used herein, an "anti-cancer agent" refers to any agent that is destructive or toxic 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.

As used herein "therapeutic activity" refers to the *in vivo* activity of a therapeutic polypeptide. Generally, the therapeutic activity is the activity that is associated with treatment of a disease or condition.

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

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

As used herein, animal includes any animal, such as, but not limited to, primates including humans, gorillas and monkeys; rodents, such as mice and rats; fowl, such as chickens; ruminants, such as goats, cows, deer, sheep; pigs and other animals. Non-human animals exclude humans as the contemplated animal. The polypeptides provided herein are from any source, animal, plant, prokaryotic and fungal. Most polypeptides are of animal origin, including mammalian origin.

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

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

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

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

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

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

As used herein, a multi-dose formulation refers to a formulation that contains multiple doses of a therapeutic agent and that can be directly administered to provide several single doses of the therapeutic agent. The doses can be administered over the course of minutes, hours, weeks, days or months. Multi-dose formulations can allow

dose adjustment, dose-pooling and/or dose-splitting. Because multi-dose formulations are used over time, they generally contain one or more preservatives to prevent microbial growth.

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

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

As used herein, an isolated or purified polypeptide or protein (*e.g.*, an isolated antibody or antigen-binding fragment thereof) or 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 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), used by those of skill in the art to assess such purity, or 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, reference to a

polypeptide, comprising "an immunoglobulin domain" includes polypeptides with one or a plurality of immunoglobulin domains.

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

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

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

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

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

**B. OVERVIEW OF THE IMMUNOSTIMULATORY BACTERIA**

Provided are modified bacteria, called immunostimulatory bacteria herein that accumulate and/or replicate in tumors and encode inhibitory RNAs, such as designed shRNAs and designed micro RNAs, that target genes whose inhibition, suppression or silencing effects tumor therapy, upon expression of the RNAs in the treated subject. Strains of bacteria for modification are any suitable for therapeutic use. The modified immunostimulatory bacteria provided herein are for use and for methods for treating cancer. The bacteria are modified for such uses and methods.

The immunostimulatory bacteria provided herein are modified by deletion or modification of bacterial genes to attenuate their inflammatory responses, and are modified to enhance anti-tumor immune responses in hosts treated with the bacteria. For example, the plasmids encoding RNAi that inhibit checkpoint genes in the host are included in the bacteria, and the bacteria can be auxotrophic for adenosine. Attenuation of the inflammatory response to the bacteria can be effected by deletion

of the *msbB* gene, which decreases TNF-alpha in the host, and/or knocking out flagellin genes. The bacteria are modified to stimulate host anti-tumor activity, for example, by adding plasmids encoding RNAi that target host immune checkpoints, and by adding nucleic acid with CpGs.

Bacterial strains can be attenuated strains or strains that are attenuated by standard methods or that by virtue of the modifications provided herein are attenuated in that their ability to colonize is limited primarily to immunoprivileged tissues and organs, particularly immune 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 disenteriae, Listeria monocytogenes, Salmonella typhi, Salmonella typhimurium, Salmonella gallinarum*, and *Salmonella enteritidis*. Other suitable bacterial species include *Rickettsia, Klebsiella, Bordetella, Neisseria, Aeromonas, Francisella, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Vibrio, Bacillus*, and *Erysipelothrix*. For example, *Rickettsia Rikettsiae, Rickettsia prowazekii, Rickettsia tsutsugamuchi, Rickettsia mooseri, Rickettsia sibirica, Bordetella bronchiseptica, Neisseria meningitidis, Neisseria gonorrhoeae, Aeromonas eucrenophila, Aeromonas salmonicida, Francisella tularensis, Corynebacterium pseudotuberculosis, Citrobacter freundii, Chlamydia pneumoniae, Haemophilus sornnus, Brucella abortus, Mycobacterium intracellulare, Legionella pneumophila, Rhodococcus equi, Pseudomonas aeruginosa, Helicobacter mustelae, Vibrio cholerae, Bacillus subtilis, Erysipelothrix rhusiopathiae, Yersinia enterocolitica, Rochalimaea quintana*, and *Agrobacterium tumerfacium.*

The bacteria accumulate by virtue of one or more properties, including, diffusion, migration and chemotaxis to immunoprivileged tissues or organs or environments, environments that provide nutrients or other molecules for which they are auxotrophic and/or environments that contain replicating cells that provide environments for entry and replication of bacteria. The immunostimulatory bacteria provided herein and species that effect such therapy include species of *Salmonella, Listeria*, and *E. coli*. The bacteria contain plasmids that encode one or more short hairpin (sh) RNA construct(s), or other RNAi modalities, whose expression inhibits or

disrupts expression of targeted genes. The shRNA constructs are expressed under control of a eukaryotic promoter, such as an RNA polymerase (RNAP) II or III promoter. Typically, RNAPIII (also referred to as POLIII) promoters are constitutive, and RNAPII (also referred to as POLII) can be regulated. In some examples, the shRNAs target the gene TREX1, to inhibit its expression. In some embodiments, the plasmids encode a plurality of RNAi molecules, such as shRNAs or microRNAs, that inhibit two or more checkpoint genes, such as shRNAs for inhibiting PD-L1, VISTA, SIRPα, CTNNB1, TGF-beta, and/or VEGF and any others known to those of skill in the art. Where a plurality of shRNAs are encoded, expression of each is under control of different promoters.

Among the bacteria provided herein, are bacteria that are modified so that they are auxotrophic for adenosine. This can be achieved by modification or deletion of genes involved in purine synthesis, metabolism, or transport. For example, disruption of the *tsx* gene in *Salmonella* species, such as *Salmonella typhi*, results in adenosine auxotrophy. Adenosine is immunosuppressive and accumulates to high concentrations in tumors; auxotrophy for adenosine improves the anti-tumor activity of the bacteria because the bacteria selectively replicate in tissues rich in adenosine.

Also provided are bacteria that are modified so that they have a defective *asd* gene. These bacteria for use *in vivo* are modified to include carrying a functional *asd* gene on the introduced plasmid; this maintains selection for the plasmid so that an antibiotic-based plasmid maintenance/selection system is not needed. Also provided is the use of *asd* defective strains that do not contain a functional asd gene on a plasmid and are thus engineered to be autolytic in the host.

Also provided are bacteria that are modified so that they are incapable of producing flagella. This can be achieved by modifying the bacteria by means of deleting the genes that encode the flagellin subunits. The modified bacteria lacking flagellin are less inflammatory and therefore better tolerated and induce a more potent anti-tumor response.

Also provided are bacteria that are modified to produce listeriolysin O, which improves plasmid delivery in phagocytic cells.

Also provided are bacteria modified to carry a low copy, CpG-containing plasmid. The plasmid further can include other modifications, and RNAi.

The bacteria also can be modified to grow in a manner such that the bacteria, if a *Salmonella* species, expresses less of the toxic SPI-1 (*Salmonella* pathogenicity island-1) genes. In *Salmonella*, genes responsible for virulence, invasion, survival, and extra intestinal spread are located in *Salmonella* pathogenicity islands (SPIs).

The bacteria include plasmids that encode RNAi, such as shRNA or microRNA, that inhibits checkpoints, such as PD-L1 or TREX1 only, or TREX1 and one or more of a second immune checkpoint. The bacteria can be further modified for other desirable traits, including for selection of plasmid maintenance, particularly for selection without antibiotics, for preparation of the strains. The immunostimulatory bacteria optionally can encode therapeutic polypeptides, including anti-tumor therapeutic polypeptides and agents.

Exemplary of the immunostimulatory bacteria provided herein are species of *Salmonella*. Exemplary of bacteria for modification as described herein are engineered strains of *Salmonella typhimurium*, such as strain YS1646 (ATCC Catalog # 202165; see, also International PCT application No Publication No. WO 99/13053, also referred to as VNP20009) that is engineered with plasmids to complement an *asd* gene knockout and antibiotic-free plasmid maintenance.

Modified immunostimulatory bacterial strains that are rendered auxotrophic for adenosine are provided herein as are pharmaceutical compositions containing such strains formulated for administration to a subject, such as a human, for use in methods of treating tumors and cancers.

The engineered immunostimulatory bacteria provided herein contain multiple synergistic modalities to induce immune re-activation of cold tumors and to promote tumor antigen-specific immune responses, while inhibiting immune checkpoint pathways that the tumor utilizes to subvert and evade durable anti-tumor immunity. Improved tumor targeting through adenosine auxotrophy and enhanced vascular disruption have improved potency, while localizing the inflammation to limit systemic cytokine exposure and the autoimmune toxicities observed with other immunotherapy modalities. Exemplary of the bacteria so-modified are *S. typhimurium* strains, including such modifications of the strain YS1646, particularly *asd*$^{-}$ strains.

For example, as provided herein, are immunostimulatory bacteria that provide for shRNA-mediated gene disruption of PD-L1. It has been shown in mice that gene

disruption of PD-L1 can improve tumor colonization. It has been shown, for example, that *S. typhimurium* infection in PD-L1 knockout mice, results in a 10-fold higher bacterial load than in wild-type mice. (see, Lee *et al.* (2010) *Immunol.* 185:2442-2449). Hence, PD-L1 is protective against *S. typhimurium* infection. Provided herein are immunostimulatory bacteria, such as *S. typhimurium*, carrying plasmids capable of RNAi-mediated gene knockdown of TREX1, PD-L1, or of PD-L1 and TREX1. Such bacteria provide anti-tumor effects due to the combination of two independent pathways that lead to enhanced and sustained anti-tumor immune responses in a single therapy.

## C. CANCER IMMUNOTHERAPEUTICS

The immunosuppressive milieu found within the tumor microenvironment (TME) is a driver of tumor initiation and progression. Cancers emerge after the immune system fails to control and contain tumors. Multiple tumor-specific mechanisms create tumor environments wherein the immune system is forced to tolerate tumors and their cells instead of eliminating them. The goal of cancer immunotherapy is to rescue the immune system's natural ability to eliminate tumors. Acute inflammation associated with microbial infection has been observationally linked with the spontaneous elimination of tumors for centuries.

### 1. Immunotherapies

Several clinical cancer immunotherapies have sought to perturb the balance of immune suppression towards anti-tumor immunity. Strategies to stimulate immunity through directly administering cytokines such as IL-2 and IFN-α have seen modest clinical responses in a minority of patients, while inducing serious systemic inflammation-related toxicities (Sharma *et al.* (2011) *Nat Rev Cancer* 11:805-812). The immune system has evolved several checks and balances to limit autoimmunity, such as upregulation of programmed cell death protein 1 (PD-1) on T cells and its binding to its cognate ligand, programmed death-ligand 1 (PD-L1), which is expressed on both antigen presenting cells (APCs) and tumor cells. The binding of PD-L1 to PD-1 interferes with $CD8^+$ T cell signaling pathways, impairing the proliferation and effector function of $CD8^+$ T cells, and inducing T cell tolerance. PD-1 and PD-L1 are two examples of numerous inhibitory "immune checkpoints," which function by downregulating immune responses. Other inhibitory immune checkpoints

include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), signal regulatory protein α (SIRPα), V-domain Ig suppressor of T cell activation (VISTA), programmed death-ligand 2 (PD-L2), indoleamine 2,3-dioxygenase (IDO) 1 and 2, lymphocyte-activation gene 3 (LAG3), Galectin-9, T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin and mucin-domain containing-3 (TIM-3, also known as hepatitis A virus cellular receptor 2 (HAVCR2)), herpesvirus entry mediator (HVEM), CD39, CD73, B7-H3 (also known as CD276), B7-H4, CD47, CD48, CD80 (B7-1), CD86 (B7-2), CD155, CD160, CD244 (2B4), B- and T-lymphocyte attenuator (BTLA, or CD272) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1, or CD66a).

Antibodies designed to block immune checkpoints, such as anti-PD-1 (for example, pembrolizumab, nivolumab) and anti-PD-L1 (for example, atezolizumab, avelumab, durvalumab), have had durable success in preventing T cell anergy and breaking immune tolerance. Only a fraction of treated patients demonstrate clinical benefit, and those that do often present with autoimmune-related toxicities (see, *e.g.*, Ribas (2015) *N Engl J Med* 373:1490-1492; Topalian *et al.* (2012) *N Engl J Med 366*:3443-3447). This is further evidence for the need for therapies, provided herein, that are more effective and less toxic.

Another checkpoint blockade strategy inhibits the induction of CTLA-4 on T cells, which binds to and inhibits co-stimulatory receptors on APCs, such as CD80 or CD86, out-competing the co-stimulatory cluster differentiation 28 (CD28), which binds the same receptors, but with a lower affinity. This blocks the stimulatory signal from CD28, while the inhibitory signal from CTLA-4 is transmitted, preventing T cell activation (see, Phan *et al.*(2003) *Proc. Natl. Acad. Sci. U.S.A.* 100:8372-8377). Anti-CTLA-4 therapy (for example, ipilimumab) have clinical success and durability in some patients, whilst exhibiting an even greater incidence of severe immune-related adverse events (see, *e.g.*, Hodi *et al.* (2010) *N Engl J Med* 363:711-723; Schadendorf *et al.* (2015) *J. Clin. Oncol.* 33:1889-1894). It also has been shown that tumors develop resistance to anti-immune checkpoint antibodies, highlighting the need for more durable anticancer therapies, and provided herein.

## 2. Adoptive Immunotherapies

In seeking to reactivate a cold tumor to become more immunogenic, a class of immunotherapies known as adoptive cell therapy (ACT) encompasses a variety of strategies to harness immune cells and reprogram them to have anti-tumor activity (Hinrichs *et al.* (2011) *Immunol. Rev.* 240:40-51). Dendritic cell-based therapies introduce genetically engineered dendritic cells (DCs) with more immune-stimulatory properties. These therapies have not been successful because they fail to break immune tolerance to cancer (see, *e.g.*, Rosenberg *et al.* (2004) *Nat. Med.* 12:1279). A method using whole irradiated tumor cells containing endogenous tumor antigens and granulocyte macrophage colony-stimulating factor (GM-CSF) to stimulate DC recruitment, known as GVAX, similarly failed in the clinic due to the lack of ability to break tumor tolerance (Copier *et al.* (2010) *Curr. Opin. Mol. Ther.* 12:647-653). A separate autologous cell-based therapy, Sipuleucel-T (Provenge), was FDA approved in 2010 for castration-resistant prostate cancer. It utilizes APCs retrieved from the patient and re-armed to express prostatic acid phosphatase (PAP) antigen to stimulate a T cell response, then re-introduced following lymphablation. Unfortunately, its broader adoption has been limited by low observed objective response rates and high costs, and its use is limited to only the early stages of prostate cancer (Anassi *et al.* (2011) *P T.* 36(4):197–202). Similarly, autologous T cell therapies (ATCs) harvest a patient's own T cells and reactivate them *ex vivo* to overcome tumor tolerance, then reintroduce them to the patient following lymphablation. ATCs have had limited clinical success, and only in melanoma, while generating serious safety and feasibility issues that limit their utility (Yee *et al.* (2013) *Clin. Cancer Res.* 19:1-3).

Chimeric antigen receptor T cell (CAR-T) therapies are T cells harvested from patients that have been re-engineered to express a fusion protein between the T cell receptor and an antibody Ig variable extracellular domain. This confers upon them the antigen-recognition properties of antibodies with the cytolytic properties of activated T cells (Sadelain (2015) *Clin. Invest.* 125:3392-400). Success has been limited to B cell and hematopoietic malignancies, at the cost of deadly immune-related adverse events (Jackson *et al.* (2016) *Nat. Rev. Clin. Oncol. 13*:370-383). Tumors can also mutate to escape recognition by a target antigen, including CD19 (Ruella *et al.*, (2016) *Comput Struct Biotechnol J.* 14: 357–362) and EGFRvIII

(O'Rourke *et al.* (2017) *Sci Transl Med.* Jul 19;9:399), thereby fostering immune escape. In addition, while CAR-T therapies are approved and are approved in the context of hematological malignancies, they face a significant hurdle for feasibility to treat solid tumors: overcoming the highly immunosuppressive nature of the solid tumor microenvironment. A number of additional modifications to existing CAR-T therapies will be required to potentially provide feasibility against solid tumors (Kakarla, *et al.* (2014) *Cancer J.* Mar-Apr; 20(2): 151–155). When the safety of CAR-Ts is significantly improved and their efficacy expanded to solid tumors, the feasibility and costs associated with these labor-intensive therapies will continue to limit their broader adoption.

**3. Cancer Vaccines and Oncolytic Viruses**

Cold tumors lack T cell and dendritic cell (DC) infiltration, and are non-T-cell-inflamed (Sharma *et al.* (2017) *Cell* 9;168(4):707-723). In seeking to reactivate a cold tumor to become more immunogenic, another class of immunotherapies harness microorganisms that can accumulate in tumors, either naturally or by virtue of engineering. These include viruses designed to stimulate the immune system to express tumor antigens, thereby activating and reprogramming the immune system to reject the tumor. Virally-based cancer vaccines have largely failed clinically for a number of factors, including pre-existing or acquired immunity to the viral vector itself, as well as a lack of sufficient immunogenicity to the expressed tumor antigens (Larocca *et al.* (2011) *Cancer J.* 17(5):359-371). Lack of proper adjuvant activation of APCs has also hampered other non-viral vector cancer vaccines, such as DNA vaccines. Oncolytic viruses, in contrast, seek to preferentially replicate in dividing tumor cells over healthy tissue, whereupon subsequent tumor cell lysis leads to immunogenic tumor cell death and further viral dissemination. The oncolytic virus Talimogene laherparepvec (T-VEC), which uses a modified herpes simplex virus in combination with the DC-recruiting cytokine GM-CSF, is FDA approved for metastatic melanoma (Bastin *et al.* (2016) *Biomedicines* 4(3):21). While demonstrating clinical benefit in some melanoma patients, and with fewer immune toxicities than with other immunotherapies, the intratumoral route of administration and manufacturing conditions have been limiting, as well as its lack of distal tumor efficacy and broader application to other tumor types. Other oncolytic virus (OV)-based vaccines, such

as those utilizing paramyxovirus, reovirus and picornavirus, among others, have met with similar limitations in inducing systemic anti-tumor immunity (Chiocca *et al.* (2014) *Cancer Immunol. Res.* 2(4):295-300). Systemic administration of oncolytic viruses presents unique challenges. Upon IV administration, the virus is rapidly diluted, thus requiring high titers that can lead to hepatotoxicity. Further, if pre-existing immunity exists, the virus is rapidly neutralized in the blood, and acquired immunity then restricts repeat dosing (Maroun *et al.* (2017) *Future Virol.* 12(4):193–213).

Of the limitations of virally-based vaccine vectors and oncolytic viruses, the greatest limitations can be the virus itself. Viral antigens have strikingly higher affinities to human T cell receptors (TCR) compared to tumor antigens (Aleksic *et al.* (2012) *Eur J Immunol.* 42(12):3174–3179). Tumor antigens, presented alongside of viral vector antigens by MHC-1 on the surface of even highly activated APCs, will be outcompeted for binding to TCRs, resulting in very poor antigen-specific anti-tumor immunity. A tumor-targeting immunostimulatory vector, as provided herein, that does not itself provide high affinity T cell epitopes can circumvent these limitations.

## D. BACTERIAL CANCER IMMUNOTHERAPY

### 1. Bacterial Therapies

The recognition that bacteria have anticancer activity goes back to the 1800s, when several physicians observed regression of tumors in patients infected with *Streptococcus pyogenes.* William Coley began the first study utilizing bacteria for the treatment of end stage cancers, and developed a vaccine composed of *S. pyogenes* and *Serratia marcescens*, which was successfully used to treat a variety of cancers, including sarcomas, carcinomas, lymphomas and melanomas. Since then, a number of bacteria, including species of *Clostridium, Mycobacterium, Bifidobacterium, Listeria,* such as, *L. monocytogenes*, and *Escherichia* species, have been studied as sources of anti-cancer vaccines (see, *e.g.*, Published International PCT application WO 1999/013053; Published International PCT application 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; Pawelek *et al.* (2003) *Lancet Oncol.4*:548-556).

Bacteria can infect animal and human cells, and some possess the innate ability to deliver DNA into the cytosol of cells, and these are candidate vectors for

gene therapy. Bacteria also are suitable for therapy because they can be administered orally, they propagate readily *in vitro* and *in vivo,* and they can be stored and transported in a lyophilized state. Bacterial genetics are readily manipulated, and the complete genomes for many strains have been fully characterized (Felgner *et al.* (2016) *mbio* 7(5):e01220-16). As a result, bacteria have been used to deliver and express a wide variety of genes, including those that encode cytokines, angiogenesis inhibitors, toxins and prodrug-converting enzymes. *Salmonella*, for example, has been used to express immune-stimulating molecules like IL-18 (Loeffler *et al.* (2008) *Cancer Gene Ther.* 15(12):787-794), LIGHT (Loeffler *et al.* (2007) *PNAS* 104(31):12879-12883), and Fas ligand (Loeffler *et al.* (2008) *J. Natl. Cancer Inst.* 100:1113-1116) in tumors. Bacterial vectors also are cheaper and easier to produce than viral vectors, and bacterial delivery is favorable over viral delivery because it can be quickly eliminated by antibiotics if necessary, rendering it a safer alternative.

To be used, however, the strains themselves must not be pathogenic or are not pathogenic after modification for use as a therapeutic. For example, in the treatment of cancer, the therapeutic bacterial strains must be attenuated or rendered sufficiently non-toxic so as to not cause systemic disease and/or septic shock, but still maintain some level of infectivity to effectively colonize tumors. Genetically modified bacteria have been described that are to be used as antitumor agents to elicit direct tumoricidal effects and/or to deliver tumoricidal molecules (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. USA* 102:755-760; Zhao, M. *et al.* (2006) *Cancer Res.* 66:7647-7652). Among these are bioengineered strains of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). These bacteria accumulate preferentially >1,000-fold greater in tumors than in normal tissues and disperse homogeneously in tumor tissues (Pawelek, J. *et al.* (1997) *Cancer Res.* 57:4537-4544; Low, K. B. *et al.* (1999) *Nat. Biotechnol.* 17:37-41). Preferential replication allows the bacteria to produce and deliver a variety of anticancer therapeutic agents at high concentrations directly within the tumor, while minimizing toxicity to normal tissues. These attenuated bacteria are safe in mice, pigs, and monkeys when administered i.v. (Zhao, M. *et al.* (2005) *Proc Natl Acad Sci USA* 102:755-760; Zhao, M. *et al.* (2006) *Cancer Res* 66:7647-7652; Tjuvajev J. *et al.*

(2001) *J. Control Release* 74:313-315; Zheng, L. *et al.* (2000) *Oncol. Res.* 12:127-135), and certain live attenuated *Salmonella* strains have been shown to be well tolerated after oral administration in human clinical trials (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; Sirard, J. C. *et al.* (1999) *Immunol. Rev.* 171:5-26). The *S. typhimurium* phoP/phoQ operon is a typical bacterial two-component regulatory system composed of a membrane-associated sensor kinase (PhoQ) and a cytoplasmic transcriptional regulator (PhoP: Miller, S. I. *et al.* (1989) *Proc Natl Acad Sci USA* 86:5054-5058; Groisman, E. A. *et al.* (1989) *Proc Natl Acad Sci USA* 86: 7077-7081). PhoP/phoQ is required for virulence, and its deletion results in poor survival of this bacterium in macrophages and a marked attenuation in mice and humans (Miller, S. I. *et al.* (1989) *Proc Natl Acad Sci USA* 86:5054-5058; Groisman, E. A. *et al.* (1989) *Proc Natl Acad Sci USA* 86: 7077-7081; Galan, J. E. and Curtiss, R. III. (1989) *Microb Pathog* 6:433-443; Fields, P. I. *et al.* (1986) *Proc Natl Acad Sci USA* 83:189-193). PhoP/phoQ deletion strains have been employed as effective vaccine delivery vehicles (Galan, J. E. and Curtiss, R. III. (1989) *Microb Pathog* 6:433-443; Fields, P. I. *et al.* (1986) *Proc Natl Acad Sci USA* 83:189-193; Angelakopoulos, H. and Hohmann, E. L. (2000) *Infect Immun* 68:213-241). Attenuated *Salmonellae* have been used for targeted delivery of tumoricidal proteins (Bermudes, D. *et al.* (2002) *Curr Opin Drug Discov Devel* 5:194-199; Tjuvajev J. *et al.* (2001) *J Control Release* 74:313-315).

Bacterially-based cancer therapies have demonstrated limited clinical benefit. A variety of bacterial species, including *Clostridium novyi* (Dang *et al.* (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98(26):15155-15160; U.S. Patent Publications Nos. 2017/0020931, 2015/0147315; U.S. Patent Nos. 7,344,710; 3,936,354), *Mycobacterium bovis* (U.S. Patent Publications Nos. 2015/0224151; US 2015/0071873), *Bifidobacterium bifidum* (Kimura *et al.* (1980) *Cancer Res.* 40:2061-2068), *Lactobacillus casei* (Yasutake *et al.* (1984) *Med Microbiol Immunol.* 173(3):113-125), *Listeria monocytogenes* (Le *et al.* (2012) *Clin. Cancer Res.* 18(3):858-868; Starks *et al.* (2004) *J. Immunol.* 173:420-427; U.S. Patent Publication No. 2006/0051380) and *Escherichia coli* (U.S. Patent No. 9,320,787) have been studied as possible agents for anticancer therapy.

The Bacillus Calmette-Guerin (BCG) strain, for example, is approved for the treatment of bladder cancer in humans, and is more effective than intravesical chemotherapy, often being used as a first-line treatment (Gardlik *et al.* (2011) *Gene therapy* 18:425-431). Another approach utilizes *Listeria monocytogenes*, a live attenuated intracellular bacterium capable of inducing potent $CD8^+$ T cell priming to expressed tumor antigens in mice (Le *et al.* (2012) *Clin. Cancer Res.* 18(3):858-868). In a clinical trial of the *Listeria*-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 (Le *et al.* (2015) *J. Clin. Oncol.* 33(12):1325-1333). These results were not replicated in a larger phase 2b study, possibly pointing to the difficulties in attempting to induce immunity to a low affinity self-antigen such as mesothelin.

Bacterial strains can be modified as described and exemplified herein to express inhibitory RNA (RNAi), such as shRNAs and microRNAs, that inhibit or disrupt TREX1 and/or PD-L1 and optionally one or more additional immune checkpoint genes. The strains can be attenuated by standard methods and/or by deletion or modification of genes, and by alteration or introduction of genes that render the bacteria able to grow *in vivo* primarily in immunoprivileged environments, such as the TME, in tumor cells and solid tumors. Strains for modification as described herein can be selected from among, for example, *Shigella, Listeria, E. coli, Bifidobacteriae* and *Salmonella*. For example, *Shigella sonnei, Shigella flexneri, Shigella disenteriae, Listeria monocytogenes, Salmonella typhi, Salmonella typhimurium, Salmonella gallinarum,* and *Salmonella enteritidis.* Other suitable bacterial species include *Rickettsia, Klebsiella, Bordetella, Neisseria, Aeromonas, Francisella, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Vibrio, Bacillus,* and *Erysipelothrix.* For example, *Rickettsia Rikettsiae, Rickettsia prowazekii, Rickettsia tsutsugamuchi, Rickettsia mooseri, Rickettsia sibirica, Bordetella bronchiseptica, Neisseria meningitidis, Neisseria gonorrhoeae, Aeromonas eucrenophila, Aeromonas salmonicida, Francisella tularensis,*

*Corynebacterium pseudotuberculosis, Citrobacter freundii, Chlamydia pneumoniae, Haemophilus sornnus, Brucella abortus, Mycobacterium intracellulare, Legionella pneumophila, Rhodococcus equi, Pseudomonas aeruginosa, Helicobacter mustelae, Vibrio cholerae, Bacillus subtilis, Erysipelothrix rhusiopathiae, Yersinia enterocolitica, Rochalimaea quintana,* and *Agrobacterium tumerfacium.* Any known therapeutic, including immunostimulatory, bacteria can be modified as described herein.

**2. Comparison of the Immune Responses to Bacteria and Viruses**

Bacteria, like viruses, have the advantage of being naturally immunostimulatory. Bacteria and viruses are known to contain conserved structures known as Pathogen-Associated Molecular Patterns (PAMPs), which are sensed by host cell Pattern Recognition Receptors (PRRs). Recognition of PAMPs by PRRs triggers downstream signaling cascades that result in the induction of cytokines and chemokines, and initiation of immune responses that lead to pathogen clearance (Iwasaki and Medzhitov (2010) *Science* 327(5963):291–295). The manner in which the innate immune system is engaged by PAMPs, and from what type of infectious agent, determines the appropriate adaptive immune response 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 bind a range of ligands, including lipopolysaccharide (TLR4), lipoproteins (TLR2), flagellin (TLR5), unmethylated CpG motifs in DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7 and TLR8) (Akira *et al.* (2001) *Nat. Immunol.* 2(8):675-680; Kawai and Akira (2005) *Curr. Opin. Immunol.* 17(4):338-344). Host surveillance of *S. typhimurium* for example, is largely mediated through TLR2, TLR4 and TLR5 (Arpaia *et al.* (2011) *Cell* 144(5):675-688). These TLRs signal through MyD88 and TRIF adaptor molecules to mediate induction of NF-kB dependent pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ (Pandey *et. al.* (2015) *Cold Spring Harb Perspect Biol* 7(1):a016246).

Another category of PRRs are the nod-like receptor (NLR) family. These receptors reside in the cytosol of host cells and recognize intracellular PAMPS. For example, *S. Typhimurium* flagellin was shown to activate the NLRC4/NAIP5

inflammasome pathway, resulting in the cleavage of caspase-1 and induction of the pro-inflammatory cytokines IL-1β and IL-18, leading to pyroptotic cell death of infected macrophages (Fink *et al.* (2007) *Cell Microbiol.* 9(11):2562–2570).

While engagement of TLR2, TLR4, TLR5 and the inflammasome induces pro-inflammatory cytokines that mediate bacterial clearance, they activate a predominantly NF-κB-driven signaling cascade that leads to recruitment and activation of neutrophils, macrophages and $CD4^+$ T cells, but not the DCs and $CD8^+$ T cells that are required for anti-tumor immunity (Lui *et al.* (2017) *Signal Transduct Target Ther.* 2:17023). In order to activate $CD8^+$ T cell-mediated anti-tumor immunity, IRF3/IRF7-dependent type I interferon signaling is critical for DC activation and cross-presentation of tumor antigens to promote $CD8^+$ T cell priming (Diamond *et al.* (2011) *J. Exp. Med.* 208(10):1989–2003; Fuertes *et al.* (2011) *J. Exp. Med.* 208(10):2005–2016). Type I interferons (IFN-α, IFN-β) are the signature cytokines induced by two distinct TLR-dependent and TLR-independent signaling pathways. The TLR-dependent pathway for inducing IFN-β occurs following endocytosis of pathogens, whereby TLR3, 7, 8 and 9 detect pathogen-derived DNA and RNA elements within the endosomes. TLRs 7 and 8 recognize viral nucleosides and nucleotides, and synthetic agonists of these, such as resiquimod and imiquimod have been clinically validated (Chi *et al.* (2017) *Frontiers in Pharmacology* 8:304). Synthetic dsRNA, such as polyinosinic:polycytidylic acid (poly (I:C)) and poly ICLC, an analog that is formulated with poly L lysine to resist RNase digestion, is an agonist for TLR3 and MDA5 pathways and a powerful inducer of IFN-β (Caskey *et al.* (2011) *J. Exp. Med.* 208(12):2357-66). TLR9 detection of endosomal CpG motifs present in viral and bacterial DNA can also induce IFN-β via IRF3. Additionally, TLR4 has been shown to induce IFN-β via MyD88-independent TRIF activation of IRF3 (Owen *et al.* (2016) *mBio.*7:1 e02051-15). It subsequently was shown that TLR4 activation of DCs was independent of type I IFN, so the ability of TLR4 to activate DCs via type I IFN is not likely biologically relevant (Hu *et al.* (2015) *Proc. Natl. Acad. Sci. U.S.A.* 112:45). Further, TLR4 signaling has not been shown to directly recruit or activate $CD8^+$ T cells.

Of the TLR-independent type I IFN pathways, one is 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 (MDA-5), and through the IFN-β promoter stimulator 1 (IPS-1) adaptor protein-mediated phosphorylation of the IRF-3 transcription factor, leading to induction of IFN-β (Ireton and Gale (2011) *Viruses* 3(6):906-919). Synthetic RIG-I-binding elements have also been discovered unintentionally in common lentiviral shRNA vectors, in the form of an AA dinucleotide sequence at the U6 promoter transcription start site. Its subsequent deletion in the plasmid prevented confounding off-target type I IFN activation (Pebernard *et al.* (2004) *Differentiation.* 72:103-111).

The second type of TLR-independent type I interferon induction pathway is mediated through Stimulator of Interferon Genes (STING), a cytosolic ER-resident adaptor protein that is now recognized as the central mediator for sensing cytosolic dsDNA from infectious pathogens or aberrant host cell damage (Barber (2011) *Immunol. Rev* 243(1):99-108). STING signaling activates the TANK binding kinase (TBK1)/IRF3 axis and the NF-kB signaling axis, resulting in the induction of IFN-β and other pro-inflammatory cytokines and chemokines that strongly activate innate and adaptive immunity (Burdette *et al.* (2011) *Nature* 478(7370):515-518). Sensing of cytosolic dsDNA through STING requires cyclic GMP-AMP synthase (cGAS), a host cell nucleotidyl transferase that directly binds dsDNA, and in response, synthesizes a cyclic dinucleotide (CDN) second messenger, cyclic GMP-AMP (cGAMP), which binds and activates STING (Sun *et al.* (2013) *Science* 339(6121):786-791; Wu *et al.* (2013) *Science* 339(6121):826-830). CDNs derived from bacteria such as c-di-AMP produced from intracellular *Listeria monocytogenes* can also directly bind murine STING, but only 3 of the 5 human STING alleles. Unlike the CDNs produced by bacteria, in which the two purine nucleosides are joined by a phosphate bridge with 3'-3' linkages, the internucleotide phosphate bridge in the cGAMP synthesized by mammalian cGAS is joined by a non-canonical 2'-3' linkage. These 2'-3' molecules bind to STING with 300-fold better affinity than bacterial 3'-3' CDNs, and thus are more potent physiological ligands of human 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; Ablasser *et al.* (2013) *Nature* 503(7477):530-534).

The cGAS/STING signaling pathway in humans may have evolved over time to preferentially respond to viral pathogens over bacterial pathogens, and this can explain why bacterial vaccines harboring host tumor antigens have made for poor $CD8^+$ T cell priming vectors in humans. TLR-independent activation of $CD8^+$ T cells by STING-dependent type I IFN signaling from conventional DCs is the primary mechanism by which viruses are detected, with TLR-dependent type I IFN production by plasmacytoid DCs operating only when the STING pathway has been virally-inactivated (Hervas-Stubbs *et al.* (2014) *J Immunol.* 193:1151-1161). Further, for bacteria such as *S. typhimurium*, while capable of inducing IFN-β via TLR4, $CD8^+$ T cells are neither induced nor required for clearance or protective immunity (Lee *et al.* (2012) *Immunol Lett.* 148(2): 138–143). The lack of physiologically relevant $CD8^+$ T epitopes for many strains of bacteria, including *S. typhimurium,* has impeded both bacterial vaccine development and protective immunity to subsequent infections, even from the same genetic strains (Lo *et al.* (1999) *J Immunol.* 162:5398-5406). Thus, bacterially-based cancer immunotherapies are biologically limited in their ability to induce type I IFN to recruit and activate $CD8^+$ T cells, necessary to promote tumor antigen cross-presentation and durable anti-tumor immunity. Hence, engineering a bacterial immunotherapy provided herein to induce viral-like TLR-independent type I IFN signaling, rather than TLR-dependent bacterial immune signaling, will preferentially induce $CD8^+$ T cell mediated anti-tumor immunity.

STING activates innate immunity in response to sensing nucleic acids in the cytosol. Downstream signaling is activated through binding of CDNs, which are synthesized by bacteria or by the host enzyme cGAS in response to binding to cytosolic dsDNA. Bacterial and host-produced CDNs have distinct phosphate bridge structures, which differentiates their capacity to activate STING. IFN-β is the signature cytokine of activated STING, and virally-induce type I IFN, rather than bacterially-induced IFN, is required for effective $CD8^+$ T cell mediated anti-tumor immunity. Immunostimulatory bacteria provided herein include those that are STING agonists.

### 3. *Salmonella* Therapy

*Salmonella* is exemplary of a bacterial genus that can be used as a cancer therapeutic. The *Salmonella* exemplified herein is an attenuated species or one that by virtue of the modifications for use as a cancer therapeutic has reduced toxicity.

**a. Tumor-tropic Bacteria**

A number of bacterial species have demonstrated preferential replication within solid tumors when injected from a distal site. These include, but are not limited to, species of *Salmonella*, *Bifodobacterium*, *Clostridium*, and *Escherichia*. The natural tumor-homing properties of the bacteria combined with the host's innate immune response to the bacterial infection is thought to mediate the anti-tumor response. This tumor tissue tropism has been shown to reduce 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 or hypoxic environments. A number of these naturally tumor-tropic bacteria have been further engineered to increase the potency of the antitumor 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).

**b. *Salmonella enterica* serovar *typhimurium***

*Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) is exemplary of a bacterial species for use as an anti-cancer therapeutic. One approach to using bacteria to stimulate host immunity to cancer has been through the Gram-negative facultative anaerobe *S. typhimurium*, which preferentially accumulates in hypoxic and necrotic areas in the body, including tumor microenvironments. *S. typhimurium* accumulates in these environments due to the availability of nutrients from tissue necrosis, the leaky tumor vasculature and their increased likelihood to survive in the immune system-evading tumor microenvironment (Baban *et al.* (2010) *Bioengineered Bugs* 1(6):385-294). *S. typhimurium* is able to grow under both aerobic and anaerobic conditions; therefore it is able to colonize small tumors that are less hypoxic and large tumors that are more hypoxic.

*S. typhimurium* is a Gram-negative, facultative pathogen that is transmitted via the fecal-oral route. It causes localized gastrointestinal infections, but also enters the bloodstream and lymphatic system after oral ingestion, infecting systemic tissues such as the liver, spleen and lungs. Systemic administration of wild-type *S. typhimurium* overstimulates TNF-α induction, leading to a cytokine cascade and septic shock,

which, if left untreated, can be fatal. As a result, pathogenic bacterial strains, such as *S. typhimurium*, must be attenuated to prevent systemic infection, without completely suppressing their ability to effectively colonize tumor tissues. Attenuation is often achieved by mutating a cellular structure that can elicit an immune response, such as the bacterial outer membrane or limiting its ability to replicate in the absence of supplemental nutrients.

*S. typhimurium* is an intracellular pathogen that is rapidly taken up by myeloid cells such as macrophages or it can induce its own uptake in in non-phagocytic cells such as epithelial cells. Once inside cells, it can replicate within a *Salmonella* containing vacuole (SCV) and can also escape into the cytosol of some epithelial cells. Many of the molecular determinants of *S. typhimurium* pathogenicity have been identified and the genes are clustered in *Salmonella* pathogenicity islands (SPIs). The two best characterized pathogenicity islands are SPI-1 which is responsible for mediating bacterial invasion of non-phagocytic cells, and SPI-2 which is required for replication within the SCV (Agbor and McCormick (2011) *Cell Microbiol.* 13(12):1858–1869). Both of these pathogenicity islands encode macromolecular structures called type three secretion systems (T3SS) that can translocate effector proteins across the host membrane (Galan and Wolf-Watz (2006) *Nature* 444:567-573).

**c. Bacterial Attenuation**

Therapeutic bacteria for administration as a cancer treatment should be attenuated. 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* (U.S. Patent Publication No. 2012/0009153) are widely 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 of the *aroA* gene, which is part of the shikimate pathway, connecting glycolysis to aromatic amino acid biosynthesis (Felgner *et al.* (2016) *MBio* 7(5):e01220-16). Deletion of *aroA* therefore results in bacterial auxotrophy for aromatic amino acids and subsequent attenuation (U.S. Patent Publication Nos. 2003/0170276, 2003/0175297, 2012/0009153, 2016/0369282,

International Patent 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 (U.S. Patent Publication No. 2016/0369282; International Patent Publication No. WO 2016/025582). For example, *S. typhimurium* strain SL7207 is an aromatic amino acid auxotroph (*aroA*⁻ mutant); strains A1 and A1-R are leucine-arginine auxotrophs. VNP20009 is a purine auxotroph (*purI*⁻ mutant). As shown herein, it is also auxotrophic for the immunosuppressive nucleoside adenosine.

Mutations that attenuate bacteria also include, but are not limited to, mutations in genes that alter the biosynthesis of lipopolysaccharide, 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 virulence factors such as *IsyA*, *pag*, *prg*, *iscA*, *virG*, *plc* and *act*; mutations that modify the stress response such as *recA*, *htrA*, *htpR*, *hsp* and *groEL*; mutations that disrupt the cell cycle such as *min*; and mutations that disrupt or inactivate regulatory functions, such as *cya*, *crp*, *phoP/phoQ*, and *ompR* (U.S. Patent Publication Nos. 2012/0009153, 2003/0170276, 2007/0298012; U.S. Patent No. 6,190,657; WO 2015/032165; Felgner *et al.* (2016) *Gut microbes* 7(2):171-177; Broadway *et al.* (2014) *J. Biotechnology* 192:177-178; Frahm *et al.* (2015) *mBio* 6(2):e00254-15; Kong *et al.* (2011) *Infection and Immunity* 79(12):5027–5038; Kong *et al.* (2012) *PNAS* 109(47):19414–19419). Ideally the genetic attenuations comprise gene deletions rather than point mutations to prevent spontaneous compensatory mutations that might result in reversion to a virulent phenotype.

**i. *msbB*⁻ Mutants**

The enzyme lipid A biosynthesis myristoyltransferase, encoded by the *msbB* gene in *S. typhimurium*, catalyzes the addition of a terminal myristyl group to the lipid A domain of lipopolysaccharide (LPS) (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. This modification significantly reduces the ability of the LPS to induce septic shock, attenuating the bacterial strain and reducing the potentially harmful production of

TNFα, thus lowering systemic toxicity. *S. typhimurium msbB* mutants maintain their ability to preferentially colonize tumors over other tissues in mice and retain anti-tumor activity, thus increasing the therapeutic index of *Salmonella* based immunotherapeutics (U.S. Patent Publication Nos. 2003/0170276, 2003/0109026, 2004/0229338, 2005/0225088, 2007/0298012).

For example, deletion of *msbB* in the *S. typhimurium* strain VNP20009 results in production of a predominantly penta-acylated LPS, which is less toxic than native hexa-acylated LPS and allows for systemic delivery without the induction of toxic shock (Lee *et al.* (2000) *International Journal of Toxicology* 19:19–25). Other LPS mutations 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 administration of higher doses.

**ii. *purI*⁻ Mutants**

Immunostimulatory bacteria that can be attenuated by rendering them auxotrophic for one or more essential nutrients, such as purines (for example, adenine), nucleosides (for example, adenosine) or amino acids (for example, arginine and leucine), are employed. In particular, in embodiments of the immunostimulatory bacteria provided herein, such as S. *typhimurium*, the bacteria are rendered auxotrophic for adenosine, which preferentially accumulates in tumor microenvironments. Hence, strains of immunostimulatory bacteria described herein are attenuated because they require adenosine for growth, and they preferentially colonize TMEs, which, as discussed below, have an abundance of adenosine.

Phosphoribosylaminoimidazole synthetase, an enzyme encoded by the *purI* gene (synonymous with the *purM* gene), is involved in the biosynthesis pathway of purines. Disruption of the *purI* gene thus renders the bacteria auxotrophic for purines. In addition to being attenuated, *purI*⁻ mutants are enriched in the tumor environment and have significant anti-tumor activity (Pawelek *et al.* (1997) *Cancer Research* 57:4537-4544). It was previously described that this colonization results from the high concentration of purines present in the interstitial fluid of tumors as a result of their rapid cellular turnover. Since the *purI*⁻ bacteria are unable to synthesize purines, they require an external source of adenine, and it was thought that this would lead to their restricted growth in the purine-enriched tumor microenvironment (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 (Low *et al.* (2003) *Methods in Molecular Medicine* Vol. 90, *Suicide Gene Therapy*:47-59), subsequent analysis of the entire genome of VNP20009 demonstrated that the purI gene is not deleted, but is disrupted by a chromosomal inversion (Broadway *et al.* (2014) *Journal of Biotechnology* 192:177–178). The entire gene is contained within two parts of the VNP20009 chromosome that is flanked by insertion sequences (one of which has an active transposase).

It is shown herein, that, *purI* mutant *S. typhimurium* strains are auxotrophic for the nucleoside adenosine, which is highly enriched in tumor microenvironments. Hence, when using VNP20009, it is not necessary to introduce any further modification to achieve 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 to prevent reversion to a wild-type gene.

**iii. Combinations of Attenuating Mutations**

A bacterium with multiple genetic attenuations by means of gene deletions on disparate regions of the chromosome is desirable for bacterial immunotherapies because the attenuation can be increased, while decreasing the possibility of reversion to a virulent phenotype by acquisition of genes by homologous recombination with a wild-type genetic material. Restoration of virulence by homologous recombination would require two separate recombination events to occur within the same organism. Ideally the combinations of attenuating mutations selected for use in an immunotherapeutic agent increases the tolerability without decreasing the potency, thereby increasing the therapeutic index. For example, disruption of the *msbB* and *purI* genes in *S. typhimurium* strain VNP20009, has been used for tumor-targeting and growth suppression, and elicits low toxicity in animal models (Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002; Bermudes *et al.* (2000) *Cancer Gene Therapy: Past Achievements and Future Challenges*, edited by Habib Kluwer Academic/Plenum Publishers, New York, pp. 57-63; Low *et al.* (2003) *Methods in Molecular Medicine*, Vol. 90, *Suicide Gene Therapy*:47-59; Lee *et al.* (2000) *International Journal of Toxicology* 19:19–25; Rosenberg *et al.* (2002) *J. Immunotherapy* 25(3):218-225; 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). When VNP20009 (*msbB*⁻/*purI*⁻) was administered to mice bearing syngeneic or human xenograft tumors, the bacteria accumulated preferentially within the extracellular components of tumors at ratios exceeding 300-1000 to 1, reduced TNFα induction, and demonstrated tumor regression and prolonged survival compared to control mice (Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002). Results from the Phase 1 clinical trial in humans, however, revealed that while VNP20009 was relatively safe and well tolerated, poor accumulation was observed in human melanoma tumors, and very little anti-tumor activity was demonstrated (Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152). Higher doses, which are required to manifest any anti-tumor activity, were not possible due to toxicity.

Thus, further improvements are needed. The immunostimulatory bacteria provided herein address this problem.

**iv. VNP20009 and Other Attenuated *S. typhimurium* Strains**

Exemplary of a therapeutic bacterium that can be modified as described herein is the strain designated as VNP20009 (ATCC # 202165, YS1646). The clinical candidate, VNP20009 (ATCC # 202165, YS1646), was at least 50,000-fold attenuated for safety by deletion of both the *msbB* and *purI* genes (Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002; Low *et al.* (2003) *Methods in Molecular Medicine*, Vol. 90, *Suicide Gene Therapy*:47-59; Lee *et al.* (2000) *International Journal of Toxicology* 19:19–25). Similar strains of *Salmonella* that are attenuated also are contemplated. As described above, deletion of *msbB* alters the composition of the lipid A domain of lipopolysaccharide, the major component of Gram-negative bacterial outer membranes (Low *et al.* (1999) *Nat. Biotechnol.* 17(1):37-41). This prevents lipopolysaccharide-induced septic shock, attenuating the bacterial strain and lowering systemic toxicity, while reducing the potentially harmful production of TNFα (Dinarello, C.A. (1997) *Chest* 112(6 Suppl):321S-329S; Low *et al.* (1999) *Nat. Biotechnol.* 17(1):37-41). Deletion of the *purI* gene renders the bacteria auxotrophic for purines, which further attenuates the bacteria and enriches it in the tumor micro environment (Pawelek *et al.* (1997) *Cancer Res.* 57:4537-4544; Broadway *et al.* (2014) *J. Biotechnology* 192:177-178).

The accumulation of VNP20009 in tumors results from a combination of factors including: the inherent invasiveness of the parental strain, ATCC14028, its ability to replicate in hypoxic environments, and its requirement for high concentrations of purines that are present in the interstitial fluid of tumors. Herein we will demonstrate that VNP20009 is also auxotrophic for the nucleoside adenosine, which can accumulate to pathologically high levels in the tumor microenvironment and contribute to an immunosuppressive tumor microenvironment (Peter Vaupel and Arnulf Mayer Oxygen Transport to Tissue XXXVII, Advances in Experimental Medicine and Biology 876 chapter 22, pp. 177-183). When VNP20009 was administered into mice bearing syngeneic or human xenograft tumors, the bacteria accumulated preferentially within the extracellular components of tumors at ratios exceeding 300-1000 to 1 and demonstrated tumor growth inhibition as well as prolonged survival compared to control mice (Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002). Results from the Phase 1 clinical trial revealed that while VNP20009 was relatively safe and well tolerated, poor accumulation was observed in human melanoma tumors, and very little anti-tumor activity was demonstrated (Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152). Higher doses, which would be required to affect any anti-tumor activity, were not possible due to toxicity that correlated with high levels of pro-inflammatory cytokines.

Other strains of *S. typhimurium* can be used for tumor-targeted delivery and therapy, such as, for example, leucine-arginine auxtroph A-1 (Zhao *et al.* (2005) *PNAS* 102(3):755-760; Yu *et al.* (2012) *Scientific Reports* 2:436; US Pat. No. 8,822,194; U.S. Patent Publication No. 2014/0178341) and its derivative AR-1 (Yu *et al.* (2012) *Scientific Reports* 2:436; Kawagushi *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; Binder *et al.* (2013) *Cancer Immunol Res.* 1(2):123-133); *aroA*⁻ mutant S. *typhimurium* strain SL7207 (Guo *et al.* (2011) *Gene therapy* 18:95-105; U.S. Patent Publication Nos. 2012/0009153, 2016/0369282 and 2016/0184456) and its obligate anaerobe derivative YB1 (WO 2015/032165; Yu *et al.* (2012) *Scientific Reports* 2:436; Leschner *et al.* (2009) *PLoS ONE* 4(8): e6692; Yu *et al.* (2012) *Scientific*

*Reports* 2:436); *aroA⁻/aroD⁻* mutant *S. typhimurium* strain BRD509, a derivative of the SL1344 (WT) strain (Yoon *et al.* (2017) *European J. of Cancer* 70:48-61); *asd⁻ /cya⁻/crp⁻* mutant *S. typhimurium* strain χ4550 (Sorenson *et al.* (2010) *Biology: Targets & Therapy* 4:61-73) and *phoP⁻/phoQ⁻ S. typhimurium* strain LH430 (WO 2008/091375).

Although VNP20009 failed to show a clinical benefit in a study involving patients with advanced melanoma, a maximum tolerated dose (MTD) was established and the treatment was safely administered to advanced cancer patients. Hence, this strain, as well as other similarly engineered bacterial strains, can be used as tumor-targeting, therapeutic delivery vehicles. Modifications provided herein provide a strategy to increase efficacy, by increasing the anti-tumor efficiency and/or the safety and tolerability of the therapeutic agent.

**v. Attenuated *S. typhimurium* Engineered To Deliver Macromolecules**

The bacterial strains are engineered to deliver therapeutic molecules. The strains herein deliver RNAi targeted and inhibitory to immune checkpoints, and also to other such targets.

While the use of VNP20009 in clinical trials of metastatic melanoma resulted in no significant changes in metastatic burden, it did demonstrate some evidence of tumor colonization. VNP20009 and other *S. typhimurium* strains have been used as vectors to deliver a wide variety of genes, such as those encoding cytokines, anti-angiogenic factors, inhibitory enzymes and cytotoxic polypeptides (U.S. Patent Publication No. 2007/0298012). For example, the delivery of cytokine-encoding LIGHT using VNP20009 inhibited growth of primary tumors as well as pulmonary metastases of carcinoma cell lines in immunocompetent mice, with no significant toxicity observed (Loeffler *et al.* (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104(31):12879-12883). In another study, VNP20009, expressing an *E. coli* cytosine deaminase gene was administered to patients who also received the prodrug 5-fluorocytosine (5-FC) orally. Two out of three patients showed intratumoral bacterial colonization for at least 15 days after initial injection, and the expressed cytosine deaminase converted the 5-FC to the anticancer drug 5-FU. No side effects from the *Salmonella* were observed, and direct IV administration of 5-FU resulted in lower tumor concentrations

of the drug than with bacterial delivery of the cytosine deaminase gene (Nemunaitis *et al.* (2003) *Cancer Gene Therapy* 10:737-744).

In other examples, attenuated *Salmonella* expressing herpes simplex virus thymidine kinase (HSV TK) demonstrated a 2.5-fold reduction in B16 melanoma tumor size via ganciclovir-mediated tumor growth suppression (Pawelek, J. *et al.* (1997) *Cancer Res* 57:4537-4544), and the C-terminal p53 peptide (Cp53) was delivered using *S. typhimurium* and inducibly-expressed in MCF7 breast cancer cells, resulting in a decrease in tumor cell population (Camacho *et al.* (2016) *Scientific Reports* 6:30591). *S. typhimurium* has also been utilized in the tumor-targeted expression of IFN-γ (Yoon *et al.* (2017) *European J. of Cancer* 70:48-61); SIINF antigen (Binder *et al.* (2013) *Cancer Immunol Res.* 1(2):123-133); *Vibrio vulnificus* flagellin B (Zheng *et al.* (2017) *Sci. Transl. Med.* 9, 9537); and truncated IL-2 (Sorenson *et al.* (2010) *Biology: Targets & Therapy* 4:61-73), for example.

*S. typhimurium* has also been modified to deliver the tumor-associated antigen (TAA) survivin (SVN) to APCs to prime adaptive immunity (U.S. Patent Publication No. 2014/0186401; 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 utilizes *Salmonella* Pathogenicity Island 2 (SPI-2) and its type III secretion system (T3SS) to deliver the TAAs into the cytosol of APCs, which then are activated to induce TAA-specific CD8+ T cells and anti-tumor immunity (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 yet to demonstrate effective tumor antigen-specific T cell priming in humans.

In addition to gene delivery, *S. typhimurium* also has been used for the delivery of small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) for cancer therapy. For example, attenuated *S. typhimurium* have been modified to express certain shRNAs, such as those that target Stat 3 and IDO1 (PCT/US2007/074272, and U.S. Patent No. 9,453,227). VNP20009 transformed with an shRNA plasmid against the immunosuppressive gene indolamine deoxygenase (IDO), successfully silenced IDO expression in a murine melanoma model, resulting in tumor cell death and significant tumor infiltration by neutrophils (Blache *et al.*

(2012) *Cancer Res.* 72(24):6447-6456). Combining this vector with the co-administration of PEGPH20 (an enzyme that depletes extracellular hyaluronan), showed positive results in the treatment of pancreatic ductal adenocarcinoma tumors (Manuel *et al.* (2015) *Cancer Immunol. Res.* 3(9):1096-1107; U.S. Patent Publication No. 2016/0184456). In another study, an *S. typhimurium* strain attenuated by a phoP/phoQ deletion and expressing a signal transducer and activator of transcription 3 (STAT3)-specific shRNA, was found to inhibit tumor growth and reduce the number of metastatic organs, extending the life of C57BL6 mice (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 (Guo *et al.* (2011) *Gene therapy* 18:95-105; U.S. Patent Publication Nos. 2009/0123426, 2016/0369282), while *S. typhimurium* strain VNP20009 has been utilized in the delivery of shRNA targeting the STAT3 (Manuel *et al.* (2011) *Cancer Res.* 71(12):4183-4191; U.S. Patent Publication Nos. 2009/0208534, 2014/0186401, 2016/0184456; WO 2008/091375; 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 (Liu *et al.* (2016) *Oncotarget* 7(16):22873-22882). Improvement of such strains is needed so that they more effectively stimulate the immune response, and have other advantageous properties, such as the immunostimulatory bacteria provided herein.

Any of the bacteria described above can be modified as described herein, such as by adding additional shRNA or microRNA encoding nucleic acids to target other checkpoints, such as TREX1. The bacteria can be modified as described herein to have reduced inflammatory effects, and, thus to be less toxic. As a result, for example, higher dosages can be administered. Any of these strains of *Salmonella*, as well as other species of bacteria, known to those of skill in the art and/or listed above and herein, can be modified as described herein, such as by introducing adenosine auxotrophy and/or shRNA for inhibiting TREX1 expression and other modifications as described herein. Exemplary are the *S. typhimurium* species described herein. It is shown herein that the *S. typhimurium* strain VNP20009 is auxotrophic for adenosine.

**4. Enhancements of Immunostimulatory Bacteria to Increase Therapeutic Index**

Provided herein are enhancements to immunostimulatory bacteria that reduce toxicity and improve the anti-tumor activity. Exemplary of such enhancements are the following. They are described with respect to *Salmonella*, particularly *S. typhimurium*; it is understood that the skilled person can effect similar enhancements in other bacterial species and other *Salmonella* strains.

**a. *asd* Gene Deletion**

The *asd* gene in bacteria encodes an aspartate-semialdehyde dehydrogenase. *asd*- mutants of *S. typhimurium* have an obligate requirement for diaminopimelic acid (DAP) which is required for cell wall synthesis and will undergo lysis in environments deprived of DAP. This DAP auxotrophy can be used for plasmid selection and maintenance of plasmid stability *in vivo* without the use of antibiotics when the *asd* gene is complemented in trans on a plasmid. Non-antibiotic-based plasmid selection systems are advantageous and allow for 1) use of administered antibiotics as 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 selection (Galán *et al.* (1990) *Gene* 28:29-35). 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 genes or interfering RNAs.

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 macromolecules to infected cells without the ability to persistently colonize host tumors. Deletion of the *asd* gene makes the bacteria auxotrophic for DAP when grown *in vitro* or *in vivo*. An example described herein, provides an *asd* deletion strain that is auxotrophic for DAP and contains a plasmid suitable for delivery of RNAi, such as shRNA or mi-RNA, that does not contain an *asd* complementing gene, resulting in a strain that is defective for replication *in vivo*. This strain is propagated *in vitro* in the presence of DAP and grows normally, and then is administered as an immunotherapeutic agent to a mammalian host where DAP is not present. The suicidal strain is able to invade host cells but is not be able to replicate due to the absence of DAP in mammalian tissues, lysing automatically and delivering its cytosolic contents (*e.g.*, plasmids or proteins). In examples provided herein, an *asd* gene deleted strain of VNP20009 was further

modified to express an LLO protein lacking its endogenous periplasmic secretion signal sequence, causing it to accumulate in the cytoplasm of the *Salmonella*. LLO is a cholesterol-dependent pore forming hemolysin from *Listeria monocytogenes* that mediates phagosomal escape of bacteria. When the autolytic strain is introduced into tumor bearing mice, the bacteria are taken up by phagocytic immune cells and enter the *Salmonella* containing vacuole (SCV). In this environment, the lack of DAP will prevent bacterial replication, and result in autolysis of the bacteria in the SCV. Lysis of the suicidal strain will then allow for release of the plasmid and the accumulated LLO that will form pores in the cholesterol-containing SVC membrane, and allow for delivery of the plasmid into the cytosol of the host cell.

**b. Adenosine Auxotrophy**

Metabolites derived from the tryptophan and ATP/adenosine pathways are major drivers in forming an immunosuppressive environment within the tumor. Adenosine, which exists in the free form inside and outside of cells, is an effector of immune function. Adenosine decreases T-cell receptor induced activation of NF-κB, and inhibits IL-2, IL-4, and IFN-γ. Adenosine decreases T-cell cytotoxicity, increases T-cell anergy, and increases T-cell differentiation to Foxp3+ or Lag-3+ regulatory (T-reg) T-cells. On NK cells, adenosine decreases IFN-γ production, and suppresses NK cell cytotoxicity. Adenosine blocks neutrophil adhesion and extravasation, decreases phagocytosis, and attenuates levels of superoxide and nitric oxide. Adenosine also decreases the expression of TNF-α, IL-12, and MIP-1α on macrophages, attenuates MHC Class II expression, and increases levels of IL-10 and IL-6. Adenosine immunomodulation activity occurs after its release into the extracellular space of the tumor and activation of adenosine receptors (ADRs) on the surface of target immune cells, cancer cells or endothelial cells. The high adenosine levels in the tumor microenvironment result in local immunosuppression, which limits the capacity of the immune system to eliminate cancer cells.

Extracellular adenosine is produced by the sequential activities of membrane associated ectoenzymes, CD39 and CD73, 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 5'AMP. 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 (Carroll and Ashcroft (2005) *Expert. Rev. Mol. Med.* 7(6):1-16). Hypoxia, which occurs in the tumor microenvironment, also inhibits adenylate kinase (AK), which converts adenosine to AMP, leading to very high extracellular adenosine concentrations. The extracellular concentration of adenosine in the hypoxic tumor microenvironment has been measured at 10- 100 μM, which is up to about 100-1000 fold higher than the typical extracellular adenosine concentration of approximately 0.1 μM (Vaupel *et al.* (2016) *Adv Exp Med Biol.* 876:177-183; 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 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 (Antonioli *et al.* (2013 *Nat. Rev. Can.* 13:842-857). Activation of one or more of $A_{2A}$, $A_{2B}$ or $A_3$ on various cells by adenosine can stimulate the production of the pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) or angiopoietin 2 (Antonioli *et al.* (2013) *Nat. Rev. Can.* 13:842-857).

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

*et al.* (2013)). Adenosine can promote tumor cell migration and metastasis, by increasing cell motility, adhesion to the extracellular matrix, and expression of cell attachment proteins and receptors to promote cell movement and motility.

The extracellular release of adenosine triphosphate (ATP) occurs from stimulated immune cells and damaged, dying or stressed cells. The NLR family pyrin domain-containing 3 (NLRP3) inflammasome, when stimulated by this extracellular release of ATP, activates caspase-1 and results in the secretion of the cytokines IL-1β and IL-18, which in turn activate innate and adaptive immune responses (Stagg and Smyth (2010) *Oncogene* 29:5346–5358). 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 (Stagg and Smyth (2010) *Oncogene* 29:5346–5358). Adenosine receptors $A_{2A}$ and $A_{2B}$ are expressed on a variety of immune cells and are stimulated by adenosine to promote cAMP-mediated signaling changes, resulting in immunosuppressive phenotypes of T-cells, B-cells, NK cells, dendritic cells, mast cells, macrophages, neutrophils, and NKT cells. As a result of this, adenosine levels can accumulate to over one hundred times their normal concentration in pathological tissues, such as solid tumors, which have been shown to overexpress ecto-nucleotidases, such as CD73. Adenosine has also been shown to promote tumor angiogenesis and development. An engineered bacterium that is auxotrophic for adenosine would thus exhibit enhanced tumor-targeting and colonization.

Immunostimulatory bacteria, such as *Salmonella typhi*, can be made auxotrophic for adenosine by deletion of the *tsx* gene (Bucarey *et al.* (2005) *Infection and Immunity* 73(10):6210–6219) or by deletion of *purD* (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 (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* deletion, 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 accumulation of adenosine. Exemplary of such bacteria, provided herein is a modified strain of *S. typhimurium* containing *purI-/msbB-* mutations to provide adenosine auxotrophy.

**c. Flagellin Deficient Strains**

Flagella are organelles on the surface of bacteria that are composed of a long filament attached via a hook to a rotary motor that can rotate in a clockwise or counterclockwise manner to provide a means for locomotion. Flagella in *S. typhimurium* are important for chemotaxis and for establishing an infection via the oral route, due to the ability to mediate motility across the mucous layer in the gastrointestinal tract. While flagella have been demonstrated to be required for chemotaxis to and colonization of tumor cylindroids *in vitro* (Kasinskas and Forbes (2007) *Cancer Res.* 67(7):3201-3209), and motility has been shown to be important for tumor penetration (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 (Stritzker *et al.* (2010) *International Journal of Medical Microbiology* 300:449–456). Each flagellar filament is composed of tens of thousands of flagellin subunits. The *S. typhimurium* chromosome contains two genes, *fliC* and *fljB*, that encode antigenically distinct flagellin monomers. Mutants defective for both *fliC* and *fljB* are nonmotile and avirulent when administered via the oral route of infection, but maintain virulence when administered parenterally.

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

Herein, *Salmonella* bacteria, *S. typhimurium*, are engineered to lack both flagellin subunits *fliC* and *fljB*, to reduce pro-inflammatory signaling. For example, as shown herein, a *Salmonella* strain lacking *msbB*, which results in reduced TNF-alpha induction, is combined with *fliC* and *fljB* knockouts. This results in a *Salmonella* strain that has a combined reduction in TNF-alpha induction and reduction in TLR5 recognition. These modifications can be combined with *msbB-*, *fliC-* and *fljB-*, and transformed with an immunostimulatory plasmid, optionally containing CpGs, and also inhibitory RNAi molecule(s), such as shRNA or miRNA, targeting an immune checkpoint, such as TREX1, PD-L1, VISTA, SIRP-alpha, TGF-beta, beta-catenin, VEGF, and combinations thereof. The resulting bacteria have reduced pro-inflammatory signaling, but robust anti-tumor activity.

For example, as provided herein, a *fliC* and *fljB* double mutant was constructed in the *asd* deleted strain of *S. typhimurium* VNP20009. VNP20009, which is attenuated for virulence by disruption of *purI/purM*, was also engineered to contain an *msbB* deletion that results in production of a lipid A subunit that is less toxigenic than wild-type lipid A. This results in reduced TNF-α production in the 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 the flagellin subunits to reduce TLR5 recognition and inflammasome induction. Deletion of the flagellin subunits combined with modification of the LPS allows for greater tolerability in the host, and directs the immuno-stimulatory response towards delivery of RNA interference against desired targets in the TME which elicit an anti-tumor response and promote an adaptive immune response to the tumor.

**d. *Salmonella* Engineered to Escape the *Salmonella* Containing Vacuole (SCV)**

*Salmonella,* such as *S. typhimurium*, are intracellular pathogens that replicate primarily in a membrane bound compartment called a *Salmonella* containing vacuole (SCV). In some epithelial cell lines and at a low frequency, *S. typhimurium* have been shown to escape into the cytosol where they can replicate. *Salmonella* engineered to escape the SCV with higher efficiency will be more efficient at delivering macromolecules, such as plasmids, as the lipid bilayer of the SCV is a potential

barrier. Provided herein are *Salmonella* and methods that have enhanced frequency of SCV escape. This is achieved by deletion of genes required for *Salmonella* induced filament (SIF) formation. These mutants have an increased frequency of SCV escape and can replicate in the cytosol.

For example, enhanced plasmid delivery using a *sifA* mutant of *S. typhimurium* has been demonstrated. The *sifA* gene encodes SPI-2, T3SS-2 secreted effector protein that mimics or activates a RhoA family of host GTPases (Ohlson *et al.* (2008) *Cell Host & Microbe* 4:434–446). Other genes encoding secreted effectors involved in SIF formation can be targeted. These include, for example, *sseJ, sseL, sopD2, pipB2, sseF, sseG, spvB,* and *steA*. Enhancing the escape of *S. typhimurium* by prevention of SIF formation releases live bacteria into the cytosol, where they can replicate.

Another method to enhance *S. typhimurium* escape from the SCV and increase the delivery of macromolecules such as plasmids, is the expression of a heterologous hemolysin that results in pore formation in, or rupture of, the SCV membrane. One such hemolysin is the Listeriolysin O protein (LLO) from *Listeria monocytogenes,* which is encoded by the *hlyA* gene. LLO is a cholesterol-dependent pore-forming cytolysin that is secreted from *L. monocytogenes* and is primarily responsible for phagosomal escape and entry into the cytosol of host cells. Secretion of LLO from *S. typhimurium* can result in bacterial escape and lead to replication in the cytosol. To prevent intact *S. typhimurium* from escaping the SCV and replicating in the cytosol, the nucleotides encoding the signal sequence can be removed from the gene. In this manner, the active LLO is contained within the cytoplasm of the *S. typhimurium* and LLO is only released when the bacteria undergo lysis. As provided herein, VNP20009 engineered to express cytoLLO to enhance delivery of plasmids for expression of interfering RNAs to targets, such as TREX1, can increase the therapeutic potency of the immunostimulatory bacteria.

**e. Deletions in *Salmonella* Genes Required for Biofilm Formation**

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 can also 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 (Di Domenico *et al.* (2017) *Int. J. Mol. Sci.* 18:1887).

*S. typhimurium* biofilm formation is regulated by CsgD. CsgD activates the *csgBAC* operon, which results in increased production of the curli fimbrial subunits CsgA and CsgB (Zakikhani *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 (Tukel *et al.* (2005) *Molecular Microbiology* 58(1):289–304). Further, 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 found in almost all bacterial species. The AdrA-mediated increase in c-di-GMP enhances expression of the cellulose synthetase gene *bcsA*, which in turn increases cellulose production via stimulation of the *bcsABZC* and *bcsEFG* operons. Reduction in the capability of immunostimulatory bacteria such as *S. typhimurium* to form biofilms can be achieved through deletion of genes involved in biofilm formation such as, for example, *csgD, csgA, csgB, adrA, bcsA, bcsB, bcsZ, bcsE, bcsF, bcsG, dsbA* or *dsbB* (Anwar *et al.* (2014) *Plos One* 9(8):e106095).

*S. typhimurium* can form biofilms in solid tumors as protection against phagocytosis by host immune cells. *Salmonella* mutants that cannot form biofilms are taken up more rapidly by host phagocytic cells and are cleared from infected tumors (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 enhance the effectiveness of plasmid delivery and gene knockdown by RNA interference as described herein. Immunostimulatory bacteria engineered to reduce biofilm formation, will increase clearance rate from

tumors/tissues and therefore increase the tolerability of the therapy, and will prevent colonization of prosthetics in patients, thereby increasing the therapeutic benefit of these strains. Adenosine mimetics can inhibit *S. typhimurium* biofilm formation, indicating that the high adenosine concentration in the tumor microenvironment can contribute to tumor-associated biofilm formation (Koopman *et al.* (2015) *Antimicrob Agents Chemother* 59:76 -84). As provided herein, live attenuated strains of bacteria, such as *S. typhimurium*, that contain a *purI* disruption (and therefore, colonize adenosine-rich tumors), and are also prevented from forming biofilms, by deletion of one or more genes required for biofilm formation, are engineered to deliver plasmids encoding interfering RNA to stimulate a robust anti-tumor immune response.

The *adrA* gene encodes a di-guanylate cyclase that produces c-di-GMP, which is required for *S. typhimurium* biofilm formation. c-di-GMP binds to and is an agonist for the host cytosolic protein STING. As described above, STING agonists are pursued as anti-cancer treatments, vaccine adjuvants, and bacteria engineered to secrete cyclic di-nucleotides for use in immunotherapies (Libanova 2012, Synlogic 2018 AACR poster). Immunostimulatory bacteria that are reduced in c-di-GMP production via the deletion of *adrA* appears to be counterintuitive, but bacterial mutants, such as *S. typhimurium* mutants that are unable to form biofilms (including an *adrA* mutant), have demonstrated reduced therapeutic potential in mouse tumor models (Crull *et al.* (2011) *Cellular Microbiology* 13(8):1223–1233). Further, several human alleles of STING are refractory to binding bacterially-produced 3'3' CDNs (Corrales *et al.* (2015) *Cell Reports* 11:1022-1023).

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 deletion of genes required for biofilm formation, and further modified to deliver interfering RNAs, promote robust anti-tumor immune responses.

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

The 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 NFκB pathway and result in the production of pro-inflammatory cytokines such as TNF-α and IL-1β, 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 (Hagar *et al.* (2015) *Cell Research* 25:149-150). The composition of lipid A and the toxigeniciy 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 of acyl chains on lipid A can also 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 (Raetz and Whitfield (2002) *Annu Rev Biochem.* 71:635–700). As described above, *msbB* mutants of *S. typhimurium* cannot undergo the terminal myristoylation of its LPS and produces predominantly penta-acylated LPS that is significantly less toxic than hexa-acylated lipid A. The modification of lipid A with palmitate is catalyzed by palmitoyl transferase (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* 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 phagolysozomes.

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 LPS (Kong *et al.* (2011) *Infection and Immunity* 79(12):5027–5038). Hexa-acylated LPS has been shown to be the most pro-inflammatory. While other groups have sought to exploit this pro-inflammatory signal, for example, by deletion of *pagP* to allow only hexa-acylated LPS to be produced (Felgner *et al.* (2016) *Gut Microbes* 7(2):171–177; (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 (Kocijancic *et al.* (2017) *Oncotarget* 8(30):49988-50001). Provided herein, is a live attenuated strain of *S. typhimurium* that can only produce penta-acylated LPS, that contains a deletion of the *msbB* gene (that prevents the terminal myristoylation of lipid A, as described above), and is further modified by deletion of *pagP* (preventing palmitoylation). A strain modified to produce penta-acylated LPS will allow for lower levels of pro-inflammatory cytokines, increased sensitivity to antimicrobial peptides, enhanced tolerability, and increased anti-tumor immunity when further modified to express interfering RNAs against immune checkpoints such as TREX1.

**g. Deletions of SPI-1 Genes**

As described above, in *Salmonella* species, such as *S. typhimurium*, pathogenesis involves a cluster of genes referred to as *Salmonella* pathogenicity islands (SPIs). SPI-1 mediates invasion of epithelial cells. SPI-1 encodes a type 3 secretion system (T3SS) that is responsible for translocation of effector proteins into the cytosol of host cells that can cause actin rearrangements that lead to uptake of *Salmonella*. The SPI-1 T3SS is essential for crossing the gut epithelial layer, but is dispensable for infection when bacteria are injected parenterally. The injection of some proteins and the needle complex itself can also induce inflammasome activation and pyroptosis of phagocytic cells. This pro-inflammatory cell death can limit the initiation of a robust adaptive immune response by directly inducing the death of antigen-presenting cells (APCs), as well as modifying the cytokine milieu to prevent the generation of memory T-cells. SPI-1 genes comprise a number of operons

including: *sitABCD, sprB, avrA, hilC, orgABC, prgKJIH, hilD, hilA, iagB, sptP, sicC, iacP, sipADCB, sicA, spaOPQRS, invFGEABCIJ,* and *invH.*

As exemplified herein, a live attenuated strain of *S. typhimurium* that contains a *purI* deletion, an *msbB* deletion, an *asd* gene deletion and is engineered to deliver plasmids encoding interfering RNA, is further modified to delete SPI-1 genes. For example, deletion of a regulatory gene (*e.g., hilA* or *invF*) required for expression of the SPI-1-associated type 3 secretion system (T3SS-1), a T3SS-1 structural gene (*e.g., invG* or *prgH*), or a T3SS-1 effector gene (e.g., *sipA* or *avrA*). This secretion system is responsible for injecting effector proteins into the cytosol of non-phagocytic host cells such as epithelial cells that cause the uptake of the bacteria. In this example, the additional deletion of the *hilA* gene from a therapeutic *Salmonella typhimurium* strain that is administered either intravenously or intratumorally focuses the *S. typhimurium* infection towards phagocytic cells that do not require the SPI-1 T3SS for uptake, and prolongs the longevity of these phagocytic cells. The *hilA* mutation also reduces the quantity of pro-inflammatory cytokines, increasing the tolerability of the therapy, as well as the quality of the adaptive immune response.

**h. Endonuclease (*endA*) Mutations to Increase Plasmid Delivery**

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

increases efficiency of intact plasmid DNA delivery, thereby increasing expression of the RNAs, such as the shRNA and/or miRNA, targeting any or two or more of the immune checkpoints, encoded in the plasmid, thereby increasing RNAi- mediated knockdown of checkpoint genes and enhancing anti-tumor efficacy.

**i. RIG-I Inhibition**

Of the TLR-independent type I IFN pathways, one is 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 (MDA-5), and through the IFN-β promoter stimulator 1 (IPS-1) adaptor protein-mediated phosphorylation of the IRF-3 transcription factor, leading to induction of type I IFN (Ireton and Gale (2011) *Viruses* 3(6):906-919). RIG-I recognizes dsRNA and ssRNA bearing 5'-triphosphates. This moiety can directly bind RIG-I, or be synthesized from a poly(dA-dT) template by the poly DNA-dependent RNA polymerase III (Pol III) (Chiu, Y. H. *et al.* (2009) *Cell* 138(3):576-91). A poly(dA-dT) template containing two AA dinucleotide sequences occurs at the U6 promoter transcription start site in a common lentiviral shRNA cloning vector. Its subsequent deletion in the plasmid prevents type I IFN activation (Pebernard *et al.* (2004) *Differentiation.* 72:103-111). A RIG-I binding sequence can be included in the plasmids provided herein; inclusion can increase immunostimulation that increases anti-tumoral activity of the immunostimulatory bacteria herein.

**j. DNase II Inhibition**

Another nuclease responsible for degrading foreign and self DNA is DNase II, an endonuclease, which resides in the endosomal compartment and degrades DNA following apoptosis. Lack of DNase II (*Dnase2a* in mice) results in the accumulation of endosomal DNA that escapes to the cytosol and activates cGAS/STING signaling (Lan YY *et al.* (2014) *Cell Rep.* 9(1):180–192). Similar to TREX1, DNase II-deficiency in humans presents with autoimmune type I interferonopathies. In cancer, dying tumor cells that are engulfed by tumor-resident macrophages prevent cGAS/STING activation and potential autoimmunity through DNase II digestion of DNA within the endosomal compartment (Ahn *et al.* (2018) *Cancer Cell* 33:862–873). Hence, embodiments of the immunostimulatory bacterial strains, as provided

herein, encode RNAi, such as shRNA or miRNA that inhibit, suppress or disrupt expression of DNase II, which can inhibit DNase II in the tumor microenvironment, thereby provoking accumulation of endocytosed apoptotic tumor DNA in the cytosol, where it can act as a potent cGAS/STING agonist.

**k. RNase H2 Inhibition**

While TREX1 and DNase II function to clear aberrant DNA accumulation, RNase H2 functions similarly to eliminate pathogenic accumulation of RNA:DNA hybrids in the cytosol. Similar to TREX1, deficiencies in RNase H2 also contribute to the autoimmune phenotype of Aicardi-Goutières syndrome (Rabe, B. (2013) *J Mol Med.* 91:1235–1240). Specifically, loss of RNase H2 and subsequent accumulation of RNA:DNA hybrids or genome-embedded ribonucleotide substrates has been shown to activate cGAS/STING signaling. (MacKenzie *et al.* (2016) *EMBO J.* Apr15;35(8):831-44). Hence, embodiments of the immunostimulatory bacterial strains, as provided herein, encode RNAi, such as shRNA or miRNA that inhibit, suppress or disrupt expression of RNAse H2, to thereby inhibit RNase H2, resulting in tumor-derived RNA:DNA hybrids and derivatives thereof, which activate cGAS/STING signaling and anti-tumor immunity.

**l. Stabilin-1/CLEVER-1 Inhibition**

Another molecule expressed primarily on monocytes and involved in regulating immunity is stabilin-1 (gene name *STAB1*, also known as *CLEVER-1*, *FEEL-1*). Stabilin-1 is a type I transmembrane protein that is upregulated on endothelial cells and macrophages following inflammation, and in particular, on tumor-associated macrophages (Kzhyshkowska *et al.* (2006) *J. Cell. Mol. Med.* 10(3):635-649). Upon inflammatory activation, stabilin-1 acts as a scavenger and aids in wound healing and apoptotic body clearance, and can prevent tissue injury, such as liver fibrosis (Rantakari *et al.* (2016) *PNAS* 113(33):9298–9303). Upregulation of stabilin-1 directly inhibits antigen-specific T cell responses, and knockdown by siRNA in monocytes was shown to enhance their pro-inflammatory function (Palani, S. *et al.* (2016) *J Immunol.* 196:115-123). Hence, embodiments of the immunostimulatory bacterial strains, as provided herein, encode RNAi, such as shRNA or miRNA that inhibit, suppress or disrupt expression of Stabilin-

1/CLEVER-1 in the tumor microenvironment, thereby enhancing the pro-inflammatory functions of tumor-resident macrophages.

**m. Bacterial Culture Conditions**

Culture conditions for bacteria can influence their gene expression. It has been documented that *S. typhimurium* can induce rapid pro-inflammatory caspase-dependent cell death of macrophages, but not epithelial cells, within 30 to 60 min of infection by a mechanism involving the SPI-1 and its associated T3SS-1 (Lundberg *et. al* (1999) *Journal of Bacteriology* 181(11):3433–3437). It is now known that this cell death is mediated by activation of the inflammasome that subsequently activates caspase-1, which promotes the maturation and release of IL-1β and IL-18 and initiates a novel form of cell death called pyroptosis (Broz and Monack (2011) *Immunol Rev.* 243(1):174–190). This pyroptotic activity can be induced by using log phase bacteria, whereas stationary phase bacteria do not induce this rapid cell death in macrophages. The SPI-1 genes are induced during log phase growth. Thus, by harvesting *S. typhimurium* to be used therapeutically at stationary phase, rapid pyroptosis of macrophages can be prevented. Macrophages are important mediators of the innate immune system and they can act to secrete cytokines that are critical for establishing appropriate anti-tumor responses. In addition, limiting pro-inflammatory cytokines such as IL-1β and IL-18 secretion will improve the tolerability of administered *S. typhimurium* therapy. As provided herein, immunostimulatory *S. typhimurium* harvested at stationary phase will be used to induce anti-tumor responses.

**E. CONSTRUCTING EXEMPLARY PLASMIDS**

The immunostimulatory bacteria provided herein are modified. They include modifications to the bacterial genome and bacterial gene expression, and also, to include plasmids that encode products that are expressed in the bacteria by including a bacterial promoter, or in the host by including an appropriate eukaryotic promoter and other regulatory regions as appropriate.

To introduce the plasmids, the bacteria are transformed using standard methods, such as electroporation with purified DNA plasmids constructed with routine molecular biology tools (DNA synthesis, PCR amplification, DNA restriction enzyme digestion and ligation of compatible cohesive end fragments with ligase).

As discussed below, the plasmids encode one or more short hairpin (sh) RNA construct(s), or other inhibitory RNA modalities, whose expression inhibits or disrupts expression of targeted genes. The RNAi, such as shRNA or microRNA constructs, are expressed under control of a eukaryotic promoter, such as an RNA polymerase (RNAP) II or III promoter. Typically, RNAPIII (also referred to as POLIII) promoters are constitutive, and RNAPII (also referred to as POLII) can be regulated. In some examples, the shRNAs target the gene TREX1, to inhibit its expression. In some embodiments the plasmids encode a plurality of shRNAs that target to inhibit two or more checkpoint genes, such as shRNAs for inhibiting PD-L1, VISTA, SIRPα, CTNNB1, TGF-beta, and/or VEGF and any others known to those of skill in the art. Where a plurality of RNAi's, such as shRNAs, are encoded, expression of each is under control of different promoters.

As provided herein, bacterial strains, such as strains of *Salmonella*, including *S. typhimurium*, are modified or identified to be auxotrophic for adenosine in the tumor microenvironment, and to carry plasmids containing genes encoding shRNAs or microRNAs capable of knocking down gene expression of TREX1, PD-L1, VISTA, SIRP-alpha, beta-catenin, TGF-beta and VEGF. *S. typhimurium* is capable of infecting multiple cell types, including both tumor cells and macrophages. For cells infected with *S. typhimurium*, the plasmid is released and capable of being transcribed by RNA polymerases. shRNAs generated are then processed and capable of interfering with target mRNA gene expression.

**1. Interfering RNAs (RNAi)**

The plasmids herein encode the RNAi nucleic acids targeting the checkpoints and other targets of interest, as described above. RNAi includes shRNA, siRNA, and microRNA. RNA interference (RNAi) allows for the sequence-selective suppression of gene expression in eukaryotic cells using small interfering RNAs (siRNAs), which are short, synthetic, dsRNA molecules with a sequence homologous to the target gene. RNAi technology provides a powerful tool for the depletion of disease-related transcripts.

**a. shRNA**

The siRNAs, which are typically about 19-29 base pairs long, function by degrading specific host mRNA sequences, precluding translation into their respective

protein products, effectively silencing the expression of the target gene. Short hairpin RNAs (shRNAs), containing a tight hairpin loop, are widely used in RNAi. shRNAs contain of two complementary RNA sequences, each 19-29 bps long, linked by a loop spacer of 4-15 nucleotides. The RNA sequence that is complementary to the target gene sequence (and is thus identical to the mRNA sequence), is known as the "sense" strand, while the strand which is complementary to the mRNA (and identical to the target gene sequence) is known as the "antisense" or "guide" strand. shRNA transcripts are processed by an RNase III enzyme known as Dicer into siRNA duplexes. The product is then loaded into the RNA-induced silencing complex (RISC) with Argonaute (Ago) proteins and other RNA-binding proteins. RISC then localizes the antisense, or "guide" strand to its complimentary mRNA sequence, which is subsequently cleaved by Ago (U.S. Patent No. 9,624,494). The use of shRNA is preferred over siRNA, because it is more cost effective, high intracellular concentrations of siRNA are associated with off-target effects, and because the concentration of siRNA becomes diluted upon cell division. The use of shRNA, on the other hand, results in stable, long-term gene knockdown, without the need for multiple rounds of transfection (Moore *et al.* (2010) *Methods Mol. Bio.* 629:141-158).

Targets of interest for RNAi, such as micro-RNA and siRNA/shRNA-mediated silencing include, but are not limited to, developmental genes such as cytokines and their receptors, cyclin kinase inhibitors, neurotransmitters and their receptors, growth/differentiation factors and their receptors; oncogenes such as *BCL2, ERBA, ERBB, JUN, KRAS, MYB, MYC*; tumor suppressor genes such as *BRCA1, BRCA2, MCC, p53*; and enzymes such as ACC synthases and oxidases, ATPases, alcohol dehydrogenases, amylases, catalases, DNA polymerases, RNA polymerases, kinases, lactases and lipases (U.S. Patent Nos. 7,732,417, 8,829,254, 8,383,599, 8,426,675, 9,624,494; U.S. Patent Publication No. 2012/0009153). Of particular interest are immune checkpoint targets, such as PD-1, PD-2, PD-L1, PD-L2, CTLA-4, IDO 1 and 2, CTNNB1 (β-catenin), SIRPα, VISTA, RNASE H2, DNase II, CLEVER-1/Stabilin-1, LIGHT, HVEM, LAG3, TIM3, TIGIT, Galectin-9, KIR, GITR, TIM1, TIM4, CEACAM1, CD27, CD40/CD40L, CD48, CD70, CD80, CD86, CD112, CD137( 4-1BB), CD155, CD160, CD200, CD226, CD244 (2B4), CD272 (BTLA), B7-H2, B7-H3, B7-H4, B7-H6, ICOS, A2aR, A2bR, HHLA2, ILT-2, ILT-4,

gp49B, PIR-B, HLA-G, ILT-2/4 and OX40/OX-40L. Other targets include MDR1, Arginase1, iNOs, IL-10, TGF-β, pGE2, STAT3, VEGF, KSP, HER2, Ras, EZH2, NIPP1, PP1, TAK1 and PLK1 (U.S. Patent Publication Nos. 2008/091375, 2009/0208534, 2014/0186401, 2016/0184456, 2016/0369282; International Patent Publication Nos. WO 2012/149364, WO 2015/002969, WO 2015/032165, WO 2016/025582).

Bacteria are attractive vectors for the tumor-targeted delivery of siRNAs and shRNAs. *Salmonella*, for example, can be used for the delivery of shRNA plasmids against genetic targets such as IDO (Blache *et al.* (2012) *Cancer Res.* 72(24):6447-6456; Manuel *et al.* (2015) *Cancer Immunol. Res.* 3(9):1096-1107; U.S. Patent Publication Nos. 2014/0186401, 2016/0184456; International Patent Publication Nos. WO 2012/149364, WO 2015/002969 ); STAT3 (Manuel *et al.* (2011) *Cancer Res.* 71(12):4183-4191; Zhang *et al.* (2007) *Cancer Res.* 67(12):5859-5864; U.S. Patent Publication Nos. 2014/0186401, 2016/0184456; International Patent Publication Nos. WO 2008/091375, WO 2012/149364, WO 2015/002969, WO 2015/032165); β-catenin (Guo *et al.* (2011) *Gene therapy* 18:95-105; International Patent Publication No. WO 2015/032165) and CTLA-4 (U.S. Patent Publication Nos. 2014/0186401, 2016/0184456; International Patent Publication Nos. WO 2012/149364, WO 2015/002969).

Expressed RNAi, such as shRNAs, mediate long-term, stable knockdown of their target transcripts for as long as the shRNAs are transcribed. RNA Pol II and III promoters are used to drive expression of shRNA constructs, depending on the type of expression required. Consistent with their normal cellular roles in producing abundant, endogenous small RNAs, Pol III promoters (such as U6 or H1) drive high levels of constitutive shRNA expression, and their transcription initiation points and termination signals (4-6 thymidines) are well defined. Pol II promoter-driven shRNAs can be expressed tissue-specifically and are transcribed as longer precursors that mimic pri-miRNAs and have cap and polyA signals that must be processed. Such artificial miRNAs/shRNAs are efficiently incorporated into RISC, contributing to a more potent inhibition of target-gene expression; this allows lower levels of shRNA expression and might prevent saturation of components in the RNAi pathway. An additional advantage of Pol II promoters is that a single transcript can simultaneously

express several miRNA and mimic shRNAs. This multiplexing strategy can be used to simultaneously knock down the expression of two or more therapeutic targets, or to target several sites in a single gene product (see, *e.g.*, U.S. Publication No. 2009/0208534).

**b. MicroRNA**

MicroRNAs (miRNAs) are short, non-coding single-stranded RNA molecules that are about or are 20-24 nucleotides long. Naturally-occurring miRNAs are involved in the post-transcriptional regulation of gene expression; miRNAs do not encode genes. miRNAs have been shown to regulate cell proliferation and survival, as well as cellular differentiation. miRNAs inhibit translation or promote RNA degradation by binding to target mRNAs that share sequence complementarity. They affect the stability and translation of mRNAs; miRNAs inhibit translation, and/or promote RNA degradation, by binding to target mRNAs that share sequence complementarity. miRNAs, which occur in eukaryotes, are transcribed by RNA Pol II into capped and polyadenylated hairpin-containing primary transcripts, known as primary miRNAs, or pri-miRNAs. These pri-miRNAs are cleaved by the enzyme Drosha ribonuclease III and its cofactor Pasha/DGCR8 into ~70 nucleotide long precursor miRNA hairpins, known as precursor miRNAs, or pre-miRNAs, which are then transported from the nucleus into the cytoplasm, and cleaved by Dicer ribonuclease III into the miRNA: miRNA* duplex, with sense and antisense strand products that are approximately 22 nucleotides long. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which recognizes and binds target mRNAs, usually at the 3'-untranslated region (UTR), through imperfect base pairing with the miRNA, resulting in the inhibition of translation, or destabilization/degradation of the target mRNA (see, *e.g.*, Auyeung *et al.* (2013) *Cell* 152(4):844-85).

As described herein, regulating gene expression by RNA interference (RNAi), often uses short hairpin RNAs (shRNAs) to inhibit, disrupt or other interfere with expression of targeted genes. While advantageously used, and used herein, in some instances, shRNAs can be poor substrates for small RNA biogenesis factors, they can be processed into a heterogeneous mix of small RNAs, and their precursor transcripts can accumulate in cells, resulting in the induction of sequence-independent, non-

specific effects and leading to *in vivo* toxicity. miRNAs are contemplated for use herein. miRNA-like scaffolds, or artificial miRNAs (amiRNAs) can be used to reduce sequence-independent non-specific effects (Watanabe *et al.* (2016) *RNA Biology* 13(1):25-33; Fellmann *et al.* (2013) *Cell Reports* 5:1704-1713). In addition to improved safety profiles, amiRNAs are more readily transcribed by Pol II than shRNAs, allowing for regulated and cell-specific expression. Artificial miRNAs (amiRNAs), in comparison to shRNAs, can effectively, and in some cases, more potently, silence gene expression without generating large amounts of inhibitory RNAs (McBride *et al.* (2008) *Proc. Natl. Acad. Sci. U.S.A.* 105(15):5868-5873). This effect was determined to be due to the more effective processing of siRNA from pre-miRNA precursors than from shRNA transcripts (Boden *et al.* (2004) *Nucl Acid Res* 32(3):1154-1158).

miRNAs have been shown to regulate several cellular processes, including cell proliferation and survival, intracellular signaling, cellular metabolism, and cellular differentiation. In 1993, the first miRNA was identified in *C. elegans* (Lee *et al.* (1993) *Cell* 75:843–854), and later, mammalian miRNAs were identified (Pasquinelli *et al.* (2000) *Nature.* 408(6808):86-89). More than 17,000 miRNAs in 142 species have been identified, with more than 1900 miRNAs identified in humans, many of which have been associated with a variety of diseases, including cancer (*e.g.,* miR-15 and miR-16 in B-CLL, miR-125b, miR-145, miR-21, miR-155 and miR-210 in breast cancer, miR-155 and let-7a in lung cancer, miR-145 in gastric cancer, miR-29b in liver cancer); viral infections (*e.g.,* miR-122 and miR-155 in HCV infection, mir-28, miR-125b, miR-150, miR-223 and miR-382 in HIV-1 infection, miR-21 and miR-223 in influenza virus infection); immune-related diseases (*e.g.,* miR-145, miR-34a, miR-155 and miR-326 in multiple sclerosis, miR-146a in systemic lupus erythematosus, miR-144, miR-146a, miR-150, miR-182, miR-103 and miR-107 in type II diabetes, miR-200a, miR-200b, miR-429, miR-122, miR-451 and miR-27 in nonalcoholic fatty liver disease, miR-29c, miR-34a, miR-155 and miR-200b in non-alcoholic steatohepatitis); and neurodegenerative diseases (*e.g.,* miR-30b, miR-30c, miR-26a, miR-133b, miR-184* and let-7 in Parkinson's disease, miR-29b-1, miR-29a and miR-9 in Alzheimer's disease) (Li and Kowdley (2012) *Genomics Proteomics Bioinformatics* 10:246-253).

Studies have shown that specific endogenous miRNAs are up-regulated or down-regulated in certain cancers. For example, miR-140 is down-regulated in non-small cell lung cancer (NSCLC) and its overexpression was found to suppress PD-L1 (Xie *et al.* (2018) *Cell Physiol. Biochem.* 46:654-663); miR-197 is downregulated in platinum-based chemotherapy resistant NSCLC, resulting in chemoresistance, tumorigenicity and metastasis (Fujita *et al.* (2015) *Mol Ther* 23(4):717-727); and several miRNAs have been found to be down-regulated in cancer cells to allow PD-L1 expression, including miR-200, miR-34a and miR-138 (Yee *et al.* (2017) *J. Biol. Chem.* 292(50):20683-20693). Several miRNAs also are upregulated, for example miR-21, miR-17 and miR-221 in lung cancer (Xie *et al.* (2018 *Cell Physiol. Biochem.* 46:654-663).

MicroRNA-103 (miR-103) was identified as the most upregulated microRNA in endothelial cells as a result of genotoxic stress and DNA damage following radiation. It was found that miR-103 led to the downregulation of the *TREX1, TREX2* and *FANCF* genes, and the decrease in TREX1 expression was identified as the major mechanism by which miR-103 mediates cell death and suppresses angiogenesis (Wilson *et al.* (2016) *Nature Communications* 7:13597). Since the loss of TREX1 results in the accumulation of ds and ssDNA, defective DNA repair, and release of cytokines, Wilson *et al.* examined whether miR-103 regulates the expression of cytokines. Results showed that miR-103 expression significantly upregulated the pro-inflammatory chemokines IP-10, RANTES, MIG, and the cytokines IL-15, IL-12 and IFN-γ, and this upregulation was due to a miR-103 mediated decrease in TREX1 levels. Studies also revealed a significant increase in costimulatory receptors CD40 and CD160, and a decrease in the numbers of PD-L1$^{+}$ macrophages and neutrophils in the 4T1 tumors. miR-103 regulation of TREX1 is therefore a potent modulator of the immune TME. Other miRNAs that target TREX1 include miR-107 (U.S. Patent No. 9,242,000), miR-27a and miR-148b (U.S. Patent No. 8,580,757). miRNA-103 can be used in the plasmids herein to inhibit TREX1.

Artificial miRNAs (amiRNAs) can be delivered to cells and used to silence target genes by creating a microRNA-based siRNA or shRNA vector (shRNAmir). The miR-30a backbone is often used in mammals, and approximately 200-300 bases of the primary miRNA transcript are included in the vector, with the miRNA hairpin

placed at the center of the fragment, and the natural miRNA stem sequence being replaced with the siRNA/shRNA-encoding sequence of interest. Viral promoters, such as CMV, MSCV and TLR promoters; cellular promoters, such as EIF-1a; inducible chimeric promoters, such as tet-CMV; and tissue-specific promoters, can be used (Chang *et al.* (2013) *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot075853). Other miRNAs that can be used include mir-16-2 (Watanabe *et al.* (2016) *RNA Biology* 13(1):25-33), miR-155 (Chung *et al.* (2006) *Nuc Acids Res* 34:e53), miR17-92 (Liu *et al.* (2008) *Nuc Acids Res* 36(9):2811-2824), miR-15a, miR-16, miR-19b, miR-20, miR-23a, miR-27b, miR-29a, miR-30b, miR-30c, miR-104, miR-132s, miR-181, miR-191, miR-223 (U.S. Patent No. 8,426,675), and Let-7 miRNA (WO 2009/006450; WO 2015/032165).

shRNAmirs are limited by the low effectiveness of computationally-predicted shRNA sequences, particularly when expressed under low or single copy conditions. Third generation artificial miRNAs, such as miR-E (based on miR-30a) and miR-3G (based on miR-16-2) have been developed, and were found to exhibit stronger gene silencing in both Pol II- and Pol III-based expression vectors in comparison to shRNAmirs, due to the enhanced processing and accumulation of precisely-defined guide RNAs. miR-E, which was developed by the discovery of the conserved CNNC motif that enhances the processing of miRNA within the stem 3p flanking sequences, is different from endogenous miR-30a in three aspects: the stem of miR-E has no bulge and has the intended guide on the opposite strand; two conserved base pairs flanking the loop were mutated from CU/GG to UA/UA; and XhoI/EcoRI restriction sites were introduced into the flanking regions for shRNA cloning (Fellmann *et al.*(2013) *Cell Reports* 5:1704-1713). miR-E was found to be more potent than miR-30a, but symmetric processing of both the 3p and 5p strands of miR-30a does not favor guide strand delivery over passenger strand delivery, which is not optimal. Additionally, cloning into miR-E using oligos longer than 100 nt is costly and time consuming (Watanabe *et al.* (2016) *RNA Biology* 13(1):25-33).

The amiRNA designated miR-16-2 (see, e.g., (Watanabe *et al.* (2016) *RNA Biology* 13(1):25-33, see FIG.1) is a third generation (3G) amiRNA scaffold alternative; it is expressed in several tissues, is naturally asymmetric (the mature strand is derived exclusively from the 5p or 3p arm of the stem), and its stem and loop

segments are small and rigid, simplifying vector cloning. miR-3G is generated by cloning the ~175 bp fragment containing the native miR-16-2 stem and loop, and the flanking 35 bps on either side of the stem, into the vector. miR-3G includes further modification of miR-16-2 by introducing cloning sites, such as MluI and EcoRI, into the 5p and 3p arm-flanking sequences, respectively, and fully base-pairing the guide (antisense) and passenger (sense) strand stem, with the exception of a mismatch at position 1 relative to the guide strand. The restriction sites allow for the generation of new targeting constructs via 88-mer duplexed DNA oligonucleotides without compromising the predicted secondary structure of the miR-16-2 hairpin and flanking elements. Additionally, one of the two CNNC motifs and the GHG motif (small RNA processing enhancers) are modified in the 3p flanking sequence of miR-16-2. siRNAs targeting the gene(s) of interest are then exchanged with the first 21 nucleotides of the mature 5p guide and 3p passenger sequences. Studies determined that miR-E and miR-3G were equally potent. miR-3G provides an attractive RNAi system, due to the smaller size of its expression cassette (~175 nts vs. ~375 for miR-E), and the simplified and cost effective single step cloning method for its production. As with shRNAs, bacteria can be used as vectors for the *in vivo* delivery of micro-RNAs. For example, it was shown that attenuated *S. typhimurium* can be used as a vector for the oral delivery of plasmids expressing miRNA against CCL22 in mice with inflammation. Downregulation of CCL22 gene expression by this method was successful both *in vitro* and *in vivo* in mouse models of atopic dermatitis (Yoon *et al.* (2012) *DNA and Cell Biology* 31(3):289-296). For purposes herein a miRNA 16-2 can be used to produce miRNAs to be used in place of the shRNA. The sequences for the shRNA can be used for design of miRNAs.

DNA encoding RNAi for disrupting and/or inhibiting and/or targeting any of selected target genes, such as any immune checkpoint described herein or known to the skilled artisan, is inserted into a microRNA backbone, such as the microRNA backbone set forth in SEQ ID NO:249, and below. Any suitable microRNA backbone known to the skilled artisan can be used; generally such backbones are based on a naturally-occurring microRNA and are modified for expression of the RNAi. Exemplary of such backbones is one based on miR-16-2 (SEQ ID NO:248). The sequence of the modified microRNA backbone is:

5'- CCGGATC AACGCCCTAG GTTTATGTTT GGATGAACTG ACATACGCGT ATCCGTC NNNNNNNNNNNNNNNNNNNN GTAG TGAAATATAT ATTAAAC NNNNNNNNNNNNNNNNNNNN TACGGTAACGCG GAATTCGCAA CTATTTTATC AATTTTTTGC GTCGAC-3' (SEQ ID NO:249), where the N's represent complementary, generally 18-26, such as 19-24, 19-22, 19-20, base pair long anti-sense and sense nucleotide sequences that target the gene to be silenced, and are inserted before and after the microRNA loop. RNAs, such as ARI-205 (SEQ ID NO:214) and ARI-206 (SEQ ID NO:215) are exemplary constructs based on the microRNA backbone of SEQ ID NO:249, that encode 21 and 22 base pair homology sequences, respectively. ARI-207 (SEQ ID NO:216) and ARI-208 (SEQ ID NO:217) are exemplary constructs based on the microRNA backbone of SEQ ID NO:249, that encode 19 base pair homology sequences. Another example, is the construct designated ARI-201, which is microRNA construct ARI-205, wherein the N's are replaced with a sequence of nucleotides targeting mouse PD-L1. The construct designated ARI-202 represents microRNA construct ARI-206, where the N's are replaced with sequences targeting mouse PD-L1. The skilled person readily can construct microRNAs for inclusion in plasmids as described and exemplified herein using the miR-16-2 backbone, or other suitable backbones known to the skilled artisan.

**2. 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 number of plasmids generally decreases the growth rate, thus possibly allowing for cells with few plasmids to dominate the culture, since they grow faster. The origin of replication also determines the plasmid's compatibility: 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.

| Origin of Replication | Copy Number | SEQ ID NO. |
|---|---|---|
| pMB1 | 15-20 | 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 |

Numerous bacterial origins of replication are known to those of skill in the art. The origin can be selected to achieve a desired copy number. Origins of replication contain sequences that are recognized as initiation sites of plasmid replication via DNA dependent DNA polymerases (Solar *et al.* (1998) *Microbiology And Molecular Biology Reviews* 62(2):434–464). Different origins of replication provide for varying plasmid copy levels within each cell and can range from 1 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, ColE1

origins, p15A, pSC101, pBR322, and others, which have low copy numbers. Such origins are well known to those of skill in the art. The pUC19 origin results in copy number of 500-700 copies per cell. The pBR322 origin has a known copy number of 15-20. 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 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 can be obtained, include, for example, pWSK29, pWKS30, pWKS129 and pWKS130 (see, 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. Those of skill in the art can identify plasmids with low or high copy number. For example, to determine experimentally if the copy number is high or low is to perform a miniprep. A high-copy plasmid should yield between 3-5 μg DNA per 1 ml LB culture; a low-copy plasmid will yield between 0.2-1 μg DNA per ml of LB culture.

Sequences of bacterial plasmids, including identification of and sequence of the origin of replication, are well known (see, *e.g.*, snapgene.com/resources/plasmid_files/basic_cloning_vectors/pBR322/).

High copy plasmids are selected for heterologous expression of proteins *in vitro* because the gene dosage is increased relative to chromosomal genes and 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 RNA interference (RNAi), such as by *S. typhimurium*, as described herein, while it would appear that a high copy plasmid would be ideally suited, therapeutically, a lower copy number is more effective.

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 interfering RNAs 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. It is shown herein, that low copy number can be advantageous.

### 3. CpG Motifs and CpG Islands

Unmethylated cytidine-phosphate-guanosine (CpG) motifs are prevalent in bacterial, but not vertebrate, genomic DNA. Pathogenic DNA and synthetic oligodeoxynucleotides (ODN) 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 ODN 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 enables 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 ODN have a phosphorothioate backbone and contain multiple CpG motifs with double palindromes that can form hairpins at their GC-rich 3′ ends (Scheiermann and Klinman (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 (Akira *et al.* (2001) *Nat Immunol.* 2(8):675-680). TLR9 recognizes hypomethylated CpG motifs in DNA of prokaryotes that do not occur naturally in mammalian DNA (McKelvey *et al.* (2011) *J Autoimmunity* 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, 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 RNAi encoded in the plasmid, such as RNAi that targets immune checkpoints, such as the shRNA or miRNA that targets TREX1, and hence, TREX1-mediated STING pathway activation, 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 RNAi described or apparent from the description herein in the immunostimulatory bacteria, and thereby enhance or improve anti-tumor immune responses in a treated subject.

Immunostimulatory CpGs can be included in the plasmids, by including a nucleic acid, typically from a bacterial gene, that encodes a gene product, and also by adding a nucleic acid that encodes CpG motifs. The plasmids herein can include CpG motifs. Exemplary CpG motifs are known (see, *e.g.*, U.S. Patent Nos. 8,232,259, 8,426,375 and 8,241,844). These include, for example, synthetic immunostimulatory oligonucleotides, between 10 and 100, 10 and 20, 10 and 30, 10 and 40, 10 and 50, 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.

**4. Plasmid Maintenance/Selection Components**

The maintenance of plasmids in laboratory settings is usually ensured by inclusion of an antibiotic resistance gene on the plasmid and use of antibiotics in growth media. As described above, the use of an *asd* deletion mutant complimented with a functional *asd* gene on the plasmid allows for plasmid selection *in vitro* without the use of antibiotics, and allows for plasmid selection *in vivo*. The *asd* gene complementation system provides for such selection (Galán *et al.* (1990) *Gene* 28:29-35). The use of the *asd* gene complementation system to maintain plasmids in the tumor microenvironment increases the potency of *S. typhimurium* engineered to deliver plasmids encoding genes or interfering RNAs.

**RNA Polymerase Promoters**

Plasmids provided herein are designed to encode interfering RNAs targeting immunological checkpoints as described above. The RNA expression cassette contains a promoter for transcription in human cells such as an H1 promoter or a U6 promoter, or a CMV promoter. U6 and H1 are RNA polymerase III (RNAP III) promoters, which are for production and processing of small RNAs. The CMV promoter is recognized by RNA polymerase II, and is more amenable for expression of long RNA stretches than is RNAP III. The promoter precedes the interfering RNA, such as an shRNA, siRNA or miRNA, as described above.

In eukaryotic cells, DNA is transcribed by three types of RNA polymerases; RNA Pol I, II and III. RNA Pol I transcribes only ribosomal RNA (rRNA) genes, RNA Pol II transcribes DNA into mRNA and small nuclear RNAs (snRNAs), and RNA Pol III transcribes DNA into ribosomal 5S rRNA (type I), transfer RNA (tRNA) (type II) and other small RNAs such as U6 snRNAs (type III). shRNAs are typically transcribed *in vivo* under the control of eukaryotic type III RNA Pol III promoters, such as the human U6 promoter, which transcribes the U6 snRNA component of the spliceosome, and the H1 human promoter, which transcribes the RNA component of RNase P. U6 and H1 promoters are more suitable than other Pol III or Pol II promoters because they are structurally simple, with a well-defined transcription start-site, and naturally drive the transcription of small RNAs. U6 and H1 promoters do not carry the sequences necessary for transcribing anything downstream from the transcription start site (Makinen *et al.* (2006) *J. Gene Med.* 8:433-441). They are thus the most straightforward promoters for use in shRNA expression.

The use of other promoters such as type II pol III tRNA promoters, while successful in expressing shRNAs, results in longer dsRNA transcripts, which can induce an interferon response. RNA pol II promoters, such as the human cytomegalovirus (CMV) promoter also may be used (US Pat. Nos. 8,202,846; 8,383,599), but are more often utilized for expression of long RNA stretches. Studies have shown that the addition of the enhancer from the CMV promoter near the U6 promoter can increase its activity, increasing shRNA synthesis and improving gene silencing (Xia *et al.* (2003) *Nucleic Acids Res.* 31(17):e100; Nie *et al.* (2010) *Genomics Proteomics Bioinformatics* 8(3):170-179). RNA pol II promoters are typically avoided in shRNA transcription due to the generation of cytoplasmic DNA,

which leads to a pro-inflammatory interferon response. In this case, a cytoplasmic DNA mediated interferon response in *S. typhimurium*-infected tumor cells has anti-tumor benefit, especially in the context of TREX1 inhibition as provided herein. Prokaryotic promoters, including T7, pBAD and pepT promoters can be utilized when transcription occurs in a bacterial cell (Guo *et al.* (2011) *Gene therapy* 18:95-105; U.S Patent Publication Nos. 2012/0009153, 2016/0369282; International Patent Publication Nos. WO 2015/032165, WO 2016/025582).

RNA pol III promoters generally are used for constitutive shRNA expression. For inducible expression, RNA pol II promoters are used. Examples include the pBAD promoter, which is inducible by L-arabinose; tetracycline-inducible promoters such as TRE-tight, IPT, TRE-CMV, Tet-ON and Tet-OFF; retroviral LTR; IPTG-inducible promoters such as LacI, Lac-O responsive promoters; LoxP-stop-LoxP system promoters (U.S. Patent No. 8,426,675; International Patent Publication No. WO 2016/025582); and pepT, which is a hypoxia-induced promoter. (Yu *et al.* (2012) *Scientific Reports* 2:436). These promoters are well known. Exemplary of these promoters are human U6 (SEQ ID NO:73) and human H1 (SEQ ID NO:74).

| SEQ ID NO. | Name | Sequence |
|---|---|---|
| 73 | human U6 RNA pol III promoter | aa ggtcgggcag gaagagggcc<br>721 tatttcccat gattccttca tatttgcata tacgatacaa ggctgttaga gagataatta<br>781 gaattaattt gactgtaaac acaaagatat tagtacaaaa tacgtgacgt agaaagtaat<br>841 aatttcttgg gtagtttgca gttttaaaat tatgttttaa aatggactat catatgctta<br>901 ccgtaacttg aaagtatttc gatttcttgg ctttatatat cttgtggaaa ggacgaaact<br>961 ag |
| 74 | human H1 RNA pol III promoter | atatttgca tgtcgctatg<br>721 tgttctggga aatcaccata aacgtgaaat gtctttggat ttgggaatct tataagttct<br>781 gtatgagacc actccctagg |

Tissue specific promoters include TRP2 promoter for melanoma cells and melanocytes; MMTV promoter or WAP promoter for breast and breast cancer cells, Villin promoter or FABP promoter for intestinal cells, RIP promoter for pancreatic beta cells, Keratin promoter for keratinocytes, Probasin promoter for prostatic epithelium, Nestin promoter or GFAP promoter for CNS cells/cancers, Tyrosine Hydroxylase S100 promoter or neurofilament promoter for neurons, Clara cell secretory protein promoter for lung cancer, and Alpha myosin promoter in cardiac cells (U.S. Patent No. 8,426,675).

### 5. DNA Nuclear Targeting Sequences

DNA nuclear targeting sequences (DTS)s such as the SV40 DTS mediate the translocation of DNA sequences through the nuclear pore complex. The mechanism of this transport is reported to be dependent on the binding of DNA binding proteins that contain nuclear localization sequences. The inclusion of a DTS on a plasmid to increase nuclear transport and expression has been demonstrated (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* (Kong *et al.* (2012) *PNAS* 109(47):19414–19419).

Rho-independent or class I transcriptional terminators such as the T1 terminator of the *rrnB* gene of *E. coli* contain sequences of DNA that form secondary structures that cause dissociation of the transcription elongation complex. Transcriptional terminators shall be included in the plasmid in order to prevent expression of interfering RNAs by the *S. typhimurium* transcriptional machinery. This ensures that expression of the encoded interfering RNA, such as shRNA, micro-RNA and siRNA, 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) a CpG island, 2) a bacterial origin of replication, 3) an *asd* gene selectable marker for plasmid maintenance, 4) one or more human interfering RNA expression cassettes, 5) DNA nuclear targeting sequence, and 6) transcriptional terminators.

## F. TUMOR TARGETING IMMUNOSTIMULATORY BACTERIA CONTAIN RNAI AGAINST EXEMPLARY IMMUNE TARGET GENES TO STIMULATE ANTI-TUMOR IMMUNITY

RNAi against any immune target can be encoded in the plasmids. These include, but are not limited to, any discussed in the disclosure herein, and any known to those of skill in the art. The following discussion describes exemplary targets. The plasmids can contain any RNAi against such targets, including, but not limited to, shRNA, siRNA and microRNA.

### 1. TREX1

In certain embodiments provided herein, the immunostimulatory bacteria encode inhibitory RNA, such as shRNA, that inhibit or disrupt or suppress *TREX1*

expression. The enzyme product encoded by *TREX1*, located upstream from cGAS, is a mediator of the type I interferon pathway. *TREX1* encodes the major 3' DNA exonuclease in mammalian cells (also called DNase III). Human TREX1 proteins are as catalytically efficient as bacterial exonucleases (Mazur and Perrino (2001) *J. Biol. Chem.* 276:17022-17029). Immunostimulatory bacterium that inhibit *TREX1* expression by processes other than RNA silencing also are contemplated herein.

For the immunostimulatory bacteria provided herein, such as those that express shRNA against *TREX1*, loss of TREX1 activity and subsequent activation of cGAS/STING-induced vascular disruption enhances tumor colonization of *S. typhimurium*. The *TREX1* gene encodes a protein that is 314 amino acids long (Mazur *et al.* (2001) *J.Biol.Chem* 276:17022-17029), exists as a homodimer, and lacks endonuclease activity. TREX1 is among several proteins involved in the repair of DNA that is damaged by exogenous genotoxic stress, including UV irradiation and DNA-damaging compounds. TREX1 can function as an editing exonuclease for DNA pol β by excising mispaired nucleotides from the 3' end (Mazur *et al.* (2001) *J.Biol.Chem* 276:17022-17029). ssDNA is degraded 3-4 times more efficiently than dsDNA (Lindahl *et al.*(2009) *Biochem Soc Trans* 37 (Pt 3), 535-538). Mutations in residues D18 and D200, frequently associated with autoimmune diseases, disable TREX1 enzyme from degrading dsDNA and reduces its ability to degrade ssDNA. TREX1 enzyme translocates from the endoplasmic reticulum to the nucleus following DNA damage, indicating its involvement in the replication of damaged DNA. Promoter activation and upregulation of TREX1 has been observed as a result of UVC exposure in mouse fibroblasts, and TREX1 null mouse cells have demonstrated hypersensitivity to UVC light (Tomicic *et al.* (2013) *Bioch. Biophys. Acta 1833*:1832-1843).

Mutations resulting in loss of TREX1 have been identified in patients with the inherited rare disease, Aicardi-Goutieres syndrome (AGS), which has phenotypic overlap with the autoimmune diseases systemic lupus erythematosus (SLE) and chilblain lupus (Aicardi and Goutieres, (2000) *Neuropediatrics* 31(3):113). Mutations in TREX1 also are associated with retinal vasculopathy with cerebral leukodystrophy. TREX1-mediated autoimmune diseases are associated with the cell's inability to prevent autoimmunity via the degradation of ssDNA and dsDNA that accumulates in the

cytoplasm. TREX1 null mice suffer from inflammatory myocarditis, resulting in circulatory failure, which is caused by chronic cytokine production (Morita *et al.* (2004) *Mol Cell Biol* 24(15):6719-6727; Yang *et al.* (2007) *Cell* 131(5):873-886; Tomicic *et al.* (2013) *Bioch. Biophys. Acta* 1833(8):1832-1843). Hence, TREX1 deficiency induces innate immunity following the cytoplasmic accumulation of DNA, resulting in an inflammatory response (Wang *et al.* (2009) *DNA Repair(Amst)8*: 1179-1189). The source of the DNA that accumulates in the cytosol of TREX1-deficient cells was found to be in part derived from endogenous retroelements that escape from the damaged nucleus, as TREX1 is known to metabolize reverse-transcribed (RT) DNA (Stetson *et al.* (2008) *Cell* 134(4):587-598). In HIV infection, HIV RT DNA accumulates in the cytosol of infected T cells and macrophages, and would normally trigger cGAS/STING activation of antiviral immunity. TREX1 digests this viral DNA and permits HIV immune escape (Yan *et al.* (2010) *Nat. Immunol.* 11(11):1005-1013). Thus, TREX1 acts as a negative regulator of STING, and can be exploited to evade detection by several retroviruses, such as murine leukemia virus (MLV), simian immunodeficiency virus (SIV), and many others (Hasan *et al.* (2014) *Front. Microbiol.* 4:393).

Like STING, TREX1 is expressed in most mammalian cell types, with the key producers of cytokines in TREX1 null mice originating from macrophages and dendritic cells (Ahn *et al.* (2014) *J. Immunol.* 193(9):4634-4642). Data indicate that TREX1 is responsible for degrading self-DNA that can leak from a damaged nucleus into the cytosol, where it would otherwise bind and activate cGAS and lead to autoimmunity (Barber (2015) *Nat. Rev. Immunol.* 15(12):760-770). In support of this, TREX1 null mice and TREX1-deficient cells that also lack cGAS are completely protected from type I interferon activation and lethal autoimmunity (Ablasser *et al.* (2014) *J. Immunol.* 192(12):5993-5997; Gray *et al.* (2015) *J. Immunol.* 195(5):1939-1943). In a negative feedback loop, type I interferon and type II IFNγ can also induce TREX1, and TREX1 thus serves to limit aberrant autoimmune activation (Tomicic *et al.* (2013) *Bioch. Biophys. Acta 1833*:1832-1843).

Lymphocytes derived from an Aicardi-Goutieres syndrome patient, containing mutated TREX1, were found to inhibit angiogenesis and the growth of neuroblastoma cells, the effect being enhanced by the presence of IFN-α (Pulliero *et al.* (2012)

*Oncology Reports* 27:1689-1694). The use of microRNA-103 also has been shown to inhibit the expression of TREX1, disrupting DNA repair and angiogenesis, and

resulting in decreased tumor growth *in vivo* (see, U.S. Patent Publication No. 2014/0127284, Cheresh *et al.*).

TREX1 is a negative regulator of macrophage activation and pro-inflammatory function. TREX1 null macrophages were found to exhibit increased TNF-α and IFN-α production, higher levels of CD86, and increased antigen presentation to T cells, as well as impaired apoptotic T cell clearance (Pereira-Lopes *et al.* (2013) *J. Immunol. 191*:6128-6135). The inability to adequately digest apoptotic DNA in TREX1 null macrophages generates high amounts of aberrant cytosolic DNA, which binds to cGAS and activates the STING pathway to produce higher levels of type I interferon (Ahn *et al.* (2014) *J. Immunol. 193*:4634-4642). Not all cell types are sensitive to the immunostimulatory effects of Trex1 knockdown, however. In a study of individual cell types, dendritic cells, macrophages, fibroblasts and keratinocytes were found to produce type I IFN upon Trex1 knockdown, while B cells, cardiomyocytes, neurons and astrocytes did not (Peschke *et al.* (2016) *J. Immunol.* 197:2157-2166). Thus, inhibiting the function of TREX1 in phagocytic cells that have engulfed S. *typhimurium* would enhance their pro-inflammatory activity, while driving an accumulation of cytosolic DNA from phagocytosed tumor cells that can then activate the cGAS/STING pathway. The use of microRNA-103 has inhibits the expression of TREX1, disrupting DNA repair and angiogenesis, and resulting in decreased tumor growth *in vivo* (see, U.S. Publication No. 2014/0127284, Cheresh *et al.*).

Studies have found that the expression of cGAS and/or STING is inhibited in over a third of colorectal cancers, while STING expression is lost in many primary and metastatic melanomas and $HPV^+$ cancers. STING signaling remains intact in all tumor-resident APCs that continuously sample the antigenic milieu of the TME, including Batf3-lineage $CD103/CD8\alpha^+$ DCs that cross-present tumor antigens to $CD8^+$ T cells, and these APCs will also readily phagocytose S. *typhimurium* or be activated by type I IFN from neighboring macrophages that have phagocytosed *S. typhimurium* containing *TREX1* gene knockdown.

Inactivation of TREX1 enhances an immune response by enabling cytosolic accumulation of dsDNA to bind to the enzyme cyclic GMP-AMP (cGAMP) synthase (cGAS), a cytosolic DNA sensor that triggers the production of type I interferons and

other cytokines through activation of the STING signaling pathway (Sun *et al.* (2013) *Science* 339(6121):786-791; Wu *et al.* (2013) *Science* 339(6121):826-830). Activation of the STING pathway has been shown to induce potent innate and adaptive antitumor immunity (Corrales *et al.* (2015) *Cell Reports 11*:1018-1030).

Hence, embodiments of the immunostimulatory bacterial strains, as provided herein, are administered to inhibit TREX1 in tumor-resident APCs and induce cGAS/STING activation, thereby activating these DCs to cross-present host tumor antigens to $CD8^+$ T cells and induce local and systemic tumor regression and durable anti-tumor immunity (Corrales *et al.* (2015) *Cell Reports 11*:1018-1030; Zitvogel *et al.*(2015) *Nat. Rev. Mol. Cell. Biol. 16*:393–405).

The clinical activity of VNP20009 was largely disappointing in part due to its poor ability to colonize human tumors, a phenomenon that was not observed in mouse models (Nemunaitis *et al.* (2003) *Cancer Gene Ther.* 10(10):737-744; Toso *et al.* (2002) *J. Clin. Oncol.* 20(1):142-152; Heimann *et al.* (2003) *J. Immunother.* 26(2):179-180). It was later revealed that the reason for the discrepancy between human and mouse tumor colonization was that orthotopically transplanted syngeneic mouse tumors are much more vascularized than human tumors. In order to more closely model the lack of human tumor vascularization in mice, autochthonous tumor models were treated with VNP20009 and found to only enable tumor colonization with pre-treatment of a vascular disrupting agent (Drees *et al.* (2015) *J of Cancer* 6(9):843-848; Drees *et al.* (2015) *Anticancer Res.* 35(2):843-849). Vascular disrupting agents such as 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) have been shown to mediate tumor collapse in mice (but not humans) by directly binding STING and inducing type I interferon signaling (Baguley (2003) *Lancet Oncol.* 4(3):141-148; Corrales and Glickman *et al.* (2015) *Cell Reports* 11(7):1018-1030). STING signaling induces TNF-$\alpha$ and IFN-$\gamma$ production, cytokines which have been shown to directly promote vascular disruption by downregulating $\alpha V\beta 3$ integrin adhesion receptors on endothelial cells (Rüegg *et al.* (1998) *Nat Medicine* 4(4):408-414). Production of innate pro-inflammatory cytokines such as TNF-$\alpha$, IL-12p40 and IFN-$\gamma$ that are induced upon STING activation are critical for activating anti-tumor immunity (Burdette *et al.* (2011) *Nature* 478(7370):515-518).

Thus, the immunostimulatory bacteria provided herein express shRNA against TREX1, and loss of TREX1 and subsequent activation of cGAS/STING-induced vascular disruption enhance tumor colonization of *S. typhimurium*.

**2. PD-L1**

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 APCs, and upon binding to PD-1 on T cells, leads to loss of $CD8^+$ T cell effector function, inducing T cell tolerance. The expression of PD-L1 is often associated with tumor aggressiveness and reduced survival in certain human cancers (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, nivolumab) and anti-PD-L1 (for example, atezolizumab, avelumab, durvalumab) antibodies have had durable success in preventing T cell anergy and breaking immune tolerance. Only a fraction of treated patients exhibit clinical benefit, and those that do often present with autoimmune-related toxicities (Ribas (2015) *N. Engl. J. Med.* 373(16):1490-1492; Topalian *et al.* (2012) *N. Engl. J. Med.* 366(26):2443-54). Besides acquiring toxicity, PD-1/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-L1 blockade, an immunostimulatory bacteria with an shRNA to PD-L1, as provided herein, will synergize with TLR activation of immune cells to both activate and potentiate anti-tumor immunity.

**3. VISTA**

Other non-redundant checkpoints in immune activation can synergize with PD-1/PD-L1 and CTLA-4, such as V-domain immunoglobulin (Ig) suppressor of T cell activation (VISTA). VISTA is expressed primarily on APCs, particularly on tumor-infiltrating myeloid cells and myeloid-derived suppressor cells (MDSC), and to a lesser extent on regulatory T cells (CD4+ Foxp3+ Tregs) (Wang *et al.* (2011) *J. Exp. Med.* 208(3):577-592). Similar to PD-L1, VISTA upregulation directly suppresses T cell proliferation and cytotoxic function (Liu *et al.* (2015) *PNAS* 112(21):6682-6687).

Monoclonal antibody targeting of VISTA was shown to remodel the tumor microenvironment in mice, increasing APC activation and enhancing anti-tumor immunity (LeMercier *et al.* (2014) *Cancer Res.* 74(7):1933-1944). Clinically, VISTA expression was shown to be upregulated on tumor-resident macrophages following treatment with anti-CTLA-4 therapy in prostate cancer, demonstrating compensatory regulation of immune checkpoints (Gao *et al.* (2017) *Nat. Med.* 23(5):551-555). The majority of VISTA expression is purported to be located in the intracellular compartment of myeloid cells, rather than on the surface, which may limit the effectiveness of the monoclonal antibody approach (Deng *et al.* (2016) *J. Immunother. Cancer* 4:86). The ability to inhibit VISTA from within the APC using a tumor-targeting bacteria containing shRNA to VISTA, as provided herein, will more efficiently and completely inhibit the T cell-suppressing function of VISTA, leading to activation of T cell-mediated anti-tumor immunity and tumor regression.

**4. SIRPα**

One mechanism by which tumor cells evade removal is to prevent their phagocytosis by innate immune cells. Phagocytosis is inhibited by surface expression of CD47, which is widely expressed on hematopoietic and non-hematopoietic cells (Liu *et al.* (2015) *PLoS ONE* 10(9):e0137345). Upon CD47 binding its receptor, signal regulatory protein alpha (SIRPα), an inhibitory signal for phagocytosis, is initiated. SIRPα is abundantly expressed on phagocytic cells, including macrophages, granulocytes and DCs. As such, the protein-protein interaction between CD47 and SIRPα represents another class of immune checkpoints unique to APCs, and tumor-resident macrophages in particular. The effectiveness of CD47 in preventing phagocytosis is evidenced by the fact that it is often upregulated in a wide variety of tumors, which allow them to avoid being phagocytosed by APCs in the tumor microenvironment (Liu *et al.* (2015) *Nat. Med.* 21(10):1209-1215). Several methods to block the CD47/SIRPα interaction have been examined, including the development of anti-CD47 or anti-SIRPα antibodies or antibody fragments, the use of small peptides that bind either protein, or the knockdown of CD47 expression (U.S. Patent Publication Nos. 2013/0142786, 2014/0242095; International Patent Publication No. WO 2015/191861; McCracken *et al.* (2015) *Clin. Cancer Res.* 21(16):3597-3601). To this end, several monoclonal antibodies that directly target SIRPα are in clinical

development, either alone or in combination with tumor-targeting antibodies (e.g. Rituximab, Daratumumab, Alemtuzumab, Cetuximab) that can enhance phagocytosis of antibody-opsonized tumor cells, in a process known as antibody-dependent cellular phagocytosis (ADCP) (McCracken *et al.* (2015) *Clin. Cancer Res.* 21(16):3597-3601; Yanagita *et al.* (2017) *JCI Insight* 2(1):e89140).

The CD47/SIRPα interaction also serves to preserve the longevity of red blood cells by preventing their phagocytic elimination (Murata *et al.* (2014) *J. Biochem.* 155(6):335-344). Thus, systemically administered therapies such as anti-CD47 antibodies that broadly disrupt this interaction have resulted in anemia toxicities (Huang *et al.* (2106) *J Thorac Dis.* 126:2610-20). Systemic SIRPα-based therapies also risk adverse events, such as organ damage by creating systemic hyperphagocytic self-eating macrophages. Using a tumor-targeting immunostimulatory bacteria containing an shRNA to SIRPα, such as provided herein, will localize the CD47/SIRPα disruption to the tumor microenvironment and eliminate these adverse events. Further, inhibition of SIRPα in the context of bacterial activation of TLR-mediated pro-inflammatory signaling pathways will potently activate these macrophages to become hyperphagocytic towards neighboring tumor cells (Bian *et al.* (2016) *PNAS.* 113(37): E5434–E5443).

**5. β-catenin**

Immune checkpoint pathways exemplify the multiple layers of regulation that exist to prevent immune hyper-activation and autoimmunity, and the difficulties in subverting these pathways to promote anti-tumor immunity. One mechanism by which tumors have evolved to be refractory to checkpoint therapies is through their lack of T cell and dendritic cell (DC) infiltration, described as non-T-cell-inflamed, or "cold tumors" (Sharma *et al.* (2017) *Cell* 9;168(4):707-723). Several tumor-intrinsic mechanisms have been identified that lead to the exclusion of anti-tumor T cells and resistance to immunotherapy. In melanoma, in particular, molecular profiling of checkpoint therapy-refractory tumors revealed a signature of elevated β-catenin and its downstream target genes, correlating with a lack of tumor-infiltrating lymphocytes (Gajewski *et al.* (2011) *Curr. Opin. Immunol.* 23(2):286-292).

*CTNNB1* is an oncogene that encodes β-catenin, and can induce the expression of the genes *c-Myc* and *cyclin D1*, resulting in tumor proliferation. Mutations in

*CTNNB1* are associated with certain cancers. Gene silencing of CTNNB1/ β-catenin using *S. typhimurium* shRNA vectors can be used in the treatment of cancer (Guo *et al.* (2011) *Gene therapy* 18:95-105; U.S. Patent Publication Nos. 2012/0009153, 2016/0369282; International Patent Publication No. WO 2015/032165). For example, shRNA silencing of *CTNNB1*, using *S. typhimurium* strain SL7207 as a delivery vector, reduced tumor proliferation and growth in SW480 xenograft mice, when compared to control cells, and reduced expression of *c-Myc* and *cyclin D1*(Guo *et al.* (2011) *Gene therapy* 18:95-105). Silencing of *CTNNB1* for the treatment of hepatoblastoma also can be achieved using miRNA, with or without antibody therapeutics against the immune checkpoints PD-1and PD-L1 (International Patent Publication No. WO 2017/005773). The use of siRNA or shRNA targeting *CTNNB1*, delivered via alternative vectors, such as liposomes, for the treatment of CTNNB1-related cancers, including adenocarcinomas and squamous cell carcinomas, also can be affected (U.S. Patent Publication Nos. 2009/0111762, 2012/0294929).

Elevated β-catenin signaling directly inhibits the chemokine CCL4 from recruiting Batf3-lineage CD103/CD8α$^+$ DCs, thereby preventing them from priming tumor antigen-specific CD8$^+$ T cells (Spranger *et al.* (2015) *Nature* 523(7559):231-235). β-catenin is the major downstream mediator of the WNT signaling pathway, a key embryonic developmental pathway that is also critical for adult tissue regeneration, homeostasis and hematopoiesis (Clevers *et al.* (2012) *Cell* 149(6):1192-1205). Excessive WNT/β-catenin signaling has been implicated in a variety of cancers (Tai *et al.* (2015) *Oncologist* 20(10):1189-1198). Accordingly, several strategies to target WNT/β-catenin signaling have been pursued, but success has been hampered by a lack of specificity to the tumor microenvironment, resulting in off-target toxicities to intestinal stem cells, bone turnover and hematopoiesis (Kahn (2014) *Nat. Rev. Drug Dis.* 13(7):513-532). The immunostimulatory bacteria provided herein overcome these problems.

For example, an advantage of using an immunostimulatory bacteria with shRNA to β-catenin as provided herein, is enhancing chemokine-mediated infiltration of T cell-priming DCs and the conversion of a cold tumor to a T-cell-inflamed tumor microenvironment, without the systemic toxicities of existing therapeutic modalities.

Further, bacterial activation of TLR innate immune signaling pathways synergize with β-catenin inhibition to further promote immune activation and anti-tumor immunity.

**6. TGF-β**

Transforming growth factor beta (TGF-β) is a pleiotropic cytokine with numerous roles in embryogenesis, wound healing, angiogenesis and immune regulation. It exists in three isoforms in mammalian cells, TGF-β1, TGF-β2 and, TGF-β3; TGF-β1 is the most predominant in immune cells (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 can also directly convert Th1 $CD4^+$ T cells to immunosuppressive Tregs, furthering promoting tumor tolerance (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 TGF-β signaling has been demonstrated in several human tumor types, including CRC, HCC, PDAC and NSCLC (Colak *et al.* (2017) *Trends in Cancer* 3:1). Systemic inhibition of TGF-β can lead to unacceptable autoimmune toxicities, and its inhibition should be localized to the tumor microenvironment. As such, a tumor-targeting immunostimulatory bacteria with RNAi, such as shRNA, to TGF-β, provided herein, or an shRNA to TGF-βRII, breaks tumor immune tolerance and stimulates anti-tumor immunity.

**7. VEGF**

Angiogenesis, or the development of new blood vessels, is an essential step for any tumor microenvironment to become established. Vascular endothelial growth factor (VEGF) is the critical mitogen for endothelial proliferation and angiogenesis, and inhibition of VEGF in the tumor microenvironment markedly decreases tumor vascularity, thereby starving the tumor of its blood supply (Kim *et al.* (1993) *Nature* 362(6423):841-4). This early research led to the development of the monoclonal antibody inhibitor of VEGF, bevacizumab (Avastin; Genentech), which in combination with chemotherapy, has become the standard of care for metastatic CRC.

Systemic administration of bevacizumab also demonstrated significant toxicities, including multiple fatalities in a Phase II trial of NSCLC, largely due to hemorrhaging. As such, several next generation anti-angiogenics have been evaluated, such as the anti-VEGF receptor 2 antibody ramucirumab (Cyramza, Imclone) and the anti-angiogenic tyrosine kinase inhibitor axitinib (Inlyta, Pfizer), yet none have been able to overcome systemic toxicity or markedly improve progression-free survival (Alshangiti *et al.* (2018) *Curr Oncol.* 25(Suppl 1):S45–S58). While the anti-tumor activity of anti-VEGF therapy has shown some promise, systemic toxicity is clearly limiting. As such, a therapy that targets only the tumor microenvironment, such as an immunostimulatory tumor-targeting bacteria with shRNA to VEGF, provided herein, delivers local anti-angiogenic therapy while preventing systemic toxicity. This therapeutic modality has the additional advantage of being taken up into myeloid cells, which predominantly produce VEGF in the tumor microenvironment, where it will have maximum impact on tumor progression (Osterberg *et al.* (2016) *Neuro-Oncology*. 18(7):939–949).

### 8. Additional Exemplary Checkpoint Targets

Exemplary checkpoint targets for which RNAi, such as micro-RNA and shRNA, can be prepared or are exemplified herein include, but are not limited to:

| 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), CD200, CD272 (BTLA), CD160 |

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

Other exemplary targets include, but are not limited to:

| Target |
|---|
| CTNNB1 (beta-catenin) |
| STAT3 |
| BCL-2 |
| MDR1 |
| Arginase1 |
| iNOS |
| TGF-β |
| IL-10<br>pGE2<br>VEGF |
| KSP<br>HER2<br>KRAS<br>TAK1<br>PLK1 |
| K-Ras (Ras) |
| Stablin-1/CLEVER-1 |
| RNase H2 |
| DNase II |

## G. COMBINATIONS OF RNAI shRNAS TO MULTIPLE IMMUNE TARGETS WITHIN A SINGLE THERAPEUTIC MODALITY AND COMBINATION THERAPY

Combinations of RNAi, such as shRNAs or microRNAs, that inhibit different targets in one bacterium, are contemplated. Combinations of such targets can be selected to act synergistically. RNAi that targets any two immune checkpoints can be combined, and introduced into the immunostimulatory bacterial hosts modified as described herein, or into therapeutic bacterial hosts of others.

### 1. TREX1 and Other Targets

In order to mitigate the induction of compensatory immune checkpoint pathways that can be upregulated upon STING activation and enhance anti-tumor immunity, the modified immunostimulatory bacteria provided herein contain short hairpin (sh)-RNA sequences against TREX1 in combination with shRNA to other

immune targets, including but not limited to PD-L1, VISTA and SIRPα. Knockdown of TREX1 and SIRPα in tumor-resident phagocytic cells enables blockade of "don't eat me" interactions with CD47 on tumor cells, as well as further enhances the susceptibility of the tumor microenvironment to *S. typhimurium* infection (Li *et al.*(2012) *J Immunol* 189(5):2537-2544), and is provided herein. The combination of enhanced phagocytosis enabled by SIRPα inhibition and simultaneous knockdown of TREX1, facilitates greater cytosolic delivery and stabilization of tumor DNA that can more potently activate cGAS/STING signaling. Notably, the anti-tumor effects of CD47/SIRPα blockade were shown to require intact STING signaling, demonstrating the potential synergy of combining TREX1-mediated STING activation with SIRPα inhibition (Liu *et al.* (2015) *Nat. Med.* 21(10):1209-1215). Knockdown of TREX1 in combination with shRNA to PD-L1, provided herein, enhances the pathogenesis and immune-stimulatory properties of the modified S. *typhimurium* (Lee *et al.* (2010) *J. Immunol.* 185(4):2442-2449), thereby igniting a more inflamed and immunogenic tumor microenvironment. shRNA targets against β-catenin and TGF-β also lead to a more T cell inflamed tumor microenvironment and synergize well with shRNA to PD-L1, and are provided herein. Combining immune activation with local checkpoint blockade within the macrophage/myeloid compartment in particular, such as through combined shRNAs to TREX1 and VISTA, provided herein, potentiates the immune response by enhancing both tumor neoantigen presentation by *S. typhimurium*-infected APCs and enhanced activation of tumor-specific T cells.

**2. TREX1 and Radiotherapy**

The success of anticancer radiotherapy depends on the induction of type I interferon-dependent innate and adaptive immunity. TREX1 has been shown to attenuate anti-tumor immunity following high levels of Gy radiation by degrading the cytosolic DNA that is produced in the damaged cancer cells, thus inhibiting the type I interferon pathway mediated by cGAS and STING (Vanpouille-Box *et al.* (2017) *Nature Communications* 8:15618). Thus, the overexpression of TREX1, or the knockout of cGAS/STING, which prevents activation of the IFN-I pathway, attenuates the abscopal tumor response upon irradiation. In order to activate STING-mediated Batf3-DC priming of $CD8^+$ T cells and achieve maximal abscopal anti-

tumor immunity, a lower dose of radiation was required that would not induce TREX1 (Vanpouille-Box *et al.* (2017) *Nature Communications* 8:15618). The downregulation of TREX1 has been shown to restore the sensitivity of tumor cells towards ionizing radiation. For example, high dose irradiation induced TREX1 expression and prevented cytoplasmic accumulation of dsDNA, thereby inhibiting abscopal tumor regression (Vanpouille-Box *et al.* (2017) *Nature Communications* 8:15618). The immunostimulatory strains provided herein that block or inhibit TREX1 expression can reduce or eliminate or blunt the expression of TREX1 upon high dose radiation treatment, significantly extending the therapeutic window.

While radiotherapy (RT) has an abscopal effect at lower doses, the lower doses are not necessarily effective. At higher doses, however, the abscopal effect is no longer observed. This is a known problem with RT. Radiotherapy has been shown to promote the upregulation of TREX1 that degrades cytosolic dsDNA, precluding IFN-β secretion secondary to cGAS/STING signaling (see, Vanpouille-Box *et al.* (2017) *Nat. Commun.* 8:15618). Hence, the immunostimulatory bacterium provided herein can be administered with RT to prevent upregulation of TREX1. Administration of an immunostimulatory bacterium, provided herein, that encodes shRNA or other product that inhibits TREX1 abrogates this response, thereby improving and complementing RT. Hence, provided herein are combination therapies in which the immunostimulatory bacteria that encode shRNA or other product that inhibit or reduce expression of TREX1 are administered with RT, either before, in conjunction with, or after, or intermittently with RT. The combination therapy of the immunostimulatory bacteria and RT therapy also can include other anti-cancer therapies, such as administration of a checkpoint inhibitor, and/or inclusion of shRNA against other checkpoints, such as PD-L1, as described herein.

### 3. TREX1 and Immunogenic Chemotherapy

Induction of TREX1 was observed following DNA-damaging UV irradiation of mouse and human fibroblasts, as well as treatment of glioma and malignant melanoma cells with the DNA alkylating agents nimustine, carmustine and fotemustine, and the topoisomerase I inhibitor topotecan. These tumor cells were re-sensitized to these anti-cancer therapeutics following siRNA knockdown of TREX1 (Tomicic *et al.* (2013) *Biochimica et Biophysica Acta 1833*:1832-1843). TREX1 was

only induced by damage agents that induce AP-1 efficiently, while agents that are weak inducers of Fos/Jun/AP-1, such as the methylating agent temozolomide and the topoisomerase II inhibitor etoposide, did not induce TREX1.

A separate study found that dsDNA accumulates and activates type I IFN upon treatment with chemotherapies that stall DNA replication in the S phase, such as cisplatin, irinotecan, doxorubicin and etoposide, but not agents that act in M phase, such as vinorelnine and paclitaxel (Wilkinson R. presented at ESMO TAT Conference 2018). S phase agents likely lead to the release of damaged DNA fragments that accumulate in the cytosol and upregulate TREX1. These chemotherapeutic agents, which include those that cause DNA strand breaks, such as nucleotide analogs, alkylating agents, platinum drugs, and intercalating agents (see, *e.g.*, Swift *et al.*(2014) *Int. J. Mol. Sci 15*:3403-3431), can induce TREX1 at levels sufficient to degrade the DNA, thereby precluding activation of the type-I interferon (IFN-I) pathway mediated via cyclic GMP-AMP (cGAMP) synthase (cGAS) and its downstream adaptor stimulator of interferon genes (STING). Treatment with the immunostimulatory bacteria provided herein can be combined with chemotherapeutic agents, and further with other checkpoint inhibitors. Hence, the immunostimulatory bacteria provided herein can advantageously be used in combination therapy with a variety of anti-cancer agents and treatments.

#### 4. Combination Therapy with Anti-Checkpoint Antibodies

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

## H. PHARMACEUTICAL PRODUCTION, COMPOSITIONS, AND FORMULATIONS

Provided herein are methods for manufacturing, 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 treatment of diseases, such as hyperproliferative diseases or condition, such as a tumor or cancer. The immunostimulatory bacteria can be administered in a single agent therapy, or can be administered in a combination therapy with a further agent or treatment. The compositions can be formulated for

single dosage administration or for multiple dosage administration. The agents can be formulated for direct administration. The compositions can be provided as a liquid or dried formulation.

## 1. Manufacturing

### a. Cell Bank Manufacturing

As the active ingredient of the immunotherapeutic described herein is composed of engineered self-replicating bacteria, the selected composition will be expanded into a series of cell banks that will be maintained for long-term storage and as the starting material for manufacturing of drug substance. Cell banks are produced under current good manufacturing practices (cGMP) in an appropriate manufacturing facility per the Code of Federal Regulations (CFR) 21 part 211 or other relevant regulatory authority. As the active agent of the immunotherapeutic is a live bacterium, the products described herein are, by definition, non-sterile and cannot be terminally sterilized. Care must be taken to ensure that aseptic procedures are used throughout the manufacturing process to prevent contamination. As such, all raw materials and solutions must be sterilized prior to use in the manufacturing process.

A master cell bank (MCB) is produced by sequential serial single colony isolation of the selected bacterial strain to ensure no contaminants are present in the starting material. A sterile culture vessel containing sterile media (can be complex media *e.g.*, LB or MSBB 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 ºC with shaking. The bacteria are then prepared for cryopreservation by suspension in a solution containing a cryoprotective agent or agents.

Examples of cryoprotective agents include: proteins such as human or bovine serum albumin, gelatin, immunoglobulins; carbohydrates including monosaccharides (galactose, D-mannose, sorbose, *etc.*) and their non-reducing derivatives (*e.g.*, methylglucoside), disaccharides (trehalose, sucrose, *etc.*), cyclodextrins, and polysaccharides (raffinose, maltodextrins, dextrans, *etc.*); amino-acids (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 may include one or more cryoprotective agents in a solution that may also 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.

Suspension of the bacteria in cryopropreservation 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 characterized to ensure identity, purity, and activity. WCB material is typically the

starting material used in production of the drug substance of biologics such as engineered bacteria.

**b. Drug Substance Manufacturing**

Drug substance is manufactured using aseptic processes under cGMP as described above. Working cell bank material is typically used as starting material for manufacturing of drug substance under cGMP, however other cell banks can be used (*e.g.*, MCB or MWCB). Aseptic processing is used for production of all cell therapies including bacterial cell-based therapies. The bacteria from the cell bank are expanded by fermentation, this can be achieved by production of a pre-culture (*e.g.*, in a shake flask) or by direct inoculation of a fermenter. Fermentation is accomplished in a sterile bioreactor or flask that can be single-use disposable or re-usable. Bacteria are harvested by concentration (*e.g.*, by centrifugation, continuous centrifugation, or tangential flow filtration). Concentrated bacteria are purified from media components and bacterial metabolites by exchange of the media with buffer (*e.g.*, by diafiltration). The bulk drug product is formulated and preserved as an intermediate (*e.g.*, by freezing or drying) or is processed directly into a drug product. Drug substance is tested for identity, strength, purity, potency, and quality.

**c. Drug Product Manufacturing**

Drug product is defined as the final formulation of the active substance contained in its final container. Drug product is manufactured using aseptic processes under cGMP. Drug product is produced from drug substance. Drug substance is thawed or reconstituted if necessary, then formulated at the appropriate target strength. Because the active component of the drug product is live, engineered bacteria, the strength is determined by the number of CFU contained within the suspension. The bulk product is diluted in a final formulation appropriate for storage and use as described below. Containers are filled, and sealed with a container closure system and the drug product is labeled. The drug product is stored at an appropriate temperature to preserve stability and is tested for identity, strength, purity, potency, and quality and released for human use if it meets specified acceptance criteria.

**2. Compositions**

Pharmaceutically acceptable compositions are prepared in view of approvals for a regulatory agency or other agency prepared in accordance with generally

recognized pharmacopeia for use in animals and in humans. The compositions can be prepared as solutions, suspensions, powders, or sustained release formulations. Typically, the compounds are formulated into pharmaceutical compositions using techniques and procedures well known in the art (see *e.g.*, Ansel *Introduction to Pharmaceutical Dosage Forms*, Fourth Edition, 1985, 126). The formulation should suit the mode of administration.

Compositions can be formulated for administration by any route known to those of skill in the art including intramuscular, intravenous, intradermal, intralesional, intraperitoneal injection, subcutaneous, intratumoral, epidural, nasal, oral, vaginal, rectal, topical, local, otic, inhalational, buccal (*e.g.*, sublingual), and transdermal administration or any route. Other modes of administration also are contemplated. Administration can be local, topical or systemic depending upon the locus of treatment. Local administration to an area in need of treatment can be achieved by, for example, but not limited to, local infusion during surgery, topical application, *e.g.,* in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant. Compositions also can be administered with other biologically active agents, either sequentially, intermittently or in the same composition. Administration also can include controlled release systems including controlled release formulations and device controlled release, such as by means of a pump.

The most suitable route in any given case depends on a variety of factors, such as the nature of the disease, the progress of the disease, the severity of the disease and the particular composition which is used. Pharmaceutical compositions can be formulated in dosage forms appropriate for each route of administration. In particular, the compositions can be formulated into any suitable pharmaceutical preparations for systemic, local intraperitoneal, oral or direct administration. For example, the compositions can be formulated for administration subcutaneously, intramuscularly, intratumorally, intravenously or intradermally. Administration methods can be employed to decrease the exposure of the active agent to degradative processes, such as immunological intervention via antigenic and immunogenic responses. Examples of such methods include local administration at the site of treatment or continuous infusion.

The immunostimulatory bacteria can be formulated into suitable pharmaceutical preparations such as solutions, suspensions, tablets, dispersible tablets, pills, capsules, powders, sustained release formulations or elixirs, for oral administrations well as transdermal patch preparation 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, 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) or frozen suspension that are thawed prior to use, dry soluble products, such as lyophilized powders, ready to be combined with a resuspension solution just prior to use, and emulsions. Dried thermostable formulations such as lyophilized formulations can be used for storage of unit doses for later use.

The pharmaceutical preparation can be in a frozen liquid form, for example a suspension. If provided in frozen liquid form, the drug product can be provided as a concentrated preparation to be thawed and diluted to a therapeutically effective concentration before use.

The pharmaceutical preparations also can be provided in a dosage form that does not require thawing or dilution for use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives, as appropriate, such as suspending agents (*e.g.*, sorbitol, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives suitable for use with

microbial therapeutics. The pharmaceutical preparations can be presented in dried form, such as lyophilized or spray-dried, for reconstitution with water or other sterile suitable vehicle before use.

Suitable excipients are, for example, water, saline, dextrose, or glycerol. The solutions can be either aqueous or nonaqueous. If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and other buffered solutions used for intravenous hydration. For intratumoral administration solutions containing thickening agents such as glucose, polyethylene glycol, and polypropylene glycol, oil emulsions and mixtures thereof may be appropriate to maintain localization of the injectant.

Pharmaceutical compositions can include carriers or other excipients. For example, pharmaceutical compositions provided herein can contain any one or more of a diluents(s), adjuvant(s), antiadherent(s), binder(s), coating(s), filler(s), flavor(s), color(s), lubricant(s), glidant(s), preservative(s), detergent(s), or sorbent(s) and a combination thereof or vehicle with which a modified therapeutic bacteria is administered. For example, pharmaceutically acceptable carriers or excipients used in parenteral preparations include aqueous vehicles, nonaqueous vehicles, isotonic agents, buffers, antioxidants, local anesthetics, suspending and dispersing agents, emulsifying agents, sequestering or chelating agents and other pharmaceutically acceptable substances. Formulations, including liquid preparations, can be prepared by conventional means with pharmaceutically acceptable additives or excipients.

Pharmaceutical compositions can include carriers such as a diluent, adjuvant, excipient, or vehicle with which the compositions are administered. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound or agent, generally in purified form or partially purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and sesame oil. Water is a typical carrier. Saline solutions and aqueous dextrose and glycerol solutions also can be employed as liquid carriers, particularly for injectable solutions. Compositions can contain along

with an active ingredient: a diluent such as lactose, sucrose, dicalcium phosphate, or carboxymethylcellulose; a lubricant, such as magnesium stearate, calcium stearate and talc; and a binder such as starch, natural gums, such as gum acacia, gelatin, glucose, molasses, polyvinylpyrrolidine, celluloses and derivatives thereof, povidone, crospovidones 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, nonaqueous 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, Ringers Injection, Isotonic Dextrose Injection, Sterile Water Injection, Dextrose and Lactated Ringers Injection. Nonaqueous parenteral vehicles include fixed oils of vegetable origin, cottonseed oil, corn oil, sesame oil and peanut oil. Isotonic agents include sodium chloride and dextrose. Buffers include phosphate and citrate. Antioxidants include sodium bisulfate. Local anesthetics include procaine hydrochloride. Suspending and dispersing agents include sodium carboxymethylcellulose, hydroxypropyl methylcellulose and polyvinylpyrrolidone. Emulsifying agents include, for example, polysorbates, such Polysorbate 80 (TWEEN 80). Sequestering or chelating agents of metal ions, such as EDTA, can be included. Pharmaceutical carriers also include polyethylene glycol and propylene glycol for water miscible vehicles and sodium hydroxide, hydrochloric acid, citric acid or lactic acid for pH adjustment. Non-anti-microbial preservatives can be included.

The pharmaceutical compositions also can contain other minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering

agents, stabilizers, solubility enhancers, and other such agents, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate and cyclodextrins. Implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained (see, *e.g.*, U.S. Pat. No. 3,710,795) also is contemplated herein. The percentage of active compound contained in such parenteral compositions is highly dependent on the specific nature thereof, as well as the activity of the compound and the needs of the subject.

**b. Dried Thermostable Formulations**

The bacteria can be dried. Dried thermostable formulations, such as lyophilized or spray dried powders and vitrified glass can be reconstituted for administration as solutions, emulsions and other mixtures. The dried thermostable formulation can be prepared from any of the liquid formulations, such as the suspensions, described above. The pharmaceutical preparations can be presented in lyophilized or vitrified form for reconstitution with water or other suitable vehicle before use.

The thermostable formulation is prepared for administration by reconstituting the dried compound with a sterile solution. The solution can contain an excipient which improves the stability or other pharmacological attribute of the active substance or reconstituted solution, prepared from the powder. The thermostable formulation is prepared by dissolving an excipient, such as dextrose, sorbitol, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agent, in a suitable buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art. Then, the drug substance is added to the resulting mixture, and stirred until it is mixed. The resulting mixture is apportioned into vials for drying. Each vial will contain a single dosage containing $1x10^5$- $1x10^{11}$ CFU per vial. After drying, the product vial is sealed with a container closure system that prevents moisture or contaminants from entering the sealed vial. The dried product can be stored under appropriate conditions, such as at -20 °C, 4 °C, or room temperature. Reconstitution of this dried formulation with water or a buffer solution provides a formulation for use in parenteral administration. The precise amount depends upon the indication treated and selected compound. Such amount can be empirically determined.

### 4. Compositions for Other Routes of Administration

Depending upon the condition treated, other routes of administration in addition to parenteral, such as topical application, transdermal patches, oral and rectal administration are also 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 gm. Tablets and capsules for rectal administration are manufactured using the same pharmaceutically acceptable substance and by the same methods as for formulations for oral administration. Formulations suitable for rectal administration can be provided as unit dose suppositories. These can be prepared by admixing the drug substance with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

For oral administration, pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulfate). The tablets can be coated by methods well-known in the art.

Formulations suitable for buccal (sublingual) administration include, for example, lozenges containing the active compound in a flavored base, usually sucrose

and acacia or tragacanth; and pastilles containing the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

Topical mixtures are prepared as described for the local and systemic administration. The resulting mixtures can be solutions, suspensions, emulsion or the like and are formulated as creams, gels, ointments, emulsions, solutions, elixirs, lotions, suspensions, tinctures, pastes, foams, aerosols, irrigations, sprays, suppositories, bandages, dermal patches or any other formulations suitable for topical administration.

The compositions can be formulated as aerosols for topical application, such as by inhalation (see, *e.g.*, U.S. Patent Nos. 4,044,126; 4,414,209 and 4,364,923, which describe aerosols for delivery of a steroid useful for treatment of lung diseases). These formulations, for administration to the respiratory tract, can be in the form of an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case, the particles of the formulation will typically have diameters of less than 50 microns, or less than 10 microns.

The compounds can be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Topical administration is contemplated for transdermal delivery and also for administration to the eyes or mucosa, or for inhalation therapies. Nasal solutions of the active compound alone or in combination with other pharmaceutically acceptable excipients also can be administered.

Formulations suitable for transdermal administration are provided. They can be provided in any suitable format, such as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches contain the active compound in an optionally buffered aqueous solution of, for example, 0.1 to 0.2 M concentration with respect to the active compound. Formulations suitable for transdermal administration also can be delivered by iontophoresis (see, *e.g.*, Tyle, P, (1986) *Pharmaceutical Research* 3(6):318-326) and typically take the form of an optionally buffered aqueous solution of the active compound.

Pharmaceutical compositions also can be administered by controlled release formulations and/or delivery devices (see *e.g.*, in U.S. Patent Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,916,899; 4,008,719; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,591,767; 5,639,476; 5,674,533 and 5,733,566).

**5. Dosages and Administration**

The compositions can be formulated as pharmaceutical compositions for single dosage or multiple dosage administration. The immunostimulatory bacteria can be included in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. For example, the concentration of the pharmaceutically active compound is adjusted so that an injection provides an effective amount to produce the desired pharmacological effect. The therapeutically effective concentration can be determined empirically by testing the immunostimulatory bacteria in known *in vitro* and *in vivo* systems such as by using the assays described herein or known in the art. For example, standard clinical techniques can be employed. *In vitro* assays and animal models can be employed to help identify optimal dosage ranges. The precise dose, which can be determinied empirically, can depend on the age, weight, body surface area, and condition of the patient or animal, the particular immunostimulatory bacteria administered, the route of administration, the type of disease to be treated and the seriousness of the disease.

Hence, it is understood that the precise dosage and duration of treatment is a function of the disease being treated and can be determined empirically using known testing protocols or by extrapolation from *in vivo* or *in vitro* test data. Concentrations and dosage values also can vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or use of compositions and combinations containing them. The compositions can be administered hourly, daily, weekly, monthly, yearly or once. Generally, dosage regimens are chosen to limit toxicity. It should be noted that the attending physician would know how to and when to terminate, interrupt or adjust therapy to lower dosage due to toxicity, or bone marrow,

liver or kidney or other tissue dysfunctions. Conversely, the attending physician would also know how to and when to adjust treatment to higher levels if the clinical response is not adequate (precluding toxic side effects).

The immunostimulatory bacteria are included in the composition in an amount sufficient to exert a therapeutically useful effect. For example, the amount is one that achieves a therapeutic effect in the treatment of a hyperproliferative disease or condition, such as cancer.

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, and oral solutions or suspensions, and oil water emulsions containing suitable quantities of the compounds or pharmaceutically acceptable derivatives thereof. Unit dose forms can be contained in vials, ampoules and syringes or individually packaged tablets or capsules. Unit dose forms can be administered in fractions or multiples thereof. A multiple dose form is a plurality of identical unit dosage forms packaged in a single container to be administered in segregated unit dose form. Examples of multiple dose forms include vials, bottles of tablets or capsules or bottles of pints or gallons. Hence, multiple dose form is a multiple of unit doses that are not segregated in packaging. Generally, dosage forms or compositions containing active ingredient in the range of 0.005% to 100% with the balance made up from non-toxic carrier can be prepared. Pharmaceutical compositions can be formulated in dosage forms appropriate for each route of administration.

The unit-dose parenteral preparations are packaged in an ampoule, a vial or a syringe with a needle. The volume of liquid solution or reconstituted powder preparation, containing the pharmaceutically active compound, is a function of the disease to be treated and the particular article of manufacture chosen for package. All preparations for parenteral administration must be sterile, as is known and practiced in the art.

As indicated, compositions provided herein can be formulated for any route known to those of skill in the art including, but not limited to, subcutaneous, intramuscular, intravenous, intradermal, intralesional, intraperitoneal injection, epidural, vaginal, rectal, local, otic, transdermal administration or any route of administration. Formulations suited for such routes are known to one of skill in the art. Compositions also can be administered with other biologically active agents, either sequentially, intermittently or in the same composition.

Pharmaceutical compositions can be administered by controlled release formulations and/or delivery devices (see, *e.g.*, in U.S. Pat. Nos. 3,536,809; 3,598,123; 3,630,200; 3,845,770; 3,847,770; 3,916,899; 4,008,719; 4,687,660; 4,769,027; 5,059,595; 5,073,543; 5,120,548; 5,354,556; 5,591,767; 5,639,476; 5,674,533 and 5,733,566). Various delivery systems are known and can be used to administer selected compositions, are contemplated for use herein, and such particles can be easily made.

**6. Packaging and Articles of Manufacture**

Also provided are articles of manufacture containing packaging materials, any pharmaceutical composition provided herein, and a label that indicates that the compositions are to be used for treatment of diseases or conditions as described herein. For example, the label can indicate that the treatment is for a tumor or 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 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. Pat. Nos. 5,323,907, 5,052,558 and 5,033,252, each of which is incorporated herein in its entirety. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, pumps, bags, vials, containers, syringes, bottles, and any

packaging material suitable for a selected formulation and intended mode of administration and treatment. Exemplary of articles of manufacture are containers including single chamber and dual chamber containers. The containers include, but are not limited to, tubes, bottles and syringes. The containers can further include a needle for intravenous administration.

The choice of package depends on the agents, and whether such compositions will be packaged together or separately. In general, the packaging is non-reactive with the compositions contained therein. In other examples, some of the components can be packaged as a mixture. In other examples, all components are packaged separately. Thus, for example, the components can be packaged as separate compositions that, upon mixing just prior to administration, can be directly administered together. Alternatively, the components can be packaged as separate compositions for administration separately.

Selected compositions including articles of manufacture thereof also can be provided as kits. Kits can include a pharmaceutical composition described herein and an item for administration provided as an article of manufacture. The compositions can be contained in the item for administration or can be provided separately to be added later. The kit can, optionally, include instructions for application including dosages, dosing regimens and instructions for modes of administration. Kits also can include a pharmaceutical composition described herein and an item for diagnosis.

## I. METHODS OF TREATMENT AND USES

The methods provided herein include methods of administering or using the immunostimulatory bacteria, for treating subjects having a disease or condition whose symptoms can be ameliorated or lessened by administration of such bacteria, such as cancer. In particular examples, the disease or condition is a tumor or a cancer. Additionally, methods of combination therapies with one or more additional agents for treatment, such as an anticancer 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. Formulations suitable for each are provided. The skilled person can establish suitable regimens and doses and select routes.

### 1. Cancers and Tumors

The immunostimulatory bacteria, combinations, uses and methods provided herein are applicable to treating all types of tumors, including cancers, particularly solid tumors including lung cancer, bladder, non-small cell lung cancer, gastric cancers, head and neck cancers, ovarian cancer, liver cancer, pancreatic cancer, kidney cancer, breast cancer, colorectal cancer, and prostate cancer. The methods also can be used for hematological cancers.

Tumors and cancers subject to treatment by the uses methods provided herein include, but are not limited to, those that originate in the immune system, skeletal system, muscles and heart, breast, pancreas, gastrointestinal tract, central and peripheral nervous system, renal system, reproductive system, respiratory system, skin, connective tissue systems, including joints, fatty tissues, and circulatory system, including blood vessel walls. Examples of tumors that can be treated with the immunostimulatory bacteria provided herein include carcinomas, gliomas, sarcomas (including liposarcoma), adenocarcinomas, adenosarcomas, and adenomas. Such tumors can occur in virtually all parts of the body, including, for example, breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head and neck, ovary, prostate, brain, pancreas, skin, bone, bone marrow, blood, thymus, uterus, testicles, cervix or liver.

Tumors of the skeletal system include, for example, sarcomas and blastomas such as osteosarcoma, chondrosarcoma, and chondroblastoma. Muscle and heat tumors include tumors of both skeletal and smooth muscles, *e.g.*, leiomyomas (benign tumors of smooth muscle), leiomyosarcomas, rhabdomyomas (benign tumors of skeletal muscle), rhabdomyosarcomas, cardiac sarcoma. Tumors of the gastrointestinal tract include *e.g.*, tumors of the mouth, esophagus, stomach, small intestine, colon and colorectal tumors, as well as tumors of gastrointestinal secretory organs such as salivary glands, liver, pancreas, and the biliary tract. Tumors of the central nervous system include tumors of the brain, retina, and spinal cord, and can also originate in associated connective tissue, bone, blood vessels or nervous tissue. Treatment of tumors of the peripheral nervous system are also contemplated. Tumors of the peripheral nervous system include malignant peripheral nerve sheath tumors. Tumors of the renal system include those of the kidneys, *e.g.*, renal cell carcinoma, as

well as tumors of the ureters and bladder. Tumors of the reproductive system include tumors of the cervix, uterus, ovary, prostate, testes and related secretory glands. Tumors of the immune system include both blood based and solid tumors, including lymphomas, *e.g.*, both Hodgkin's and non-Hodgkin's. Tumors of the respiratory system include tumors of the nasal passages, bronchi and lungs. Tumors of the breast include, *e.g.*, both lobular and ductal carcinoma.

Other examples of tumors that can be treated by the immunostimulatory bacteria and methods provided herein include Kaposi's sarcoma, CNS neoplasms, neuroblastomas, capillary hemangioblastomas, meningiomas and cerebral metastases, melanoma, gastrointestinal and renal carcinomas and sarcomas, rhabdomyosarcoma, glioblastoma (such as glioblastoma multiforme) and leiomyosarcoma. Examples of other cancer that can be treated as provided herein include but are not limited to lymphoma, blastoma, neuroendocrine tumors, mesothelioma, schwannoma, meningioma, melanoma, and leukemia or lymphoid malignancies. Examples of such cancers include hematologic malignancies, such as Hodgkin's lymphoma; non-Hodgkin's lymphomas (Burkitt's lymphoma, small lymphocytic lymphoma/chronic lymphocytic leukemia, mycosis fungoides, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, marginal zone lymphoma, hairy cell leukemia and lymphoplasmacytic leukemia), tumors of lymphocyte precursor cells, including B-cell acute lymphoblastic leukemia/lymphoma, and T-cell acute lymphoblastic leukemia/lymphoma, thymoma, tumors of the mature T and NK cells, including peripheral T-cell leukemias, adult T-cell leukemia/T-cell lymphomas and large granular lymphocytic leukemia, Langerhans cell histocytosis, myeloid neoplasias such as acute myelogenous leukemias, including AML with maturation, AML without differentiation, acute promyelocytic leukemia, acute myelomonocytic leukemia, and acute monocytic leukemias, myelodysplastic syndromes, and chronic myeloproliferative disorders, including chronic myelogenous leukemia; tumors of the central nervous system such as glioma, glioblastoma, neuroblastoma, astrocytoma, medulloblastoma, ependymoma, and retinoblastoma; solid tumors of the head and neck (*e.g.*, nasopharyngeal cancer, salivary gland carcinoma, and esophageal cancer), lung (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung), digestive system (*e.g.*, gastric or stomach

cancer including gastrointestinal cancer, cancer of the bile duct or biliary tract, colon cancer, rectal cancer, colorectal cancer, and anal carcinoma), reproductive system (*e.g.,* testicular, penile, or prostate cancer, uterine, vaginal, vulval, cervical, ovarian, and endometrial cancer), skin (*e.g.*, melanoma, basal cell carcinoma, squamous cell cancer, actinic keratosis, cutaneous melanoma), liver (*e.g.*, liver cancer, hepatic carcinoma, hepatocellular cancer, and hepatoma), bone (*e.g.,* osteoclastoma, and osteolytic bone cancers) additional tissues and organs (*e.g.*, pancreatic cancer, bladder cancer, kidney or renal cancer, thyroid cancer, breast cancer, cancer of the peritoneum, and Kaposi's sarcoma), tumors of the vascular system (*e.g.*, angiosarcoma and hemangiopericytoma), Wilms' tumor, retinoblastoma, osteosarcoma and Ewing's sarcoma.

**2. Administration**

In practicing the uses and methods herein, immunostimulatory bacteria provided herein can be administered to a subject, including a subject having a tumor or having neoplastic cells, or a subject to be immunized. One or more steps can be performed prior to, simultaneously with or after administration of the immunostimulatory bacteria to the subject including, but not limited to, diagnosing the subject with a condition appropriate for administering immunostimulatory bacteria, determining the immunocompetence of the subject, immunizing the subject, treating the subject with a chemotherapeutic agent, treating the subject with radiation, or surgically treating the subject.

For embodiments that include administering immunostimulatory bacteria to a tumor-bearing subject for therapeutic purposes, the subject typically has previously been diagnosed with a neoplastic condition. Diagnostic methods also can include determining the type of neoplastic condition, determining the stage of the neoplastic conditions, determining the size of one or more tumors in the subject, determining the presence or absence of metastatic or neoplastic cells in the lymph nodes of the subject, or determining the presence of metastases of the subject.

Some embodiments of therapeutic methods for administering immunostimulatory bacteria to a subject can include a step of determination of the size of the primary tumor or the stage of the neoplastic disease, and if the size of the primary tumor is equal to or above a threshold volume, or if the stage of the

neoplastic disease is at or above a threshold stage, an immunostimulatory bacterium is administered to the subject. In a similar embodiment, if the size of the primary tumor is below a threshold volume, or if the stage of the neoplastic disease is at or below a threshold stage, the immunostimulatory bacterium is not yet administered to the subject; such methods can include monitoring the subject until the tumor size or neoplastic disease stage reaches a threshold amount, and then administering the immunostimulatory bacterium to the subject. Threshold sizes can vary according to several factors, including rate of growth of the tumor, ability of the immunostimulatory bacterium to infect a tumor, and immunocompetence of the subject. Generally the threshold size will be a size sufficient for an immunostimulatory bacterium to accumulate and replicate in or near the tumor without being completely removed by the host's immune system, and will typically also be a size sufficient to sustain a bacterial infection for a time long enough for the host to mount an immune response against the tumor cells, typically about one week or more, about ten days or more, or about two weeks or more. Exemplary threshold stages are any stage beyond the lowest stage (*e.g.*, Stage I or equivalent), or any stage where the primary tumor is larger than a threshold size, or any stage where metastatic cells are detected.

Any mode of administration of a microorganism to a subject can be used, provided the mode of administration permits the immunostimulatory bacteria to enter a tumor or metastasis. Modes of administration can include, but are not limited to, intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intratumoral, multipuncture, inhalation, intranasal, oral, intracavity (*e.g.*, administering to the bladder via a catheter, administering to the gut by suppository or enema), aural, rectal, and ocular administration.

One skilled in the art can select any mode of administration compatible with the subject and the bacteria, and that also is likely to result in the bacteria reaching tumors and/or metastases. The route of administration can be selected by one skilled in the art according to any of a variety of factors, including the nature of the disease, the kind of tumor, and the particular bacteria contained in the pharmaceutical composition. Administration to the target site can be performed, for example, by

ballistic delivery, as a colloidal dispersion system, or systemic administration can be performed by injection into an artery.

The dosage regimen can be any of a variety of methods and amounts, and can be determined by one skilled in the art according to known clinical factors. A single dose can be therapeutically effective for treating a disease or disorder in which immune stimulation effects treatment. Exemplary of such stimulation is an immune response, that includes, but is not limited to, one or both of a specific immune response and non-specific immune response, both specific and non-specific responses, innate response, primary immune response, adaptive immunity, secondary immune response, memory immune response, immune cell activation, immune cell proliferation, immune cell differentiation, and cytokine expression.

As is known in the medical arts, dosages for a subject can depend on many factors, including the subject's species, size, body surface area, age, sex, immunocompetence, and general health, the particular bacteria to be administered, duration and route of administration, the kind and stage of the disease, for example, tumor size, and other compounds such as drugs being administered concurrently. In addition to the above factors, such levels can be affected by the infectivity of the bacteria and the nature of the bacteria, as can be determined by one skilled in the art. In the present methods, appropriate minimum dosage levels of bacteria can be levels sufficient for the bacteria to survive, grow and replicate in a tumor or metastasis. Exemplary minimum levels for administering a bacterium to a 65 kg human can include at least about $5 \times 10^6$ colony forming units (CFU), at least about $1 \times 10^7$ CFU, at least about $5 \times 10^7$ CFU, at least about $1 \times 10^8$ CFU, or at least about $1 \times 10^9$ CFU. In the present methods, appropriate maximum dosage levels of bacteria can be levels that are not toxic to the host, levels that do not cause splenomegaly of 3x or more, levels that do not result in colonies or plaques in normal tissues or organs after about 1 day or after about 3 days or after about 7 days. Exemplary maximum levels for administering a bacterium to a 65 kg human can include no more than about $5 \times 10^{11}$ CFU, no more than about $1 \times 10^{11}$ CFU, no more than about $5 \times 10^{10}$ CFU, no more than about $1 \times 10^{10}$ CFU, or no more than about $1 \times 10^9$ CFU.

The methods and uses provided herein can include a single administration of immunostimulatory bacteria to a subject or multiple administrations of

immunostimulatory bacteria to a subject or others of a variety of regimens, including combination therapies with other anti-tumor therapeutics and/or treatments. These include, cellular therapies, such as administration of modified immune cells, CAR-T therapy, CRISPR therapy, checkpoint inhibitors, such as antibodies, and chemotherapeutic compounds, such as nucleoside analogs, surgery and radiotherapy.

In some embodiments, a single administration is sufficient to establish immunostimulatory bacteria in a tumor, where the bacteria can colonize and can cause or enhance an anti-tumor response in the subject. In other embodiments, the immunostimulatory bacteria provided for use in the methods herein can be administered on different occasions, separated in time typically by at least one day. Separate administrations can increase the likelihood of delivering a bacterium to a tumor or metastasis, where a previous administration may have been ineffective in delivering the bacterium to a tumor or metastasis. In embodiments, separate administrations can increase the locations on a tumor or metastasis where bacterial colonization/proliferation can occur or can otherwise increase the titer of bacteria accumulated in the tumor, which can increase eliciting or enhancing a host's anti-tumor immune response.

When separate administrations are performed, each administration can be a dosage amount that is the same or different relative to other administration dosage amounts. In one embodiment, all administration dosage amounts are the same. In other embodiments, a first dosage amount can be a larger dosage amount than one or more subsequent dosage amounts, for example, at least 10x larger, at least 100x larger, or at least 1000x larger than subsequent dosage amounts. In one example of a method of separate administrations in which the first dosage amount is greater than one or more subsequent dosage amounts, all subsequent dosage amounts can be the same, smaller amount relative to the first administration.

Separate administrations can include any number of two or more administrations, including two, three, four, five or six administrations. One skilled in the art readily can determine the number of administrations to perform, or the desirability of performing one or more additional administrations, according to methods known in the art for monitoring therapeutic methods and other monitoring methods provided herein. Accordingly, the methods provided herein include methods

of providing to the subject one or more administrations of a immunostimulatory bacteria, where the number of administrations can be determined by monitoring the subject, and, based on the results of the monitoring, determining whether or not to provide one or more additional administrations. Deciding whether or not to provide one or more additional administrations can be based on a variety of monitoring results, including, but not limited to, indication of tumor growth or inhibition of tumor growth, appearance of new metastases or inhibition of metastasis, the subject's anti-bacterial antibody titer, the subject's anti-tumor antibody titer, the overall health of the subject and the weight of the subject.

The time period between administrations can be any of a variety of time periods. The time period between administrations can be a function of any of a variety of factors, including monitoring steps, as described in relation to the number of administrations, the time period for a subject to mount an immune response, the time period for a subject to clear bacteria from normal tissue, or the time period for bacterial colonization/proliferation in the tumor or metastasis. In one example, the time period can be a function of the time period for a subject to mount an immune response; for example, the time period can be more than the time period for a subject to mount an immune response, such as more than about one week, more than about ten days, more than about two weeks, or more than about a month; in another example, the time period can be less than the time period for a subject to mount an immune response, such as less than about one week, less than about ten days, less than about two weeks, or less than about a month. In another example, the time period can be a function of the time period for bacterial colonization/proliferation in the tumor or metastasis; for example, the time period can be more than the amount of time for a detectable signal to arise in a tumor or metastasis after administration of a microorganism expressing a detectable marker, such as about 3 days, about 5 days, about a week, about ten days, about two weeks, or about a month.

The methods used herein also can be performed by administering compositions, such as suspensions and other formulations, containing the immunostimulatory bacteria provided herein. Such compositions contain the bacteria and a pharmaceutically acceptable excipient or vehicle, as provided herein or known to those of skill in the art.

As discussed above, the uses and methods provided herein also can include administering one or more therapeutic compounds, such as anti-tumor compounds or other cancer therapeutics, to a subject in addition to administering immunostimulatory bacteria to the subject. The therapeutic compounds can act independently, or in conjunction with the immunostimulatory bacteria, for tumor therapeutic effects. Therapeutic compounds that can act independently include any of a variety of known chemotherapeutic compounds that can inhibit tumor growth, inhibit metastasis growth and/or formation, decrease the size of a tumor or metastasis, eliminate a tumor or metastasis, without reducing the ability of the immunostimulatory bacteria to accumulate in a tumor, replicate in the tumor, and cause or enhance an anti-tumor immune response in the subject. Examples of such chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; antibiotics such as aclacinomycins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carubicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY 117018, onapristone, and toremifene (Fareston); anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; aziridines such as benzodepa, carboquone, meturedepa, and uredepa; ethylenimines and methylmelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylol melamine; folic acid replenisher such as folinic acid; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide

hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; proteins such as arginine deiminase and asparaginase; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; taxanes, such as paclitaxel and docetaxel and albuminated forms thereof (*i.e.*, *nab*-paclitaxel and *nab*-docetaxel), topoisomerase inhibitor RFS 2000; thymidylate synthase inhibitor (such as Tomudex); additional chemotherapeutics including aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatrexate; defosfamide; demecolcine; diaziquone; difluoromethylornithine (DMFO); eflornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2', 2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; Navelbine; Novantrone; teniposide; daunomycin; aminopterin; Xeloda; ibandronate; CPT-11; retinoic acid; esperamycins; capecitabine; and topoisomerase inhibitors such as irinotecan. Pharmaceutically acceptable salts, acids or derivatives of any of the above can also be used.

Therapeutic compounds that act in conjunction with the immunostimulatory bacteria include, for example, compounds that increase the immune response eliciting properties of the bacteria, *e.g.*, by increasing expression of the RNAi, such as shRNA and miRNA, that inhibit, suppress or disrupt expression of the checkpoint genes, such as PD-L1, or TREX1 or other checkpoint genes, or compounds that can further augment bacterial colonization/proliferation. For example, a gene expression-altering compound can induce or increase transcription of a gene in a bacterium, such as an exogenous gene, *e.g.*, encoding shRNA that inhibit, suppress or disrupt expression of

one or more checkpoint genes, thereby provoking an immune response. Any of a wide variety of compounds that can alter gene expression are known in the art, including IPTG and RU486. Exemplary genes whose expression can be up-regulated include proteins and RNA molecules, including toxins, enzymes that can convert a prodrug to an anti-tumor drug, cytokines, transcription regulating proteins, shRNA, siRNA, and ribozymes. In other embodiments, therapeutic compounds that can act in conjunction with the immunostimulatory bacteria to increase the colonization/proliferation or immune response eliciting properties of the bacteria are compounds that can interact with a bacteria-expressed gene product, and such interaction can result in an increased killing of tumor cells or an increased anti-tumor immune response in the subject. A therapeutic compound that can interact with a bacteria-expressed gene product can include, for example a prodrug or other compound that has little or no toxicity or other biological activity in its subject-administered form, but after interaction with a bacteria-expressed gene product, the compound can develop a property that results in tumor cell death, including but not limited to, cytotoxicity, ability to induce apoptosis, or ability to trigger an immune response. A variety of prodrug-like substances are known in the art, including ganciclovir, 5-fluorouracil, 6-methylpurine deoxyriboside, cephalosporin-doxorubicin, 4-[(2-chloroethyl)(2-mesuloxyethyl)amino]benzoyl-L-glutamic acid, acetominophen, indole-3-acetic acid, CB1954, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycampotothecin, bis-(2-chloroethyl)amino-4-hydroxyphenylaminomethanone 28, 1-chloromethyl-5-hydroxy-1,2-dihyro-3H-benz[e]indole, epirubicin-glucoronide, 5'-deoxy5-fluorouridine, cytosine arabinoside, and linamarin.

### 3. Monitoring

The methods provided herein can further include one or more steps of monitoring the subject, monitoring the tumor, and/or monitoring the immunostimulatory bacteria administered to the subject. Any of a variety of monitoring steps can be included in the methods provided herein, including, but not limited to, monitoring tumor size, monitoring the presence and/or size of metastases, monitoring the subject's lymph nodes, monitoring the subject's weight or other health indicators including blood or urine markers, monitoring anti-bacterial antibody titer,

monitoring bacterial expression of a detectable gene product, and directly monitoring bacterial titer in a tumor, tissue or organ of a subject.

The purpose of the monitoring can be simply for assessing the health state of the subject or the progress of therapeutic treatment of the subject, or can be for determining whether or not further administration of the same or a different immunostimulatory bacterium is warranted, or for determining when or whether or not to administer a compound to the subject where the compound can act to increase the efficacy of the therapeutic method, or the compound can act to decrease the pathogenicity of the bacteria administered to the subject.

In some embodiments, the methods provided herein can include monitoring one or more bacterially expressed genes. Bacteria, such as those provided herein or otherwise known in the art, can express one or more detectable gene products, including but not limited to, detectable proteins.

As provided herein, measurement of a detectable gene product expressed in a bacterium can provide an accurate determination of the level of bacteria present in the subject. As further provided herein, measurement of the location of the detectable gene product, for example, by imaging methods including tomographic methods, can determine the localization of the bacteria in the subject. Accordingly, the methods provided herein that include monitoring a detectable bacterial gene product can be used to determine the presence or absence of the bacteria in one or more organs or tissues of a subject, and/or the presence or absence of the bacteria in a tumor or metastases of a subject. Further, the methods provided herein that include monitoring a detectable bacterial gene product can be used to determine the titer of bacteria present in one or more organs, tissues, tumors or metastases. Methods that include monitoring the localization and/or titer of bacteria in a subject can be used for determining the pathogenicity of bacteria since bacterial infection, and particularly the level of infection, of normal tissues and organs can indicate the pathogenicity of the bacteria. The methods that include monitoring the localization and/or titer of immunostimulatory bacteria in a subject can be performed at multiple time points and, accordingly, can determine the rate of bacterial replication in a subject, including the rate of bacterial replication in one or more organs or tissues of a subject; accordingly, methods that include monitoring a bacterial gene product can be used for

determining the replication competence of the bacteria. The methods provided herein also can be used to quantitate the amount of immunostimulatory bacteria present in a variety of organs or tissues, and tumors or metastases, and can thereby indicate the degree of preferential accumulation of the bacteria in a subject; accordingly, the bacterial gene product monitoring can be used in methods of determining the ability of the bacteria to accumulate in tumor or metastases in preference to normal tissues or organs. Since the immunostimulatory bacteria used in the methods provided herein can accumulate in an entire tumor or can accumulate at multiple sites in a tumor, and can also accumulate in metastases, the methods provided herein for monitoring a bacterial gene product can be used to determine the size of a tumor or the number of metastases present in a subject. Monitoring such presence of bacterial gene product in tumor or metastasis over a range of time can be used to assess changes in the tumor or metastases, including growth or shrinking of a tumor, or development of new metastases or disappearance of metastases, and also can be used to determine the rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases, or the change in the rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases. Accordingly, monitoring a bacterial gene product can be used for monitoring a neoplastic disease in a subject, or for determining the efficacy of treatment of a neoplastic disease, by determining rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases, or the change in the rate of growth or shrinking of a tumor, or development of new metastases or disappearance of metastases.

Any of a variety of detectable proteins can be detected by monitoring, exemplary of which are any of a variety of fluorescence proteins (*e.g.*, green fluorescence proteins), any of a variety of luciferases, transferring or other iron binding proteins; or receptors, binding proteins, and antibodies, where a compound that specifically binds the receptor, binding protein or antibody can be a detectable agent or can be labeled with a detectable substance (*e.g.*, a radionuclide or imaging agent).

Tumor and/or metastasis size can be monitored by any of a variety of methods known in the art, including external assessment methods or tomographic or magnetic

imaging methods. In addition to the methods known in the art, methods provided herein, for example, monitoring bacterial gene expression, can be used for monitoring tumor and/or metastasis size.

Monitoring size over several time points can provide information regarding the increase or decrease in size of a tumor or metastasis, and can also provide information regarding the presence of additional tumors and/or metastases in the subject. Monitoring tumor size over several time points can provide information regarding the development of a neoplastic disease in a subject, including the efficacy of treatment of a neoplastic disease in a subject.

The methods provided herein also can include monitoring the antibody titer in a subject, including antibodies produced in response to administration of immunostimulatory bacteria to a subject. The bacteria administered in the methods provided herein can elicit an immune response to endogenous bacterial antigens. The bacteria administered in the methods provided herein also can elicit an immune response to exogenous genes expressed by the bacteria. The bacteria administered in the methods provided herein also can elicit an immune response to tumor antigens. Monitoring antibody titer against bacterial antigens, bacterially expressed exogenous gene products, or tumor antigens can be used to monitor the toxicity of the bacteria, monitoring the efficacy of treatment methods, or monitoring the level of gene product or antibodies for production and/or harvesting.

Monitoring antibody titer can be used to monitor the toxicity of the bacteria. Antibody titer against a bacteria can vary over the time period after administration of the bacteria to the subject, where at some particular time points, a low anti-(bacterial antigen) antibody titer can indicate a higher toxicity, while at other time points a high anti-(bacterial antigen) antibody titer can indicate a higher toxicity. The bacteria used in the methods provided herein can be immunogenic, and can, therefore, elicit an immune response soon after administering the bacteria to the subject. Generally, immunostimulatory bacteria against which the immune system of a subject can mount a strong immune response can be bacteria that have low toxicity when the subject's immune system can remove the bacteria from all normal organs or tissues. Thus, in some embodiments, a high antibody titer against bacterial antigens soon after administering the bacteria to a subject can indicate low toxicity of the bacteria.

In other embodiments, monitoring antibody titer can be used to monitor the efficacy of treatment methods. In the methods provided herein, antibody titer, such as anti-(tumor antigen) antibody titer, can indicate the efficacy of a therapeutic method such as a therapeutic method to treat neoplastic disease. Therapeutic methods provided herein can include causing or enhancing an immune response against a tumor and/or metastasis. Thus, by monitoring the anti-(tumor antigen) antibody titer, it is possible to monitor the efficacy of a therapeutic method in causing or enhancing an immune response against a tumor and/or metastasis.

In other embodiments, monitoring antibody titer can be used for monitoring the level of gene product or antibodies for production and/or harvesting. As provided herein, methods can be used for producing proteins, RNA molecules or other compounds, particularly RNA molecules such as shRNA, by expressing an exogenous gene in a microorganism that has accumulated in a tumor. Monitoring antibody titer against the protein, RNA molecule or other compound can indicate the level of production of the protein, RNA molecule or other compound by the tumor-accumulated microorganism, and also can directly indicate the level of antibodies specific for such a protein, RNA molecule or other compound.

The methods provided herein also can include methods of monitoring the health of a subject. Some of the methods provided herein are therapeutic methods, including neoplastic disease therapeutic methods. Monitoring the health of a subject can be used to determine the efficacy of the therapeutic method, as is known in the art. The methods provided herein also can include a step of administering to a subject an immunostimulatory bacterium, as provided herein. Monitoring the health of a subject can be used to determine the pathogenicity of an immunostimulatory bacterium administered to a subject. Any of a variety of health diagnostic methods for monitoring disease such as neoplastic disease, infectious disease, or immune-related disease can be monitored, as is known in the art. For example, the weight, blood pressure, pulse, breathing, color, temperature or other observable state of a subject can indicate the health of a subject. In addition, the presence or absence or level of one or more components in a sample from a subject can indicate the health of a subject. Typical samples can include blood and urine samples, where the presence or absence or level of one or more components can be determined by performing, for example, a

blood panel or a urine panel diagnostic test. Exemplary components indicative of a subject's health include, but are not limited to, white blood cell count, hematocrit, and c-reactive protein concentration.

The methods provided herein can include monitoring a therapy, where therapeutic decisions can be based on the results of the monitoring. Therapeutic methods provided herein can include administering to a subject immunostimulatory bacteria, where the bacteria can preferentially accumulate in a tumor and/or metastasis, and where the bacteria can cause or enhance an anti-tumor immune response. Such therapeutic methods can include a variety of steps including multiple administrations of a particular immunostimulatory bacterium, administration of a second immunostimulatory bacterium, or administration of a therapeutic compound. Determination of the amount, timing or type of immunostimulatory bacteria or compound to administer to the subject can be based on one or more results from monitoring the subject. For example, the antibody titer in a subject can be used to determine whether or not it is desirable to administer an immunostimulatory bacterium and, optionally, a compound, the quantity of bacteria and/or compound to administer, and the type of bacteria and/or compound to administer, where, for example, a low antibody titer can indicate the desirability of administering an additional immunostimulatory bacterium, a different immunostimulatory bacterium, and/or a therapeutic compound such as a compound that induces bacterial gene expression or a therapeutic compound that is effective independent of the immunostimulatory bacteria.

In another example, the overall health state of a subject can be used to determine whether or not it is desirable to administer an immunostimulatory bacterium and, optionally, a compound, the quantity of bacterium or compound to administer, and the type of bacterium and/or compound to administer where, for example, determining that the subject is healthy can indicate the desirability of administering additional bacteria, different bacteria, or a therapeutic compound such as a compound that induces bacterial gene (*e.g.*, shRNA that inhibits one or more checkpoint gene(s)) expression. In another example, monitoring a detectable bacterially expressed gene product can be used to determine whether it is desirable to administer an immunostimulatory bacterium and, optionally, a compound, the

quantity of bacterium and/or compound to administer, and the type of bacterium and/or compound to administer where, for example, determining that the subject is healthy can indicate the desirability of administering additional bacteria, different bacteria, or a therapeutic compound such as a compound that induces bacterial gene (*e.g.*, shRNA that inhibits one or more checkpoint gene(s)) expression. Such monitoring methods can be used to determine whether or not the therapeutic method is effective, whether or not the therapeutic method is pathogenic to the subject, whether or not the bacteria have accumulated in a tumor or metastasis, and whether or not the bacteria have accumulated in normal tissues or organs. Based on such determinations, the desirability and form of further therapeutic methods can be derived.

In another example, monitoring can determine whether or not immunostimulatory bacteria have accumulated in a tumor or metastasis of a subject. Upon such a determination, a decision can be made to further administer additional bacteria, a different immunostimulatory bacterium and, optionally, a compound to the subject.

## J. EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

**Summary of exemplary engineered immunostimulatory bacterial strains and nomenclature:**

| Strain # | Plasmid | Strain Background | RNAi Targets | Alternate name |
|---|---|---|---|---|
| AST-100 | None | YS1646 | none | VNP 20009 |
| AST-101 | None | YS1646-ASD | none | ASD (*asd* gene knockout) |
| AST-102 | pEQU6 | YS1646 | none | YS1646 (pEQU6 – plasmid) |
| AST-103 | pEQU6 | YS1646 | Scrambled (shRNA) | YS1646 (pEQU6-shSCR) |
| AST-104 | pEQU6 | YS1646 | muTREX1 (shRNA) ARI-108 | YS1646 (pEQU6-shTREX1) |
| AST-105 | pEQU6 | YS1646 | muPD-L1 (shRNA) ARI-115 | YS1646 (pEQU6-shPDL1) |
| AST-106 | pEQU6 | YS1646 | muTREX1 (microRNA) ARI-203 | YS1646 (pEQU6-miTREX1) |
| AST-107 | pATI-U6 | YS1646-ASD | Scrambled (shRNA) | ASD (pATI-shSCR) |

| Strain # | Plasmid | Strain Background | RNAi Targets | Alternate name |
|---|---|---|---|---|
| AST-108 | pATI-U6 | YS1646-ASD | muTREX1 (shRNA) ARI-108 | ASD (pATI-shTREX1) |
| AST-109 | pATIKAN-U6 | YS1646-ASD | Scrambled (shRNA) | ASD (pATIKan-shSCR) |
| AST-110 | pATIKAN-U6 | YS1646-ASD | muTREX1 (shRNA) ARI-108 | ASD (pATIKan-shTREX1) |
| AST-111 | None | YS1646-ASD-fljb-fliC | None | ASD/FLG (*asd* and flagellin knockout) |
| AST-112 | pATI-U6 | YS1646-ASD-fljb-fliC | muTREX1 (shRNA) ARI-108 | ASD/FLG (pATI-shTREX1) |
| AST-113 | pATI-U6 | YS1646-ASD-fljb-fliC | muTREX1 (shRNA) ARI-108 | ASD/FLG (pATI-U6 Kan shTREX1) |
| AST-114 | None | YS1646-ASD-LLO | None | ASD/LLO (*asd* knockout / cytoLLO knock-in) |
| AST-115 | pATI-U6 | YS1646-ASD-LLO | muTREX1 (shRNA) ARI-108 | ASD/LLO (pATIKan-shTREX1) |
| AST-116 | pATIKanpBRori-U6 | YS1646-ASD | Scrambled | ASD (pATIKanLow-shSCR) |
| AST-117 | pATIKanpBRori-U6 | YS1646-ASD | muTREX1 (shRNA) ARI-108 | ASD (pATIKanLow-shTREX1) |
| AST-118 | pATIKanpBRori-U6 | YS1646-ASD-fljb-fliC | muTREX1 (shRNA) ARI-108 | ASD/FLG (pATIKanLow-shTREX1) |
| AST-119 | pATIKanpBRori-U6 | YS1646-ASD-pMTL-LLO | muTREX1 (shRNA) ARI-108 | ASD/LLO (pATIKanLow-shTREX1) |
| AST-120 | pEQU6 | YS1646-ASD-pMTL-LLO | muTREX1 (microRNA) ARI-203 | ASD/LLO(pEQU6-miTREX1) Suicidal |
| AST-121 | pEQU6 | YS1646 | muVISTA ARI-157 | YS1646 (pEQU6-shVISTA) |
| AST-122 | pEQU6 | YS1646 | muTGF-beta ARI-149 | YS1646 (pEQU6-TGF-beta) |
| AST-123 | pEQU6 | YS1645 | muBeta-Catenin ARI-166 | YS1646 (pEQU6-Beta-Catenin) |

## Example 1

### Salmonella *asd* Gene Knockout Strain Engineering

Strain AST-101was prepared. It is an attenuated *Salmonella typhimurium* derived from YS1646 (which can be purchased from ATCC, Catalog # 202165) that has been engineered to be *asd*$^-$ (an *asd* gene knockout). In this example, the *Salmonella typhimurium* strain YS1646 *asd*$^-$ gene deletion was engineered using

modifications of the method of Datsenko and Wanner (*Proc Natl Acad Sci USA 97*:6640–6645 (2000)) as outlined in **Fig. 1**, and described below.

**Introduction of the Lambda Red helper plasmid into YS1646**

The YS1646 strain was prepared to be electrocompetent as described previously (Sambrook J., (1998), *Molecular Cloning, A Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory*) by growing a culture in LB and concentrating 100-fold and washing three times with ice-cold 10% glycerol. The electrocompetent strain was electroporated with the Lambda red helper plasmid pKD46 (SEQ ID NO:218 ) using a 0.2 cm gap cuvette at the following settings: 2.5 kV, 186 ohms, 50 μF. Transformants carrying pKD46 were grown in 5 mL SOC medium with ampicillin and 1mM L-arabinose at 30 °C and selected on LB agar plates containing ampicillin. A YS1646 clone containing the lambda red helper plasmid pKD46 then was made electrocompetent, as described above for YS1646.

**Construction of *asd* gene knockout cassette**

The *asd* gene from the genome of YS1646 (Broadway *et al.* (2014) J. *Biotechnology* 192:177-178) was used for designing the *asd* gene knockout cassette. A plasmid containing 204 and 203 bp of homology to the left hand and right hand regions, respectively, of the *asd* gene, was transformed into DH5-alpha competent cells. A kanamycin gene cassette flanked by lox P sites was cloned into this plasmid. The *asd* gene knockout cassette then was PCR amplified using primers asd-1 and asd-2 (**Table 1**) and gel purified.

**Execution of *asd* gene deletion**

The YS1646 strain carrying plasmid pKD46 was electroporated with the gel-purified linear *asd* gene knock-out cassette. Electroporated cells were recovered in SOC medium and plated onto LB Agar plates supplemented with Kanamycin (20 μg/mL) and diaminopimelic acid (DAP, 50 μg/ml). During this step, lambda red recombinase induces homologous recombination of the chromosomal *asd* gene with the *kan* cassette (due to the presence of homologous flanking sequences upstream and downstream of the chromosomal *asd* gene), and knockout of the chromosomal copy of the *asd* gene occurs. The presence of the disrupted *asd* gene in the selected kanamycin resistant clones was confirmed by PCR amplification with primers from the YS1646 genome flanking the sites of disruption (primer asd-3) and from the

multi-cloning site (primer scFv-3) (**Table 1**). Colonies were also replica plated onto LB plates with and without supplemental DAP to demonstrate DAP auxotrophy. All clones with the *asd* gene deletion were unable to grow in the absence of supplemental DAP, demonstrating DAP auxotrophy.

**Table 1. Primer information**

| Primer name | Primer sequence | SEQ ID NO. |
|---|---|---|
| asd-1 | ccttcctaacgcaaattccctg | 219 |
| asd-2 | ccaatgctctgcttaactcctg | 220 |
| asd-3 | gcctcgccatgtttcagtacg | 221 |
| asd-4 | ggtctggtgcattccgagtac | 222 |
| scFv-3 | cataatctgggtccttggtctgc | 223 |

**Kanamycin gene cassette removal**

The *kan* selectable marker was removed by using the Cre/loxP site-specific recombination system. The YS1646 *asd*⁻ gene $Kan^R$ mutant was transformed with pJW168 (a temperature sensitive plasmid expressing the cre recombinase, SEQ ID NO:224). $Amp^R$ colonies were selected at 30 °C; pJW168 was subsequently eliminated by growth at 42 °C. A selected clone (AST-101) then was tested for loss of *kan* by replica plating on LB agar plates with and without kanamycin, and confirmed by PCR verification using primers from YS1646 genome flanking the sites of disruption (primer asd-3 and asd-4, for primer sequence, see **Table 1**).

**Characterization of the *asd* deletion mutant strain AST-101**

The *asd* mutant AST-101 was unable to grow on LB agar plates at 37 °C, but was able to grow on LB plates containing 50 µg/mL diaminopimelic acid (DAP). The *asd* mutant growth rate was evaluated in LB liquid media and it was unable to grow in liquid LB but was able to grow in LB supplemented with 50 µg/mL DAP, as determined by measuring absorbance at 600 nM.

**Sequence confirmation of the AST-101 *asd* locus sequence after *asd* gene deletion**

The AST-101 *asd* gene deletion strain was verified by DNA sequencing using primer asd-3 and asd-4. Sequencing of the region flanking the *asd* locus was performed and the sequence confirmed that the *asd* gene was deleted from the YS1646 chromosome.

## Example 2

### Design and Characterization of Exemplary shRNAs

In order to generate recombinant *Salmonella typhimurium* transformed with plasmids encoding shRNAs against desired target genes, a set of 6 shRNAs were designed against each of human PD-L1, SIRP-alpha, beta-catenin, VISTA, TREX1, and VEGF. A total of 9 shRNAs were designed against human TGF-beta isoform 1. The shRNAs were subcloned into the pEQU6 vector (SEQ ID NO:41), for a total of 45 shRNAs.

**Proteins targeted by shRNA**

| SEQ ID NO. | Protein |
|---|---|
| 31 | Human PD-L1 |
| 32 | Human CTNNB1 |
| 33 | Human SIRP-alpha |
| 34 | Human TREX1 |
| 35 | Human VISTA |
| 193 | Human TGF-beta, isoform 1 |
| 194 | Human VEGF |

The target sequences in each gene are as follows:

| SEQ ID NO. | Target | Target Sequence | Reference |
|---|---|---|---|
| 1 | Human PD-L1 | gtagagtatggtagcaata | ARI-122 |
| 2 | Human PD-L1 | gccgactacaagcgaatta | ARI-123 |
| 3 | Human PD-L1 | gacaagcagtgaccatcaa | ARI-124 |
| 4 | Human PD-L1 | gaatcaacacaacaactaa | ARI-125 |
| 5 | Human PD-L1 | gcacatcctccaaatgaaa | ARI-126 |
| 6 | Human PD-L1 | gtagcactgacattcatct | ARI-127 |
| 7 | Human CTNNB1 | gacagactgccttcaaatt | ARI-168 |
| 8 | Human CTNNB1 | gcagctggaattctttcta | ARI-169 |
| 9 | Human CTNNB1 | gactaccagttgtggttaa | ARI-170 |
| 10 | Human CTNNB1 | ggacacagcagcaatttgt | ARI-171 |
| 11 | Human CTNNB1 | ggatgttcacaaccgaatt | ARI-172 |
| 12 | Human CTNNB1 | gccacaagattacaagaaa | ARI-173 |
| 13 | Human SIRP-alpha | gccaggtgaggaagttcta | ARI-174 |
| 14 | Human SIRP-alpha | gagctggctcctggtgaat | ARI-175 |
| 15 | Human SIRP-alpha | gctgagaacactggatcta | ARI-176 |
| 16 | Human SIRP-alpha | gaagaatgccagagaaata | ARI-177 |
| 17 | Human SIRP-alpha | ggacacaaatgatatcaca | ARI-178 |
| 18 | Human SIRP-alpha | ggagtatgccagcattcag | ARI-179 |

| SEQ ID NO. | Target | Target Sequence | Reference |
|---|---|---|---|
| 19 | Human TREX1 | gcagcgcatgggcgtcaat | ARI-109 |
| 20 | Human TREX1 | ggcccaaggaagagctata | ARI-110 |
| 21 | Human TREX1 | gcaccatcaggcccatgta | ARI-111 |
| 22 | Human TREX1 | gccacaaccaggaacacta | ARI-112 |
| 23 | Human TREX1 | gcaggggtaccaaggatct | ARI-113 |
| 24 | Human TREX1 | gccacactgtatggactat | ARI-114 |
| 25 | Human VISTA | gatgtgaccttctacaaga | ARI-195 |
| 26 | Human VISTA | gaccaccatggcaacttct | ARI-196 |
| 27 | Human VISTA | ggtgcagacaggcaaagat | ARI-197 |
| 28 | Human VISTA | gtgcctgcatcgtaggaat | ARI-198 |
| 29 | Human VISTA | gcaacattcaagggattga | ARI-199 |
| 30 | Human VISTA | gtccctgactctccaaact | ARI-200 |
| 195 | Human TGF-beta isoform 1 | gaaacccacaacgaaatct | ARI-180 |
| 196 | Human TGF-beta isoform 1 | gtacacacagcatatatat | ARI-181 |
| 197 | Human TGF-beta isoform 1 | ctgctgaggctcaagttaa | ARI-182 |
| 198 | Human TGF-beta isoform 1 | gtggagctgtaccagaaat | ARI-183 |
| 199 | Human TGF-beta isoform 1 | gactcgccagagtggttat | ARI-184 |
| 200 | Human TGF-beta isoform 1 | gagccgtggaggggaaatt | ARI-185 |
| 201 | Human TGF-beta isoform 1 | cctgtgacagcagggataa | ARI-186 |
| 202 | Human TGF-beta isoform 1 | gccctggacaccaactatt | ARI-187 |
| 203 | Human TGF-beta isoform 1 | ccctgtacaaccagcataa | ARI-188 |
| 204 | Human VEGF | gagatcgagtacatcttca | ARI-189 |
| 205 | Human VEGF | gcagattatgcggatcaaa | ARI-190 |
| 206 | Human VEGF | gatagagcaagacaagaaa | ARI-191 |
| 207 | Human VEGF | ggagaaagcatttgtttgt | ARI-192 |
| 208 | Human VEGF | gatccgcagacgtgtaaat | ARI-193 |
| 209 | Human VEGF | gcgaggcagcttgagttaa | ARI-194 |

To generate each shRNA, a pair of designed oligonucleotides was synthesized to form a cassette encoding the shRNA. The oligonucleotides were allowed to anneal to each other to form the cassette and ligated to linearized pEQU6 vector that was predigested with the restriction enzymes Spe1 and Xho1. The linked DNA fragments were transformed into *E. coli* cells and the positive clones were selected with restriction enzyme digestion. The shRNA sequences were purified and sequenced. Six sequences for RNA interference were selected from different cDNA-coding regions and analyzed by a BLAST search to ensure that they did not have significant sequence homology with other genes. The six exemplary shRNA encoding sequences are as follows:

| SEQ ID NO | Target Protein | shRNA-encoding Sequence |
|---|---|---|
| 36 | Human PD-L1 | gtagagta tggtagcaat atctagagta ttgctaccat actctac |
| 37 | Human CTNNB1 | g acagactgcc ttcaaatttc tagagaattt gaaggcagtc tgtc |
| 38 | Human SIRP-alpha | g ccaggtgagg aagttctatc tagagtagaa cttcctcacc tggc |
| 39 | Human TREX1 | g cagcgcatgg gcgtcaattc tagagattga cgcccatgcg ctgc |
| 40 | Human VISTA | g accaccatgg caacttcttc tagagagaag ttgccatggt ggtc |

The sequences of the resulting vectors, designated pEQU6-shPDL1-shRNA, pEQU6-shPDL1-H1-shCTNNB1, pEQU6-shPDL1-H1-shSIRP-alpha, pEQU6-shPDL1-H1-shTREX1, and pEQU6-shPDL1-H1-shVISTA, are set forth in SEQ ID NOs: 43-47. Each shRNA then is individually screened to identify the best shRNA against each target protein. The plasmid used for screening contains a bacterial origin of replication, a kanamycin resistance marker, and a human U6 promoter sequence, followed by the individual shRNA, which then is followed by a terminator poly-T sequence. The vector can employ an H1 promoter instead of a U6 promoter. U6 and H1 are RNA polymerase III promoters, which generally are used for production and processing of small RNAs (see, Sequence Listing). Each shRNA was designed to hybridize with a 19 nucleotide overlap to the target sequence, and contains a 7 nucleotide loop-spacer, followed by the reverse complement of the initial target sequence. The shRNA designs are not limited to these nucleotide lengths. Complementary shRNA sequences range from 19-29 nucleotides (the "sense" sequence derived from the target gene), followed by a loop spacer of 4-15 nucleotides, and then completed with a 19-29 nucleotide sequence, which is the "antisense" sequence of the primary target sequence.

A second vector was used to achieve knockdown of gene expression for separate targets. This vector uses a second promoter, H1, which is separated by a length of at least 75 nucleotides, which can be from about 60-100, from the U6 promoter, in order to achieve effective gene knockdown by both target shRNAs. As an example, one particular vector carries shRNA sequences to PD-L1 and SIRP-alpha, with the anti-PD-L1 shRNA under the U6 promoter, followed by an anti-SIRP-alpha shRNA under an H1 promoter. Multiple targeting shRNAs can be added to a plasmid by utilizing additional promoters, such as U6 or H1 promoters from orthologous species.

In order to identify the top performing shRNAs against each target, individual shRNAs subcloned into pEQU6 were tested for their ability to knockdown gene expression. First, HEK293 cells are co-transfected with both the pEQU6 plasmid (encoding a distinct shRNA sequence) and a cDNA expression plasmid (expressing target protein cDNA under a CMV promoter). For example, the pEQU6 plasmid encoding shRNA to PD-L1, clone 1, is co-transfected with a PD-L1 cDNA expressing

plasmid. shRNA-mediated knockdown of gene expression is measured by Western blot and qPCR. Commercially available cDNAs are available from GE/Dharmacon or Origene, and are subcloned into a CMV expression vector that results in a fused HA tag to the C-terminus of the target protein. This allows for uniform measurement of gene knockdown using an anti-HA antibody-HRP fusion. The cDNA molecules correspond to portions of the cDNA encoding genes.

In addition to shRNAs targeting human genes, shRNAs for use for testing in *in vivo* models are provided. shRNAs are generated that target orthologous murine genes, in order to test in syngeneic murine transplant and autochthonous murine tumor models. Murine targeting shRNA sequences (SIGMA) are subcloned into the pEQU6 vector described above and characterized for gene knockdown propensity by Western blot and qPCR. Furthermore, a combination of shRNAs against PD-L1 and TREX1 were subcloned into pEQU6-H1 (SEQ ID NO:42), with the shRNA against PD-L1 under the U6 promoter and the shRNA against TREX1 under the H1 promoter. For use in the mouse models the following shRNA-encoding sequences were designed:

| SEQ ID NO. | Target (mouse) | shRNA encoding sequence (SIGMA) | Reference |
|---|---|---|---|
| 75 | muPD-L1 | ccggccgaaatgatacacaattcgactcgagtcgaattgtgtatcatttcggttttg | ARI-115 |
| 76 | muSIRP-alpha | ccggccacaactggaatgtcttcatctcgagatgaagacattccagttgtggttttt | ARI-138 |
| 77 | muTREX1-clone1 | ccggacaaccaacctaaggccacatctcgagatgtggccttaggttggttgttttttg | ARI-101 |
| 78 | muTREX1-clone2 | ccggcctagatggtaccttctgtgtctcgagacacagaaggtaccatctaggttttttg | ARI-102 |

For screening individual shRNA performance against each target, the positive control for Western blot corresponds to beta-tubulin expression, and the negative control for both Western blot and qPCR screening corresponds to a scrambled shRNA that lacks homology to any mammalian sequences. Each shRNA is individually tested by western blot. For qPCR gene expression, knockdown is quantified as % gene knockdown, and triplicate testing with error bars is generated.

Western blot screening was performed as follows. First, the co-transfection experiment was setup with the target gene expression plasmid (pCMV-cDNA-HA) and each of 6 designed shRNA vectors, as individual reactions, using Lipofectamine 2000 (Invitrogen). The chart below describes the component of each reaction. 48

hours after transfection, cells were lysed in SDS-PAGE buffer and subjected to 4-20% SDS-PAGE gel electrophoresis and Western blot analyses. The Western blot was carried out using the anti-HA-antibody purchased from Santa Cruz Biotechnology at a 1:1000 dilution. The membranes were detected by ECL reagents. For each 6-well:

| | 293 cells | cDNA | shRNA 1 | shRNA 2 | shRNA 3 | shRNA 4 | shRNA 5 | shRNA6 |
|---|---|---|---|---|---|---|---|---|
| DNA | | 1.0 µg | 1.0 µg | 1.0 µg | 1.0 µg | 1.0 µg | 1.0 µg | 1.0 µg |
| pEQ-shRNA | | | 2.0 µg | 2.0 µg | 2.0 µg | 2.0 µg | 2.0 µg | 2.0 µg |
| pEQ-scramble-shRNA | 3.0 µg | 2.0 µg | | | | | | |
| Total DNA | 3.0 µg | 3.0 µg | 3.0 µg | 3.0 µg | 3.0 µg | 3.0 µg | 3.0 µg | 3.0 µg |

The gene silencing assessment by qPCR was performed as follows. First, the co-transfection experiment was setup with the target gene expression plasmid pCMV-cDNA-HA and 6 shRNA vectors using Lipofectamine™ 2000 (Invitrogen). The chart below describes the component of each reaction. The cDNA to shRNA ratio is 1:6. 48 hours after transfection, RNA was extracted using the RNeasy Plus kit (Qiagen). cDNA was synthesized from mRNA using oligo(dT)$_{20}$ primer, SuperScript™ IV reverse transcriptase (ThermoFisher) and 100 ng of total RNA. The real time PCR assay was performed with PowerUP™ SYBR™ master mix (ThermoFisher) on an Applied Biosystems StepOne™ Real-Time PCR System against cDNA-HA and GAPDH (endogenous control) targets. For each 6-well:

| | 293 cells | cDNA | shRNA 1 | shRNA 2 | shRNA 3 | shRNA 4 | shRNA 5 | shRNA6 |
|---|---|---|---|---|---|---|---|---|
| cDNA | | 0.2 µg | 0.2 µg | 0.2 µg | 0.2 µg | 0.2 µg | 0.2 µg | 0.2 µg |
| pEQ-shRNA | | | 1.2 µg | 1.2 µg | 1.2 µg | 1.2 µg | 1.2 µg | 1.2 µg |
| pEQ-plasmid control | 1.2 µg | 1.2 µg | | | | | | |
| Total DNA | 1.2 µg | 2.2 µg | 2.2 µg | 2.2 µg | 2.2 µg | 2.2 µg | 2.2 µg | 2.2 µg |

The shRNA-mediated gene knockdown with these shRNAs were functionally characterized. See, *Methods Mol Biol.* (2010) 629:141–158 for a description of the methods used. Using the human PD-L1 gene as a reference, a set of 6 shRNAs were designed with a 19 base pair complementary region to the PD-L1 gene (SEQ ID NO: 31), and cloned into the pEQU6 screening vector (SEQ ID NO:41) behind the U6 promoter, utilizing the cloning strategy that is described above. Each shRNA

construct was screened for disruption of human PD-L1 gene expression by using the qPCR and western blot protocols described above. As shown in **Fig. 2A**, several shRNAs were effective at knocking down PD-L1 gene expression. ARI-123 (SEQ ID NO:2) resulted in the highest potency, with approximately 75% knockdown of human PD-L1 gene expression. This was confirmed by western blot (**Fig. 2B**), where ARI-123 demonstrated >99% knockdown of PD-L1 gene expression. In addition, ARI-122 (SEQ ID NO:1) showed > 99% knockdown of PD-L1 gene expression by Western blot.

A set of 6 shRNAs with 19 bp complementary regions were designed to disrupt the expression of the human TREX1 gene (SEQ ID NO:34), and cloned into the pEQU6 screening vector (SEQ ID NO:41) behind the U6 promoter in the manner described above. As shown in **Fig. 3A**, ARI-109 (SEQ ID NO:19), ARI-110 (SEQ ID NO:20), ARI-111 (SEQ ID NO:21) and ARI-114 (SEQ ID NO:24) all showed approximately 70% knockdown of TREX1 gene expression by qPCR. Western blot analysis was used to confirm the gene disruption findings identified by qPCR (**Fig. 3B**). Both ARI-110 (SEQ ID NO:20) and ARI-114 (SEQ ID NO:24) showed a high degree of gene knockdown, 85.5% and 76.1%, respectively.

Using the human beta-catenin gene (SEQ ID NO:32) as a reference, a set of 6 shRNAs were designed with a 19 base complementary region to the beta-catenin gene and cloned into the pEQU6 screening vector (SEQ ID NO:41) behind the U6 promoter as described above. Each shRNA construct was screened for disruption of human beta-catenin gene expression by both qPCR and Western blot. As shown in **Fig. 4A**, several shRNAs were effective at knocking down beta-catenin gene expression. ARI-169 (SEQ ID NO:8) demonstrated >75% knockdown of human beta-catenin gene expression. In the Western blot analysis (**Fig. 4B**) ARI-169 (SEQ ID NO:8), ARI-170 (SEQ ID NO:9), ARI-171 (SEQ ID NO:10), and ARI-172 (SEQ ID NO:11), each showed >99% knockdown of beta-catenin gene expression.

The human SIRP-alpha gene (SEQ ID NO:33) was also screened for shRNAs that disrupt gene expression. A set of 6 shRNAs with 19 bp complementary regions were designed and cloned into the pEQU6 screening vector (SEQ ID NO:41) behind the U6 promoter as described above. As shown in **Fig. 5A**, several shRNA constructs were able to significantly knockdown SIRP-alpha gene expression. ARI-175 (SEQ ID

NO:14), ARI-176 (SEQ ID NO:15), and ARI-177 (SEQ ID NO:16) all showed approximately greater than 70% knockdown of SIRP-alpha gene expression by qPCR. In the Western blot analysis (**Fig. 5B**), a high degree of knockdown was observed for several constructs: ARI-175 (>95% knockdown), ARI-176 (>80% knockdown), and ARI-177 (approximately 90% knockdown), which was consistent with the findings by these three constructs when screened by qPCR.

Using the human TGF-beta isoform 1 gene (SEQ ID NO:193) as a reference, a set of nine shRNAs were designed and cloned into the pEQU6 screening vector (SEQ ID NO:41) behind the U6 promoter as described above. Each shRNA construct was screened for disruption of human TGF-beta isoform 1 gene expression by qPCR. As shown in **Fig. 6**, several shRNAs were effective at knocking down TGF-beta gene expression. ARI-181 (SEQ ID NO:196) was the most potent shRNA, with approximately >85% knockdown of human TGF-beta gene expression. This was followed by ARI-183 (SEQ ID NO:198), which showed approximately 75% knockdown of TGF-beta gene expression.

A set of 6 shRNAs with 19 bp complementary regions were designed to disrupt the expression of human VEGF (SEQ ID NO:194), and cloned into the pEQU6 screening vector (SEQ ID NO:41) behind the U6 promoter as described above. As shown in **Fig. 7**, several shRNA constructs possessed a high degree of knockdown efficiency against VEGF gene expression, when assessed by qPCR. ARI-189 (SEQ ID NO:204), ARI-190 (SEQ ID NO:205), and ARI-191 (SEQ ID NO:206) all showed approximately equal to, or greater than, 70% knockdown of VEGF gene expression by qPCR. In addition, ARI-193 (SEQ ID NO:208) showed greater than 80% knockdown of VEGF gene expression. Western blot analysis was used to confirm the gene disruption findings identified by qPCR, with ARI-189 (SEQ ID NO:204), ARI-190 (SEQ ID NO:205), ARI-191 (SEQ ID NO:206), ARI-193 (SEQ ID NO:208) all showing very faint VEGF Western blot bands as individual lanes on a gel when compared to a positive control, a VEGF lane that lacked a cognate shRNA to VEGF in the transfection reaction. Therefore, the findings from the Western blot analysis confirmed the findings from the qPCR reaction.

Using the human VISTA gene as a reference (SEQ ID NO:35), a set of six shRNAs were designed and cloned into the pEQU6 screening vector (SEQ ID NO:41)

behind the U6 promoter as described above. Each shRNA construct was screened for disruption of VISTA gene expression in a qPCR knockdown experiment. As shown in **Fig. 8A**, several shRNAs were effective at knocking down human VISTA gene expression. ARI-195 (SEQ ID NO:25) and ARI-196 (SEQ ID NO:26) were the most potent shRNAs, with approximately 80% and 65% knockdown of human VISTA gene expression, respectively. These results were confirmed by Western blot analysis, which demonstrated nearly complete knockdown (approximately 99%) for ARI-195 and ARI-196 (**Fig. 8B**).

**Combination RNAi**

Combined RNAi knockdown of two separate gene targets by separate shRNAs expressed from the same plasmid was tested using an engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs each targeting PD-L1 (ARI-123, SEQ ID NO:2) and TREX1 (ARI-114, SEQ ID NO:24) were subcloned to generate the combination RNAi ARI-134 (SEQ ID NO:210). ARI-134 then was tested for the ability to simultaneously express two separate shRNAs *in situ*, that can each individually knockdown expression of their respective targets (PD-L1 and TREX1). As a control, knockdown of human PD-L1 expression in HEK293 cells by ARI-134 was compared to ARI-123 (the single RNAi targeting solely PD-L1 (SEQ ID NO:2)), and knockdown of human TREX1 in HEK 293 cells by ARI-134 was compared to ARI-114 (a single RNAi solely targeting TREX1 (SEQ ID NO:24)). Whereas the ARI-123 knockdown had 27.6% of wild type human PD-L1 gene expression, knockdown of human PD-L1 by ARI-134 (the combination vector) was improved with 11.8% of wild type human PD-L1 gene expression (**Fig. 9A**). Likewise, whereas human TREX1 knockdown with ARI-114 had 16% of wild type TREX1 expression, the knockdown of human TREX1 with ARI-134 was 100% (**Fig. 9B**). When knockdown against PD-L1 and TREX1 by ARI-134 was analyzed by Western blot, there was no detectable expression of either human PD-L1 or human TREX1 versus their respective positive controls (individual human PD-L1 and human TREX1 expression reactions lacking any RNAi). Therefore, the combination RNAi ARI-134 is able to knockdown expression of PD-L1 and TREX1.

Similarly, the individual RNAis, each targeting PD-L1 (ARI-123, SEQ ID NO:2) and SIRP-alpha (ARI-175, SEQ ID NO:14) described above, were subcloned

into an engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42) to generate the combination RNAi, ARI-135 (SEQ ID NO:211). ARI-135 was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of PD-L1 and SIRP-alpha. As a control, knockdown of human PD-L1 expression in HEK293 cells by ARI-135 was compared to ARI-123 (a single RNAi solely targeting PD-L1 alone (SEQ ID NO:2), described above). Likewise, knockdown of human SIRP-alpha in HEK 293 cells by ARI-135 was compared to ARI-175 (a single RNAi targeting SIRP-alpha alone (SEQ ID NO:14), described above). Knockdown of PD-L1 by both ARI-123 and ARI-135 resulted in approximately 20% of wild type human PD-L1 gene expression (**Fig. 10A**). Likewise, knockdown of SIRP-alpha with both ARI-175 and ARI-135 resulted in <20% wild type SIRP-alpha expression (**Fig. 10B**). When knockdown against both PD-L1 and SIRP-alpha by ARI-135 was analyzed by Western blot, there was no detectable expression of either human PD-L1 or human SIRP-alpha versus their respective positive controls (human PD-L1 and human SIRP-alpha expression reactions lacking any RNAi). Therefore, the combination RNAi ARI-135 is able to knockdown expression of PD-L1 and SIRP-alpha.

Next, the individual RNAi's, each targeting PD-L1 (ARI-123, SEQ ID NO:2) and beta-catenin (ARI-169, SEQ ID NO:8) described above, were subcloned into the engineered combination RNAi plasmid carrying the U6 and H1 promoter (SEQ ID NO:42) to generate the combination RNAi ARI-136 (SEQ ID NO:212). ARI-136 then was tested for the ability to simultaneously express two separate RNAi's *in situ* that can each individually knockdown expression of PD-L1 and beta-catenin. As a control, knockdown of human PD-L1 expression in HEK293 cells by ARI-136 was compared to ARI-123 (the single RNAi targeting PD-L1 alone (SEQ ID NO:2), described above). Likewise, knockdown of human beta-catenin in HEK 293 cells by ARI-136 was compared to ARI-169 (the single RNAi targeting beta-catenin alone (SEQ ID NO:8), described above). Knockdown of PD-L1 by ARI-123 resulted in approximately 20% of wild type human PD-L1 gene expression (**Fig. 11A**). Knockdown of PD-L1 by ARI-136 resulted in approximately 10% of wild type human PD-L1 gene expression, which is approximately two-fold better than ARI-123 (**Fig. 11A**). Knockdown of beta-catenin with ARI-136 and ARI-169 resulted in

approximately 30% of wild type beta-catenin expression (**Fig. 11B**). When knockdown against PD-L1 and beta-catenin by ARI-136 was analyzed by Western blot, there was no detectable expression of either human PD-L1 or human beta-catenin versus their respective positive controls (human PD-L1 and human beta-catenin expression reactions lacking any RNAi). Therefore, the combination RNAi ARI-136 is able to knockdown expression of PD-L1 and beta-catenin.

The individual RNAi's, each targeting PD-L1 (ARI-123, SEQ ID NO:2) and VISTA (ARI-195, SEQ ID NO:25) described above, were subcloned into an engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42) to generate the combination RNAi, ARI-137 (SEQ ID NO:213). ARI-137 was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of PD-L1 and VISTA. As a control, knockdown of human PD-L1 expression in HEK293 cells by ARI-137 was compared to ARI-123 (a single RNAi solely targeting PD-L1 alone (SEQ ID NO:2), described above). Likewise, knockdown of human VISTA in HEK 293 cells by ARI-137 was compared to ARI-195 (a single RNAi targeting VISTA alone, described above, SEQ ID NO:25). Knockdown of PD-L1 by both ARI-123 and ARI-137 resulted in approximately 20% of wild type human PD-L1 gene expression (**Fig. 12A**). Likewise, knockdown of VISTA with both ARI-195 and ARI-137 resulted in less than, or approximately equal to, 20% wild type VISTA expression (**Fig. 12B**). When knockdown against PD-L1 and VISTA by ARI-137 was analyzed by Western blot, there was no detectable expression of either human PD-L1 or human VISTA versus their respective positive controls (human PD-L1 and human VISTA expression reactions lacking any RNAi). Therefore, the combination RNAi ARI-137 is able to knockdown expression of PD-L1 and VISTA.

In addition to human targets, combined RNAi knockdown of two mouse gene targets by separate shRNAs expressed from the same plasmid was tested using the engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs each targeting mouse PD-L1 (ARI-115, SEQ ID NO:75) and mouse TREX1 (ARI-108) were subcloned to generate the combination RNAi ARI-128. ARI-128 then was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of their respective targets (mouse PD-L1

and mouse TREX1). As a control, knockdown of mouse PD-L1 expression in HEK293 cells by ARI-128 was compared to ARI-115 (the single RNAi targeting solely targeting PD-L1 (SEQ ID NO:75)), and knockdown of mouse TREX1 in HEK 293 cells by ARI-128 was compared to ARI-108 (a single RNAi solely targeting TREX1). Whereas the ARI-115 knockdown had 22.8% of wild type mouse PD-L1 gene expression, knockdown of mouse PD-L1 by ARI-128 (the combination vector) was improved, allowing only 14.0% of wild type mouse TREX1 gene expression (**Fig. 13A**). Knockdown of mouse TREX1 with either ARI-108 or ARI-128 was very efficient (6.6% and 11.3%, respectively, of wild-type mouse TREX1 expression) (**Fig. 13B**). When knockdown against both mouse PD-L1 and mouse TREX1 by ARI-128 was analyzed by Western blot, there was no detectable expression of either mouse PD-L1 or mouse TREX1 versus their respective positive controls (individual mouse PD-L1 and mouse TREX1 expression reactions lacking any RNAi).

A combination RNAi was generated for targeting mouse PD-L1 and mouse SIRP-alpha using the engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs each targeting mouse PD-L1 (ARI-115, SEQ ID NO:75) and mouse SIRP-alpha (ARI-138, SEQ ID NO:76) were subcloned to generate the combination RNAi ARI-129. ARI-129 then was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of their respective targets (mouse PD-L1 and mouse SIRP-alpha). As a control, knockdown of mouse PD-L1 expression in HEK293 cells by ARI-129 was compared to ARI-115 (the single RNAi targeting solely targeting PD-L1), and knockdown of mouse SIRP-alpha in HEK 293 cells by ARI-129 was compared to ARI-138 (a single RNAi solely targeting SIRP-alpha). ARI-115 and ARI-129 had knockdown of approximately 20% or less of wild type mouse PD-L1 gene expression (**Fig. 14A**). Knockdown of mouse SIRP-alpha with either ARI-138 or ARI-129 was approximately 25% or less of wild-type mouse SIRP-alpha expression (**Fig. 14B**). When knockdown against both mouse PD-L1 and mouse SIRP-alpha by ARI-129 was analyzed by Western blot, there was no detectable expression of either mouse PD-L1 or mouse SIRP-alpha versus their respective positive controls (individual mouse PD-L1 and mouse SIRP-alpha expression reactions lacking any RNAi).

Next, a combination RNAi was generated for targeting mouse PD-L1 and mouse VISTA using the engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs each targeting mouse PD-L1 (ARI-115, SEQ ID NO:75) and mouse VISTA (ARI-157) were subcloned to generate the combination RNAi ARI-132. ARI-132 then was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of their respective targets (mouse PD-L1 and mouse VISTA). As a control, knockdown of mouse PD-L1 expression in HEK293 cells by ARI-132 was compared to ARI-115 (the single RNAi targeting solely targeting PD-L1), and knockdown of mouse VISTA in HEK 293 cells by ARI-132 was compared to ARI-157 (a single RNAi solely targeting VISTA). Both ARI-115 and ARI-132 had knockdown of approximately 20% or less of wild type mouse PD-L1 gene expression (**Fig. 15A**). Knockdown of mouse VISTA with either ARI-157 or ARI-132 was approximately 30% or less of wild-type mouse VISTA expression (**Fig. 15B**). When knockdown against both mouse PD-L1 and mouse VISTA by ARI-132 was analyzed by Western blot, there was no detectable expression of either mouse PD-L1 or mouse VISTA versus their respective positive controls (individual mouse PD-L1 and mouse VISTA expression reactions lacking any RNAi).

A combination of RNAi was generated for targeting mouse TREX1 and mouse SIRP-alpha using the engineered plasmid carrying a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs, one targeting mouse TREX1 (ARI-108) and the other targeting mouse SIRP-alpha (ARI-138, SEQ ID NO:76), were subcloned to generate the combination RNAi designated ARI-131. ARI-131 was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of the respective targets (mouse TREX1 and mouse SIRP-alpha). As a control, knockdown of mouse TREX1 expression in HEK293 cells by ARI-131 was compared to ARI-108 (the single RNAi targeting solely targeting TREX1), and knockdown of mouse SIRP-alpha in HEK 293 cells by ARI-131 was compared to ARI-138 (a single RNAi solely targeting SIRP-alpha). ARI-108 and ARI-131 had knockdown of approximately 20% or less of wild type mouse TREX1 gene expression (**Fig. 16A**). Knockdown of mouse SIRP-alpha with

either ARI-138 or ARI-131 was approximately 25% or less than wild-type mouse SIRP-alpha expression (**Fig. 16B**).

A combination RNAi was generated that targets mouse PD-L1 and mouse beta-catenin using the engineered plasmid carrying a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs each targeting mouse PD-L1 (ARI-115, SEQ ID NO:75) and mouse beta-catenin (ARI-166) were subcloned to generate the combination RNAi ARI-133. ARI-133 then was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of their respective targets (mouse PD-L1 and mouse beta-catenin). As a control, knockdown of mouse PD-L1 expression in HEK293 cells by ARI-133 was compared to ARI-115 (the single RNAi targeting solely targeting PD-L1), and knockdown of mouse beta-catenin in HEK 293 cells by ARI-133 was compared to ARI-166 (a single RNAi solely targeting beta-catenin). ARI-115 and ARI-133 had knockdown of approximately 25% or less of wild type mouse PD-L1 gene expression (**Fig. 17A**). Knockdown of mouse beta-catenin with either ARI-166 or ARI-133 was approximately 25% or less of wild-type mouse beta-catenin expression (**Fig. 17B**). When knockdown against mouse PD-L1 and mouse beta-catenin by ARI-133 was analyzed by Western blot, there was no detectable expression of either mouse PD-L1 or mouse beta-catenin versus their respective positive controls (individual mouse PD-L1 and mouse beta-catenin expression reactions lacking any RNAi).

Next, a combination RNAi was generated for targeting mouse TREX1 and mouse VISTA using the engineered plasmid carrying both a U6 and H1 promoter (SEQ ID NO:42). Individual shRNAs each targeting mouse TREX1 (ARI-108) and mouse VISTA (ARI-157) were subcloned to generate the combination RNAi ARI-130. ARI-130 then was tested for the ability to simultaneously express two separate shRNAs *in situ* that can each individually knockdown expression of their respective targets (mouse TREX1 and mouse VISTA). As a control, knockdown of mouse TREX1 expression in HEK293 cells by ARI-130 was compared to ARI-108 (a RNAi targeting solely targeting TREX1), and knockdown of mouse VISTA in HEK 293 cells by ARI-130 was compared to ARI-157 (a single RNAi solely targeting VISTA). Both ARI-108 and ARI-130 had knockdown of approximately 30% or less of wild type mouse TREX1 gene expression (**Fig. 18A**). Knockdown of mouse VISTA with

either ARI-157 or ARI-130 was approximately 30% or less of wild-type mouse VISTA expression (**Fig.18B**). When knockdown against both mouse TREX1 and mouse VISTA by ARI-130 was analyzed by Western blot, there was no detectable expression of either mouse TREX1 or mouse VISTA versus their respective positive controls (individual mouse TREX1 and mouse VISTA expression reactions lacking any RNAi).

**Micro RNA (mi-RNA)**

A microRNA construct, ARI-205 (SEQ ID NO:214), was used to generate a mouse PD-L1 targeting microRNA, ARI-201, by inserting RNAi targeting mouse PD-L1 into the microRNA backbone of SEQ ID NO:249, and compared to the PD-L1 targeting shRNA construct ARI-115 (SEQ ID NO:75) by qPCR and Western blot analysis, as described above. Whereas ARI-115 knockdown was 26.6% of wild-type PD-L1 expression, knockdown by ARI-201 was improved, with 14.6% of PD-L1 expression (**Fig. 19A**). By Western blot, ARI-115 was able to knockdown PD-L1 to 15.8% of wild type PD-L1 expression, and knockdown by ARI-201 was improved, with 10.5% of PD-L1 expression (**Fig. 19B**).

A microRNA was generated against mouse TREX1, ARI-203, based on the microRNA construct described above, ARI-205 (SEQ ID NO:214), using oligonucleotide synthesis, overlapping PCR and restriction digest cloning, and tested by qPCR. Whereas ARI-108, a shRNA that targets mouse TREX1, had a gene knockdown efficiency of 22.3% versus wild-type TREX1, ARI-203 possessed a knockdown efficiency of 5.9% (**Fig. 20**). Therefore, the microRNA was approximately three to four-fold improved in its knockdown efficiency of mouse TREX1, when compared to the shRNA.

A large microRNA construct, ARI-206 (SEQ ID NO:215), requiring expression under an RNA polymerase II promoter, was constructed for testing knockdown of target genes and testing by qPCR and Western blot analysis. A mouse TREX1 targeting version of this microRNA, ARI-204, was tested against ARI-108, the mouse TREX1 targeting shRNA described above. ARI-204 and ARI-108 were able to efficiently knock down expression of mouse TREX1 (22.5% and 24.1% of wild type mouse TREX1 expression, respectively, **Fig. 21A**). The activity of ARI-204 mouse TREX1 targeting microRNA was slightly improved over the ARI-108 mouse

TREX1 targeting shRNA, when assessed for knockdown of mouse TREX1 gene expression by Western blot (11.1% for ARI-204, versus 21.4% for ARI-108, **Fig. 21B**).

A mouse PD-L1 targeting version of microRNA construct ARI-206, ARI-202, was tested against ARI-115, the mouse PD-L1 targeting shRNA described above. ARI-202 and ARI-115 were able to efficiently knock down expression of mouse PD-L1 (10.0 and 11.2% of wild type mouse PD-L1 expression, respectively, **Fig. 22A**). The ARI-202 mouse PD-L1 targeting microRNA was slightly improved over the ARI-115 mouse PD-L1 targeting shRNA, when assessed for knockdown of mouse PD-L1 gene expression by Western blot (8.7% for ARI-202, versus 13.8% for ARI-115, **Fig. 22B**).

The shRNA gene knockdown can be directly measured in tumor cell lines that are known to overexpress the target gene. For example, the following are known tumor cell lines with high PD-L1 expression: PC-3 (prostate), MDA-MB-231 (breast), and ASPC-1 (pancreatic) (Grenga *et al.* (2014) *J. ImmunoTherapy of Cancer* 2(Suppl 3):P102). Cells can be stimulated with IFN-gamma to see induction of PD-L1 expression. The U937 tumor cell line overexpresses SIRP-alpha (Irandoust *et al.* (2013) *PLoS ONE* 8(1):e52143). Simultaneous knockdown of gene expression against PD-L1 and SIRP-alpha can be performed in U937 cells induced with IFN-gamma.

The microRNA constructs above, ARI-205 (SEQ ID NO:214) and ARI-206 (SEQ ID NO:215) encode 21 and 22 base pair homology sequences, respectively. Alternatively, microRNA constructs can be used that encode 19 base pair homology sequences, for example, ARI-207 (SEQ ID NO: 216) and ARI-208 (SEQ ID NO:217). The individual microRNAs against target genes can be generated by gene synthesis, PCR amplification with primers containing restriction sites and subcloning into the expression vector with matched restriction enzyme generated overhangs.

## Example 3

## Modified *Salmonella typhimurium* Targets Demonstrate Robust Tumor Growth Inhibition in Multiple Syngeneic Murine Tumor Models

**TREX1**

Delivery of an shRNA to TREX1, following tumor microenvironment uptake of systemically administered attenuated *Salmonella*, results in activation of STING-

mediated anti-tumor immunity and tumor growth inhibition. To assess the ability of AST-104 (strain YS1646 transformed with pEQU6-shTREX1) to induce tumor growth inhibition in a murine colon carcinoma model, 6-8 week-old female BALB/c mice (8 mice per group) were inoculated subcutaneously (SC) in the right flank with CT26 murine colon carcinoma ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were intravenously (IV) injected twice, four days apart, with $1x10^7$ CFUs of AST-104, or AST-102 (strain YS1646 transformed with pEQU6 plasmid control), and compared to PBS control. Six hours following the first IV dose, mice were bled, and plasma was collected and assessed for pro-inflammatory cytokines, using the Mouse Inflammation Cytometric Bead Array kit and analyzed by FACS (BD Biosciences).

As shown in **Fig. 23**, the control strain, AST-102 demonstrated modest tumor control, compared to PBS (18% tumor growth inhibition (TGI), $p$ = ns at day 25). However, the shTREX1-containing strain, AST-104, demonstrated significant tumor growth inhibition compared to PBS (66% TGI, $p = 0.01$ at day 25, calculated over the average of 8 animals per group), and significant tumor control compared to AST-102 ($p = 0.02$ at day 28). The percent tumor growth inhibition (TGI) is calculated as 1-(mean test tumor volume/mean control tumor volume) x 100.

**Activation of Pro-inflammatory Cytokines**

**TREX1**

The level of systemic serum cytokines at 6 hours post IV injection were assessed. The immune-activating cytokines TNF-alpha, IL-12, and interferon-gamma, elicited by AST-104 (containing an shTREX1 plasmid that includes the *asd* complementation in the plasmid; *asd* contains CpG elements) were significantly higher, compared to the AST-102 plasmid control (also containing CpG from the *asd*) and PBS groups (**Fig. 24A**). IL-10, a cytokine known to suppress immunity (see, *e.g.*, Wang *et al.* (2012) *Scand J Immunol.* 3:273–281), trended lower in the shTREX1 group compared to the plasmid control (**Fig. 24B**). These data demonstrate that inhibiting TREX1 activates known STING pathway-induced cytokines that promote anti-tumor immunity and potent tumor growth inhibition in a murine model of colon carcinoma.

To assess the ability of AST-104 (containing an shTREX1 plasmid with CpG elements) to induce tumor growth inhibition in a separate aggressive murine colon carcinoma model, as well as a checkpoint therapy-resistant melanoma model, 6-8 week-old female C57BL/6 mice (10 mice per group) were inoculated SC in the right flank with MC38 colon carcinoma cells or B16.F10 melanoma cells (5 and 2 x $10^5$ cells, respectively, in 100 µL PBS). Mice bearing established flank tumors were IV injected twice, four days apart, with 5x$10^6$ CFUs of AST-104, or AST-102, and compared to PBS control.

As shown in **Fig. 25**, strain AST-104, containing shRNA to TREX1, induced potent tumor growth inhibition of MC38 tumors (85% TGI, $p < 0.0001$, day 28), and significant tumor growth inhibition compared to the plasmid control ($p = 0.049$, day 28). Similarly, as shown in **Fig. 26**, AST-104 induced highly significant tumor growth inhibition in B16.F10 melanoma compared to PBS (83% TGI, $p = 0.0012$, day 24), and greater tumor growth inhibition compared to plasmid control strain AST-102, which had significant efficacy in this model compared to PBS ($p = 0.019$, day 24). These results also show that plasmids containing CpG elements, in combination with shTREX1-mediated STING activation demonstrate synergy and efficacy, and have the benefit of systemic, instead of intratumoral, administration.

In summary, in multiple aggressive murine tumor models, the addition of a plasmid encoding shRNA against TREX1 in the YS1646 strain significantly enhanced anti-tumor responses compared to the YS1646 strain containing a control plasmid. These data demonstrate the potency of activating the STING pathway through systemic administration of an immunostimulatory tumor-targeting bacteria.

**PD-L1**

The immune system has evolved several checks and balances to limit autoimmunity. Programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) are two examples of numerous inhibitory "immune checkpoints," which function by downregulating immune responses. The binding of PD-L1 to PD-1 interferes with $CD8^+$ T cell signaling pathways, impairing the proliferation and effector function of $CD8^+$ T cells, and inducing T cell tolerance (Topalian *et al.* (2012) *N Engl J Med 366*:3443-3447).

Tumor colonization of a modified *Salmonella typhimurium* strain delivering shRNA to knockdown the PD-L1 gene disrupts its binding to PD-1, and its inhibition of $CD8^+$ T cell function. PD-L1/PD-1 checkpoint inhibition synergizes well with the immunostimulatory *S. typhimurium* containing CpG plasmid DNA, all in one therapeutic modality. To demonstrate the *in vivo* efficacy of the YS1646 strain containing a plasmid encoding shRNA to PD-L1 (AST-105), this strain, in comparison to the AST-102 strain (containing a control plasmid that also contains CpG motifs) in a murine colon carcinoma model was evaluated. For this experiment, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right flank with CT26 murine colon carcinoma ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected twice, four days apart, with $5x10^6$ CFUs of AST-105, AST-102, or IV administration of anti-PD-L1 antibody (4 mg/kg, BioXCell clone 10F.9G2). Six hours following the first IV dose, mice were bled, and plasma was collected and assessed for pro-inflammatory cytokines using the Mouse Inflammation Cytometric Bead Array kit and analyzed by FACS (BD Biosciences).

As shown in **Fig. 27**, treatment with strain AST-105 demonstrated statistically significant tumor control compared to treatment with the plasmid-containing control strain AST-102 (69% TGI, $p = 0.05$, day 25). Tumor growth inhibition was also greater for treatment with AST-105 (expressing shPD-L1) than from systemic administration of an anti-PD-L1 antibody (68% TGI vs. anti-PD-L1).

Comparing the production of innate pro-inflammatory cytokines at 6 hours post IV injection, the cytokines elicited by strain AST-105 were significantly higher compared to the anti-PD-L1 antibody ($p < 0.05$, **Fig. 28**), and much higher than those from AST-102. These data demonstrate that inhibiting PD-L1 within the tumor microenvironment, compared to systemic administration of anti-PD-L1 antibody, uniquely activates potent pro-inflammatory cytokines that induce anti-tumor immunity and promote tumor growth inhibition in a murine model of colon carcinoma.

## Example 4

## Intratumoral Administration of Modified *S. typhimurium* shTREX1 Provides Distal Tumor Colonization and Complete Anti-tumor Responses in a Dual Flank Murine Colon Carcinoma Model

A hallmark of inducing adaptive immunity to a tumor is the ability to induce regression of a distal, untreated tumor. To assess the ability of the YS1646 strain containing the pEQU6 shRNA plasmids to induce primary and distal tumor growth inhibition in a dual flank murine colon carcinoma model, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right and left flanks with CT26 murine colon carcinoma (2 $x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were intratumorally (IT) injected twice, four days apart, into the right flank tumor with $5x10^6$ CFUs of AST-104, (pEQU6 shTREX1 in YS1646), AST-105 (pEQU6 shPD-L1 in YS1646) or AST-102 (plasmid control in YS1646), and compared to PBS control.

As shown in **Fig. 29**, IT injection of AST-104 and AST-105 induced significant tumor growth inhibition in the injected tumor, compared to the PBS control (AST-105 – 60.5% TGI, $p = 0.03$; AST-104 – 61.4% TGI, $p = 0.03$ day 25). Unlike AST-105, only AST-104 induced significant growth inhibition of the distal, untreated tumor compared to PBS (60% TGI, $p < 0.0001$, day 25), and significant distal tumor growth inhibition compared to AST-102 containing the plasmid control ($p = 0.004$, day 25). The AST-104 strain also demonstrated significant tumor regression and increased survival compared to PBS control ($p = 0.0076$, Log-rank (Mantel-Cox) test) with 2/10 complete remissions (**Fig. 30**).

To determine whether the bacteria colonize injected, as well as distal tumors, tumor-bearing mice treated with AST-104 were sacrificed and tumors were collected. Injected and distal tumors were transferred to M tubes and were homogenized in PBS using a gentleMACS™ Dissociator (Miltenyi Biotec). Tumor homogenates were serially diluted and plated on LB agar plates and incubated at 37 °C for colony forming unit (CFU) determination. As shown in **Fig. 31**, the distal tumor was colonized to the same extent as the injected tumor, indicating that the engineered *Salmonella* strains dosed with an intratumoral route of administration are able to transit and colonize distal lesions. These data demonstrate the potency of administering an immunostimulatory bacteria IT with the ability to systemically colonize distal tumor lesions preferentially over other organs, and the potency of activating the STING Type I Interferon pathway, leading to systemic tumor regression and complete remissions.

## Example 5

## Modified *S. typhimurium* Strains with Plasmids Containing Cpg Elements Demonstrate Enhanced Anti-Tumor Activity Compared To YS1646 Parental Strain

Toll-like receptors (TLRs) are key receptors for sensing pathogen-associated molecular patterns (PAMPs) and activating innate immunity against pathogens (Akira *et al.* (2001) *Nat Immunol.* 2(8):675-680). Of these, TLR9 is responsible for recognizing hypomethylated CpG motifs in pathogenic DNA which do not occur naturally in mammalian DNA (McKelvey *et al.* (2011) *J Autoimmunity* 36:76). Recognition of CpG motifs upon phagocytosis of pathogens into endosomes in immune cell subsets induces IFR7-dependent type I interferon signaling and activates innate and adaptive immunity. It is shown herein, that the *S. typhimurium* strain YS1646 carrying modified *Salmonella typhimurium* plasmids containing CpG motifs (YS1646 pEQU6 Scramble) similarly activate TLR9 and induce type I IFN-mediated innate and adaptive immunity, as compared to the YS1646 strain without a plasmid.

The CpG motifs in the engineered plasmids used here are shown in **Table 2**. The pEQU6 shSCR (non-cognate shRNA) plasmid in strain AST-103 possesses 362 CpG motifs, indicating that *Salmonella*-based plasmid delivery can be immuno-stimulatory and have an anti-tumor effect, when compared to the same *Salmonella* lacking transformation with this plasmid. To assess the ability of CpG-containing plasmids within YS1646 to induce tumor growth inhibition in a murine colon carcinoma model, 6-8 week-old female BALB/c mice (9 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected weekly with three doses of $5x10^6$ CFUs of YS1646 (AST-100) or YS1646 containing an shRNA scrambled plasmid with CpG motifs (AST-103), and compared to PBS control.

**Table 2. CpG motifs in the engineered plasmids**

| Sequence name | Number of CpG Motifs | SEQ ID NO. |
|---|---|---|
| pBR322 Origin | 80 | 243 |
| pEQU6 (shSCR) | 362 | 244 |
| Asd Gene ORF | 234 | 242 |
| pATI-2.0 | 538 | 245 |

As shown in **Fig. 32**, the YS1646 (AST-100) strain demonstrated modest tumor control (32% TGI, $p$ = ns, day 28) as compared to PBS. The AST-103 strain, that varies from YS1646 only by the addition of the CpG-containing plasmid encoding a non-cognate scrambled shRNA, demonstrated highly significant tumor growth inhibition compared to YS1646 alone, untransformed and therefore lacking a plasmid ($p$ = 0.004, day 32).

The *asd* gene possesses 234 CpG motifs (**Table 2**), indicating that a plasmid containing it can have immunostimulatory properties. As shown in **Fig. 46**, AST-109 (YS1646-ASD with scrambled shRNA) had 51% tumor growth inhibition vs PBS alone, indicative of a strong immuno-stimulatory effect.

These data demonstrate the potent immunostimulatory properties of plasmid DNA containing TLR9-activating CpG motifs within a tumor-targeting attenuated strain of *S. typhimurium.*

**Example 6**

**The Modified *Salmonella typhimurium* Strains Containing MicroRNA Inhibition Demonstrate Enhanced Anti-Tumor Activity Compared To shRNA**

Superior TREX1 gene knockdown was achieved *in vitro* with microRNA ARI-203 (see Example 2, **Fig. 20**). The microRNA strain AST-106 was generated by transforming YS1646 with ARI-203, pEQU6 plasmid encoding a microRNA (miRNA) against TREX1. AST-106 was compared to the shRNA strain, AST-104 (YS1646 transformed with pEQU6 shTREX1). *In vivo* potency in a murine colon carcinoma model was tested. For this experiment, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected weekly on day 8, day 15 and day 23 with $5x10^6$ CFUs of AST-104 or AST-106 and compared to PBS control.

As shown in **Fig. 33**, both versions of the TREX1 knockdown strains demonstrated significant tumor growth inhibition compared to PBS control (AST-104 58% TGI, $p$ = 0.014; AST-106 77% TGI, $p$ = 0.003, day 17), with the AST-106 miTREX1 exhibiting the most potent tumor control after the second dose, which was significantly better than the shTREX1 strain AST-104 ($p$ =0.036, day 17). These data demonstrate that the microRNA based inhibitory RNAs can deliver more potent gene

knockdown *in vivo* and outperform the shRNA-based inhibitory RNAs in a tumor growth inhibition model.

**Example 7**

**Vector Synthesis**

**Complementation of *asd* deletion by *asd* expression from plasmids**

A plasmid (pATIU6) was chemically synthesized and assembled (SEQ ID NO:225). The plasmid contained the following features: a high copy (pUC19) origin or replication, a U6 promoter for driving expression of a short hairpin, an ampicillin resistance gene flanked by HindIII restriction sites for subsequent removal, and the *asd* gene containing 85 base pairs of sequence upstream of the start codon (SEQ ID NO:246). Into this vector, shRNAs targeting murine TREX1 or a scrambled, non-cognate shRNA sequence were introduced by restriction digestion with SpeI and XhoI and ligation and cloning into *E. coli* DH5-alpha. The resulting plasmids, designated pATI-shTREX1 and pATI-shSCR, respectively, were amplified in *E. coli* and purified for transformation into the *asd* knockout strain AST-101 by electroporation and clonal selection on LB amp plates to produce strains AST-108, and AST-107, respectively. *asd*- mutants complemented with pATIU6-derived plasmids were able to grow on LB agar and liquid media in the absence of DAP.

In a subsequent iteration, the ampicillin resistance gene (AmpR) from pATI-shTREX1 was replaced with a kanamycin resistance gene. This was accomplished by digestion of pATI-shTREX1 plasmid with HindIII followed by gel purification to remove the AmpR gene. PCR amplification of the kanamycin resistance (KanR) gene using primers APR-001 and APR-002 (SEQ ID NO:226 and SEQ ID NO:227), digestion with HindIII and ligation into the gel purified, digested pATIU6 plasmid.

In subsequent iterations, a single point mutation was introduced into the pATIKan plasmid at the pUC19 origin of replication using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) and the primers APR-003 (SEQ ID NO:228) and APR-004 (SEQ ID NO:229) to change the nucleotide T at position 148 to a C. This mutation makes the origin of replication homologous to the pBR322 origin of replication in order to reduce the plasmid copy number.

| Primer ID | Description | Sequence | SEQ ID NO |
|---|---|---|---|
| APR-001 | Kan primerF | AAAAAAGCTTGCAGCTCTGGCCCGTG | 226 |

| | | | |
|---|---|---|---|
| APR-002 | Kan PrimerR | AAAAAAGCTTTTAGAAAAACTCATCGAGCATCAAATGA | 227 |
| APR-003 | pATI ori T148CF | ACACTAGAAGgACAGTATTTGGTATCTG | 228 |
| APR-004 | pATI ori T148CR | AGCCGTAGTTAGGCCACC | 229 |

**pATI2.0**

A plasmid was designed and synthesized that contains the following features: a pBR322 origin of replication, an SV40 DNA nuclear targeting sequence (DTS), an rrnB terminator, a U6 promoter for driving expression of shRNAs followed by flanking restriction sites for cloning the promoter and shRNAs or microRNAs, the *asd* gene, an rrnG terminator, and a kanamycin resistance gene flanked by HindIII sites for curing and a multicloning site (SEQ ID NO:247). In addition, a plasmid was designed and synthesized for expression of two separate shRNA or microRNAs. This plasmid contains the following features: a pBR322 origin of replication, an SV40 DNA nuclear targeting sequence (DTS), an rrnB terminator, a U6 promoter for driving expression of shRNAs followed by flanking restriction sites for cloning the promoter and shRNAs or microRNAs, an H1 promoter for driving the expression of a 2nd shRNA or microRNA, a 450 bp randomly generated stuffer sequence placed between the H1 and U6 promoters, the *asd* gene, an rrnG terminator, and a kanamycin resistance gene flanked by HindIII sites for curing and a multicloning site (SEQ ID NO:245).

## Example 8

## *S. typhimurium* Flagellin Knockout Strain Engineering by Deletion of the *Flic* and *Fljb* Genes

In the example herein, *S. typhimurium* strains were engineered to lack both flagellin subunits *fliC* and *fljB* to reduce pro-inflammatory signaling. Deletions of *fliC* and *fljB* were sequentially engineered into the chromosome of the *asd* gene deleted strain of YS1646 (AST-101).

**Deletion of *fliC***

In this example, *fliC* was deleted from the chromosome of the AST-101 strain using modifications of the method of Datsenko and Wanner (*Proc Natl Acad Sci USA* 97:6640–6645 (2000)) as described in detail in Example 1 and schematically depicted in **Fig. 34**. Synthetic *fliC* gene homology arm sequences were ordered that contained

224 and 245 bases of homologous sequence flanking the *fliC* gene, cloned into a plasmid called pSL0147 (SEQ ID NO:230). A kanamycin gene cassette flanked by cre/lox p sites then was cloned into pSL0147, the *fliC* gene knockout cassette was then PCR amplified with primer flic-1 (SEQ ID NO:232) and flic-2 (SEQ ID NO:233) and gel purified and introduced into the AST-101 strain carrying the temperature sensitive lambda red recombination plasmid pKD46 by electroporation. Electroporated cells were recovered in SOC+DAP medium and plated onto LB Agar plates supplemented with Kanamycin (20 μg/mL) and diaminopimelic acid (DAP, 50 μg/ml). Colonies were selected and screened for insertion of the knockout fragment by PCR using primers flic-3 (SEQ ID NO:234) and flic-4 (SEQ ID NO:235). pKD46 then was cured by culturing the selected kanamycin resistant strain at 42 °C and screening for loss of ampicillin resistance. The Kanamycin resistance marker then was cured by electroporation of a temperature sensitive plasmid expressing the Cre recombinase (pJW1680) and $Amp^R$ colonies were selected at 30 °C; pJW168 was subsequently eliminated by growing cultures at 42 °C. Selected *fliC* knockout clones were then tested for loss of kanamycin marker by PCR using primers flanking the sites of disruption (flic-3 and flic-4) and evaluation of the electrophoretic mobility on agarose gels.

**Deletion of *fljB***

*fljB* was then deleted in the *asd/fliC* deleted YS1646 strain using modifications of the methods described above. Synthetic *fljB* gene homology arm sequences that contained 249 and 213 bases of the left hand and right hand sequence, respectively, flanking the *fliC* gene, were synthesized and cloned into a plasmid called pSL0148 (SEQ ID NO:231). A kanamycin gene cassette flanked by cre/loxP sites then was cloned into pSL0148 and the *fljB* gene knockout cassette then was PCR amplified with primer fljb-1 (SEQ ID NO:236) and fljb-2 (SEQ ID NO:237) and gel purified and introduced into AST-101 carrying the temperature sensitive lambda red recombination plasmid pKD46 by electroporation. The kanamycin resistance gene then was cured by cre-mediated recombination as described above, and the temperature-sensitive plasmids were cured by growth at non-permissive temperature. The *fliC* and *fljB* gene knockout sequences were amplified by PCR using primers flic-3 and flic-4 or fljb-3 (SEQ ID NO:238) and fljb-4 (SEQ ID NO:239), and verified by

DNA sequencing. This *asd*$^-$ / *fliC*$^-$/ *fljB*$^-$ mutant derivative of YS1646 was designated AST-111.

**Primer sequence information**

| Primer name | Primer sequence | SEQ ID NO. |
|---|---|---|
| flic-1 | CGTTATCGGCAATCTGGAGGC | 232 |
| flic-2 | CCAGCCCTTACAACAGTGGTC | 233 |
| flic-3 | GTCTGTCAACAACTGGTCTAACGG | 234 |
| flic-4 | AGACGGTCCTCATCCAGATAAGG | 235 |
| fljb-1 | TTCCAGACGACAAGAGTATCGC | 236 |
| fljb-2 | CCTTTAGGTTTATCCGAAGCCAGAATC | 237 |
| fljb-3 | CACCAGGTTTTTCACGCTGC | 238 |
| fljb-4 | ACACGCATTTACGCCTGTCG | 239 |

***In vitro* characterization of engineered *S. typhimurium* flagellin knockout strain**

The YS1646 derived *asd* mutant strain harboring the deletions of both *fliC* and *fljB*, herein referred to as AST-111 or ASD/FLG, was evaluated for swimming motility by spotting 10 microliters of overnight cultures onto swimming plates (LB containing 0.3% agar and 50 mg/mL DAP). While motility was observed for YS1646 and the *asd* deleted strain AST-101, no motility was evident with the *asd/fliC/fljB*-deleted strain AST-111. The AST-111 strain then was electroporated with pATIshTREX1 (a plasmid containing an *asd* gene and an shRNA targeting TREX1), to produce AST-112, and its growth rate in the absence of DAP was assessed. As shown in **Fig. 35** ASD/FLG (pATI-shTREX1) strain AST-112 was able to replicate in LB in the absence of supplemental DAP, and grew at a rate comparable to the *asd* strain containing pATIshTREX1(AST-108). These data demonstrate that the elimination of flagellin does not decrease the fitness of *S. typhimurium in vitro*.

Elimination of flagellin subunits decreases pyroptosis in macrophages. To demonstrate this, $5x10^5$ mouse RAW-dual™ macrophage cells (InvivoGen, San Diego, Ca.) were infected with the *asd/fliC/fljB* deleted strain harboring a low copy shTREX1 plasmid, designated AST-118, or the *asd* deleted strain harboring the same plasmid (AST-117) at an MOI of approximately 100 in a gentamycin protection assay. After 24 hours of infection, culture supernatants were collected and assessed for lactate dehydrogenase release as a marker of cell death using a Pierce™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, Ma.). AST-117 induced

75% maximal LDH release, while AST-118 induced 54% maximal LDH release, demonstrating that the deletion of the flagellin genes reduce the *S. typhimurium*-induced pyroptosis.

**ASD/FLG knockout strain containing shTrex1 plasmid demonstrates enhanced anti-tumor activity, enhanced interferon gamma responses, and increased tumor colonization in mice compared to parental *asd* strain.**

To assess the impact of the flagellin knockout strains administered in a murine model of colon carcinoma, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 μL PBS). Mice bearing established flank tumors were IV injected with three weekly doses of $5 x10^6$ CFUs of the ASD/FLG strain containing the pATIKan-shTREX1 plasmid (AST-113) or the ASD strain with the same pATIKan-shTREX1 plasmid (AST-110), and compared to PBS control. Six hours following the first IV dose, mice were bled, and plasma was collected and assessed for pro-inflammatory cytokines using the Mouse Inflammation Cytometric Bead Array kit and analyzed by FACS (BD Biosciences).

As shown in **Fig. 36**, The AST-113 strain, incapable of making flagella and containing the pATIshTrex1 plasmid (ASD/FLG pATI-shTREX1), demonstrated enhanced tumor control compared to the parental ASD pATI-shTREX1 strain AST-110, and significant tumor control compared to the PBS control (54% TGI, $p = 0.02$, day 17).

Comparing the levels of systemic serum cytokines at 6 hours post IV injection, the cytokines elicited by the AST-113 strain were comparable for TNF-α and IL-6 as compared to the parental AST-110 strain capable of making flagella. The levels of the potent anti-tumor immune cytokine IFN-γ were significantly higher with AST-113 compared to AST-110, indicating that the flagellin deficient strain can provide for superior anti-tumor potency over the parental *asd* knockout strain (**Fig. 37**).

At 35 days post tumor implantation (12 days after the last dose of engineered *Salmonella* therapy), three mice per group were euthanized, and tumors were homogenized and plated on LB plates to enumerate the number of colony forming units (CFUs) per gram of tumor tissue as described above. As shown in **Fig. 38**, the AST-113 strain, deleted of *fliC* and *fljB* and containing the pATIshTREX1 plasmid, was able to colonize tumors at least as well as the strain that only had the *asd* gene

deletion and contained the same plasmid (AST-110). AST-113 colonized tumors with a mean of 1.2 x$10^7$ CFU per gram of tissue compared with a mean of 2.1 x $10^6$ cfu/g of tumor for AST-110, indicating that the absence of flagellin can lead to an increased tumor colonization by greater than 5 times that of strains with a functional flagella. Together, these data demonstrate that, contrary to the expectation from the art, not only is the flagella not required for tumor colonization, but its loss can enhance tumor colonization and anti-tumor immunity.

## Example 9

### *S. typhimurium* Engineered to Express cytoLLO for Enhanced Plasmid Delivery

In this example, the *asd* deleted strain of YS1646 described in Example 1 (AST-101) was further modified to express the listeriolysin O (LLO) protein lacking the signal sequence that accumulates in the cytoplasm of the *Salmonella* strain (referred to herein as cytoLLO). LLO is a cholesterol-dependent pore-forming cytolysin that is secreted from *Listeria monocytogenes* and mediates phagosomal escape of bacteria. A gene encoding LLO, with codons 2-24 deleted, was synthesized with codons optimized for expression in *Salmonella*. The sequence of the open reading frame of cytoLLO is in SEQ ID NO:240. The cytoLLO gene was placed under control of a promoter that induces transcription in *S. typhimurium* (SEQ ID NO: 241, reproduced below). The cytoLLO expression cassette was inserted in single copy into the knockout-out *asd* locus of the *asd* deleted strain AST-101 using modifications of the method of Datsenko and Wanner (*Proc Natl Acad Sci USA* (2000) 97:6640–6645), as described in Example 1.

**Sequence of promoter driving expression of cytoLLO**

| | | |
|---|---|---|
| LLO promoter | attatgtcttgacatgtagtgagtgggctggtataatgcagcaag | SEQ ID NO: 241 |

The *asd* deleted strain with the cytoLLO expression cassette inserted at the *asd* locus (referred to herein as ASD/LLO or AST-114) was further modified by electroporation with a pATI plasmid encoding an *asd* gene that allows the strain to grow in the absence of exogenous DAP and selects for plasmid maintenance, and also contains a U6 promoter driving expression of shTREX1 as described in Example 7 (referred to herein as ASD/LLO (pATI-shTREX1) or AST-115). As shown in **Fig. 39**, the ASD/LLO (pATI-shTREX1) strain AST-115 grew at a comparable rate to the *asd*

deleted strain containing the same plasmid (pATI-shTREX1), AST-110, demonstrating that the LLO knock-in does not impact bacterial fitness *in vitro*.

**_S. typhimurium_ engineered to produce cytoLLO demonstrate potent anti-tumor activity**

To determine whether the *cytoLLO* gene knock-in provided anti-tumor efficacy, the ASD/LLO (pATI-shTREX1) strain AST-115 was evaluated in a murine model of colon carcinoma. For this study, 6-8 week-old female BALB/c mice (8 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected with a single dose of 5 $x10^6$ CFUs of AST-115, and compared to PBS control.

As shown in **Fig. 40**, the addition of the *cytoLLO* gene into the *asd* strain ASD/LLO (pATI-shTREX1) demonstrated highly significant tumor control compared to PBS control (76% TGI, $p = 0.002$, day 28), and comparable efficacy after a single dose to previous studies where the TREX1 shRNA plasmid containing strains were given at multiple doses. These data demonstrate the *cytoLLO*-mediated advantage of delivering more plasmid into the cytosol, resulting in greater gene knockdown, thereby improving the therapeutic efficacy of RNAi against targets such as TREX1.

## Example 10

## Adenosine Auxotrophic Strains of *S. typhimurium*

Strains provided herein are engineered to be auxotrophic for adenosine. As a result, they are attenuated *in vivo* because they are unable to replicate in the low adenosine concentrations of normal tissue, therefore colonization occurs primarily in the solid tumor microenvironment where adenosine levels are high. The *Salmonella* strain YS1646 (AST-100) is a derivative of the wild type strain ATCC14028, and was engineered to be auxotrophic for purine due to disruption of the *purI* gene (Low *et al.*, (2004) *Methods Mol. Med* 90:47-60). Subsequent analysis of the entire genome of YS1646 demonstrated that the *purI* gene (synonymous with *purM*) was not in fact deleted, but was instead disrupted by a chromosomal inversion (Broadway *et al.* (2014) *J.Biotechnol. 20*:177-178), and that the entire gene is still contained within two parts of the YS1646 chromosome that is flanked by insertion sequences (one of which has an active transposase). The presence of the complete genetic sequence of the *purI* gene disrupted by means of a chromosomal reengagement leaves open the possibility

of reversion to a wild type gene. While it has previously been demonstrated that purine auxotrophy of YS1646 was stable after serial passage *in vitro*, it was not clear what the reversion rate is (Clairmont *et al.* (2000) *J. Infect. Dis.* 181:1996-2002).

It is shown herein that, when provided with adenosine, YS1646 is able to replicate in minimal medium; whereas the wild-type parental strain ATCC14028 can grow in minimal media that is not supplemented with adenosine. YS1646 was grown overnight in LB medium washed with M9 minimal medium and diluted into M9 minimal media containing no adenosine, or increasing concentrations of adenosine. Growth was measured using a SpectraMax® M3 spectrophotometer (Molecular Devices) at 37 °C, reading the $OD_{600}$ every 15 minutes.

As shown in **Fig. 41**, YS1646 was able to replicate when adenosine was provided at concentrations ranging from 11 to 300 micromolar, but was completely unable to replicate in M9 alone or M9 supplemented with 130 nanomolar adenosine. These data demonstrate that *purI* mutants are able to replicate in concentrations of adenosine that are found in the tumor microenvironment, but not at concentrations found in normal tissues. Engineered adenosine auxotrophic strains exemplified herein include strains wherein all, or portions of the *purI* open reading frame are deleted from the chromosome to prevent reversion to wild-type. Such gene deletions can be achieved utilizing the lambda red system as described in Example 1.

*Salmonella* strains containing a *purI* disruption, further engineered to contain an *asd* gene deletion (ASD) as described in Example 1, or *asd* gene deletion further engineered to have deletions of *fliC* and *fljB* and (ASD/FLG), as described in Example 8, or *asd* mutants further engineered to express cytoLLO (ASD/cLLO) as described in Example 9 and complemented with a low copy number plasmid (pATIlow) expressing *asd* as described in Example 7 (Strains AST-117, AST-118, and AST-119, respectively), were also evaluated for growth in M9 minimal media. The data in **Fig. 42** show that each strain was able to replicate when adenosine was provided at concentrations ranging from 11 to 300 micromolar, but was completely unable to replicate in M9 alone or M9 supplemented with 130 nanomolar adenosine.

**Example 11**

**Characterization and use of the *asd* Gene Complementation System *in vitro***

**Growth of Strains with *asd* Gene Complementation**

To assess fitness of the bacterial strains containing plasmids, growth curves were performed in LB liquid media using a Spectramax plate reader at 37 °C, reading the $OD_{600}$ every 15 minutes. As Shown in **Fig. 43,** YS1646 containing a low copy plasmid pEQU6-shTREX1 (AST-104) grew comparably to YS1646 that did not contain a plasmid (AST-100). An *asd* mutant strain harboring a high copy shTREX1 plasmid with an *asd* gene that can complement the *asd* auxotrophy (AST-110) was able to replicate in LB in the absence of DAP, but grew slower than YS1646. An *asd* deleted strain containing an shTREX-1 expression plasmid with low copy number origin of replication and an *asd* gene that can complement the *asd* auxotrophy (pATIlow-shTREX1), strain AST-117, grew at a faster rate than AST-110. These data demonstrate that low copy number plasmids that complement the *asd* gene auxotrophy are superior to high copy number plasmids, as they allow for more rapid replication rates of *S. typhimurium in vitro*.

**Intracellular growth of *asd* complemented strains**

To measure fitness of the *asd* mutants complemented with *asd* on high and low copy plasmids, the ability of bacterial strains to replicate intracellularly in mouse tumor cell lines was assessed using a gentamycin protection assay. In this assay, mouse melanoma B16.F10 cells or mouse colon cancer CT26 cells were infected with *asd* mutant *Salmonella* strains containing plasmids that contain a complementary *asd* gene and have either a high copy origin of replication, AST-110 (ASD pATI-shTREX1) or a low copy origin of replication, AST-117 (ASD pATI low copy-shTREX1). Cells were infected at a multiplicity of approximately 5 bacteria per cell for 30 minutes, then cells were washed with PBS, and medium containing gentamicin was added to kill extracellular bacteria. Intracellular bacteria are not killed by gentamicin, as it cannot cross the cell membrane. At various time points after infection, cell monolayers were lysed by osmotic shock with water and the cell lysates were diluted and plated on LB agar to enumerate surviving colony forming units (CFU).

As shown in **Fig. 44**, the *asd* mutant strain complemented with a high copy plasmid, AST-110, had an initial decline in CFU, but was able to grow in B16.F10

cells but not in CT26 cells, demonstrating that the *asd* gene complementation system is sufficient to support growth inside mammalian tumor cells. The *asd* mutant strain containing the low copy plasmid, AST-117, was able to invade and replicate in both cell types, demonstrating that *asd* gene complementation on a low copy plasmid allows for robust *asd* mutant growth inside mammalian cells. The strain with low copy plasmid replicated to higher numbers in both tumor cell types compared to the strain with a high copy plasmid. This demonstrates that *Salmonella* strains with low copy plasmids have enhanced fitness over strains with high copy plasmids.

**Plasmid maintenance in tumors using *asd* complementation system**

In this example, CT26 tumor-bearing mice were treated with YS1646 containing a plasmid that expresses an shRNA targeting TREX1 (pEQU6-TREX1), strain AST-104, or an *asd* deleted strain of YS1646 containing a plasmid with a functional *asd* gene and an shRNA targeting TREX1 (pATI-shTREX1), strain AST-110. At 12 days after the final *Salmonella* injection, tumors were homogenized, and homogenates were serially diluted and plated on LB agar plates to enumerate the total number of CFUs present, or on LB plates containing kanamycin to enumerate the number of kanamycin resistant colonies.

As shown in **Fig. 45**, *S. typhimurium* that did not have selective pressure to maintain the shRNA plasmid, AST-104, demonstrated plasmid loss, as the percent kanamycin resistant (KanR) colonies was less than 10%. The strain that used the *asd* gene complementation system for plasmid maintenance, AST-110, had nearly identical numbers of kanamycin resistant and kanamycin sensitive CFUs. These data demonstrate that the *asd* gene complementation system is sufficient to maintain the plasmid in the context of the tumor microenvironment in mice.

**Enhanced anti-tumor efficacy using *asd* complementation system**

The *asd* complementation system is designed to prevent plasmid loss and potentiate the anti-tumor efficacy of the inhibitory RNA delivery by *S. typhimurium* strains *in vivo*. To test this, *asd* deleted strains containing shTREX1 plasmid (AST-110) or scrambled control (AST-109) that contain a functional *asd* gene cassette were compared to YS1646 containing pEQU6-shTREX1 (AST-104, a plasmid that lacks an *asd* gene cassette and therefore does not have a mechanism for plasmid maintenance) for anti-tumor efficacy in a murine colon carcinoma model. For this experiment, 6-8

week-old female BALB/c mice (8 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected twice, on day 8 and day 18, with $5x10^6$ CFUs of AST-109 (ASD transformed with pATI-shScramble), AST-110 (ASD transformed with pATI-shTREX1), or AST-104 (YS1646 transformed with pEQU6-shTREX1) and compared to PBS control.

As shown in **Fig. 46**, the YS1646 strain AST-104 demonstrated tumor control compared to PBS (70% TGI, day 28) despite its demonstrated plasmid loss over time. The *asd* strain containing the scramble control in a pATI plasmid with the *asd* gene complementation system (AST-109) demonstrated tumor control compared to PBS (51% TGI, day 25), indicating that maintained delivery of CpG plasmids stimulates an anti-tumor response. The *asd* strain containing plasmid with the *asd* gene complementation system and shTREX1 (AST-110) demonstrated the highest tumor growth inhibition compared to PBS (82% TGI, $p = 0.002$, day 25). These data demonstrate that improved potency is achieved by preventing plasmid loss using the *asd* complementation system and delivery of shTREX1, as compared to YS1646 containing plasmids without gene complementation systems or shTREX1.

***S. typhimurium* strains with low copy plasmids demonstrate superior anti-tumor efficacy and tumor colonization compared to high copy plasmids**

In order to compare the anti-tumor efficacy of the low copy shTREX1 plasmid with the *asd* complementation system, relative to the high copy shTREX1plasmid in a murine model of colon carcinoma, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected with two weekly doses of 5 $x10^6$ CFUs of AST-117 (ASD (pATI Low-shTREX1)) or AST-110 (ASD (pATI-shTREX1) and were compared to PBS injections as a negative control. As shown in **Fig. 47**, the strain with the low copy plasmid, AST-117, demonstrated superior anti-tumor efficacy compared to the strain with the high copy plasmid AST-110 (High 59 %TGI, Low 79%TGI, $p = 0.042$, day 25).

At the end of this tumor growth inhibition study, 4 mice from each group were euthanized, and tumors and spleens were homogenized as described above to evaluate tumor colonization and tumor to spleen colonization ratios. As shown in **Fig. 48A**, the

strain containing the low copy plasmid, AST-117, colonized tumors at a level greater than 100 times higher than the strain with the high copy plasmid, AST-110. When the ratio of colonies recovered from tumor and spleen were calculated, AST-117 had a greater than 10-fold higher tumor to spleen colonization ratio compared to AST-110 (**Fig. 48B**), demonstrating that the strain with the low copy plasmid had greater specificity for tumor colonization than the strain with the high copy plasmid. These data demonstrate a previously unknown attribute that *S typhimurium* engineered to deliver plasmids encoding interfering RNAs have improved tumor colonizing capabilities and anti-tumor efficacy when the plasmids have low copy number origins of replication.

## Example 12

## *S. typhimurium* Harvested at Log vs Stationary Phase

**Production of log vs stationary injection stocks**

It has been demonstrated that the *Salmonella* pathogenicity island-1 (SPI-1) genes of *Salmonella typhimurium* are induced during logarithmic growth (Lundberg *et al.* (1999) *Journal Of Bacteriology* 181:3433–3437). This pathogenicity island is essential for uptake in non-phagocytic cells, such as epithelial cells, or cells derived from solid tumors. Induction of SPI-1 genes during late log has also been demonstrated to result in rapid pyroptosis (caspase-1-dependent proinflammatory programmed cell death) of macrophages (Fink *et al.* (2007) *Cell Microbiol.* 9(11): 2562–2570).

To determine the optimal phase of growth for production of *Salmonella typhimurium*-based immunotherapy, strains were produced by growing overnight cultures in LB at 37 °C with agitation. The overnight cultures were diluted into fresh LB in disposable shaker flasks and grown until the $OD_{600}$ reached 1.0 for late-log phase, or until the culture stopped increasing in OD for stationary phase (approximately 2 hours). The cultures were washed in PBS and suspended in a volume of PBS + 15% glycerol that result in a stock concentration $OD_{600}$ of 1.0 for cryopreservation to produce injection stocks at approximately $1 \times 10^9$ CFU/mL. The injection stocks were then stored at -80 °C.

**Modified *S. typhimurium* strains grown to stationary phase demonstrate equivalent anti-tumor potency with and superior tolerability compared to strains grown to log phase**

To determine the impact that the phase of culture at harvest has on *in vivo* activity, log vs stationary phase cultures of the modified *Salmonella typhimurium* strains were evaluated in a murine model of colon carcinoma. 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were IV injected with three weekly doses of $5 x10^6$ CFUs of AST-104 (YS1646 transformed with pEQU6-shTREX1) strains harvested at log or stationary phase, and compared to PBS control. Six hours following the first IV dose, mice were bled, and plasma was collected and assessed for pro-inflammatory cytokines using the Mouse Inflammation Cytometric Bead Array kit and analyzed by FACS (BD Biosciences).

As shown in **Fig. 49A**, the AST-104 log and AST-104 stationary phase injection stocks demonstrated comparable anti-tumor efficacy compared to the PBS control group (log - 67% TGI, $p = 0.04$, stationary – 77% $p = 0.01$, day 28), with the stationary phase injection stock demonstrating slightly better tumor growth inhibition. Comparing the levels of systemic serum cytokines at 6 hours post IV injection, the inflammatory cytokines elicited by the log phase injection stock were significantly higher for both TNF-α ($p = 0.007$), and IL-6 ($p = 0.016$), compared to the AST-104 stationary phase strain (**Fig. 49B**). These data demonstrate that growing bacterial therapeutic strains to stationary phase prior to IV administration can significantly reduce inflammatory toxicity and can improve tumor growth inhibition, indicating that the therapeutic index can be improved with material harvested at stationary phase.

## Example 13

## Engineering of an Autolytic *S. typhimurium* Strain for Delivery of RNAi

As described above, the *asd* gene in *S. typhimurium* encodes aspartate semialdehyde dehydrogenase. Deletion of this gene renders the bacteria auxotrophic for diaminopimelic acid (DAP) when grown *in vitro* or *in vivo*. This example employs an *asd* deletion strain (described in Example 1) that is auxotrophic for DAP and contains a plasmid suitable for delivery of RNAi that does not contain an *asd*-

complementing gene so that the strain 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 mammalian hosts where DAP is not present, which results in autolysis of the bacteria. Autolytic strains are able to invade host cells, but are not able to replicate due to the absence of DAP in mammalian tissues; this combination of attributes allows for RNAi-mediated gene knockdown and increased safety relative to replicating strains.

In this example, the *asd* deleted strain of YS1646 (AST-101, described in Example 1) was further modified to express cytoLLO to generate strain AST-114 (described in Example 9), was electroporated to contain a plasmid encoding ARI-203 (a microRNA targeting TREX1, described in Example 2), to make strain AST-120 (ASD/LLO (pEQU6-miTREX1)). When this strain is introduced into tumor bearing mice, the bacteria are taken up by host cells and enter the *Salmonella* containing vacuole (SCV). In this environment, the lack of DAP prevents replication, and result in lysis of the bacteria in the SCV. Lysis of AST-120 allows for release of the plasmid, and the accumulated cytoLLO that form pores in the cholesterol-containing SVC membrane, resulting in efficient delivery of the plasmid into the cytosol of the host cell.

The ability of the autolytic strain AST-120, to replicate in LB in the presence or absence of DAP was assessed using a SpectraMax® M3 spectrophotometer (Molecular Devices) at 37 °C, reading the $OD_{600}$ every 15 minutes. As shown in **Fig. 50**, AST-120 is able to grow robustly in LB supplemented with 50 µg/mL DAP, but cannot replicate in LB alone.

**Increased attenuation of autolytic *S. typhimurium* in mice**

To determine whether the autolytic strain AST-120, engineered to deliver cytoLLO and a microRNA targeting TREX1, was attenuated for virulence, a median lethal dose ($LD_{50}$) study was performed. Increasing doses of AST-120, ranging from $1x10^6$ to $5x10^7$ CFU, were administered IV to C57BL/6 mice (a strain of mouse that is highly sensitive to LPS). After IV administration, AST-120 was well tolerated at all doses with transient weight loss observed after a single dose. A second dose was administered 7 days after the first dose and one mouse out of four, at the highest dose level ($5x10^7$ CFU), was found moribund and required euthanasia. All other mice

administered AST-120 experienced transient weight loss, but recovered. These data indicate that the $LD_{50}$ for the autolytic strain of *S. typhimurim* delivering a micro-RNA targeting TREX1 (AST-120) is greater than $5x10^7$ CFU. The $LD_{50}$ for the VNP20009 strain is known to be approximately $5 x10^6$ in C57BL/6 mice (Lee *et al.* (2000) *International Journal of Toxicology* 19:19–25), demonstrating that AST-120 is at least 10-fold attenuated compared to VNP20009.

**Antitumor activity of autolytic *S. typhimurium***

To determine whether the autolytic strain AST-120, engineered to deliver cytoLLO and a microRNA targeting TREX1, was able to provide an anti-tumor response, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right flank with CT26 ($2x10^5$ cells in 100 μL PBS). Mice bearing established flank tumors were IV injected with a single dose of $5 x10^6$ CFUs of the autolytic strain AST-120 (ASD/LLO (pEQU6-miTREX1)) and compared to mice treated with PBS as a control. As shown in **Fig. 51**, an antitumor response was detected after only a single dose, compared to animals treated with PBS alone (52.4%TGI, $p = 0.02$, day 17). Together, these data demonstrate that *S. typhimurium* engineered to be autolytic by means of DAP auxotrophy and engineered to contain a plasmid for delivery of RNAi targeting TREX1, are exquisitely attenuated and can elicit an anti-tumor response.

## Example 14

## Exemplary strains engineered for increased tolerability

***adrA* or *csgD* deletion**

In this example, a live attenuated strain of *Salmonella typhimurium* that contains a *purI* deletion, an *msbB* deletion, an *asd* gene deletion and is engineered to deliver plasmids encoding interfering RNA, is further modified to delete *adrA*, a gene required for *Salmonella typhimurium* biofilm formation. *Salmonella* that cannot form biofilms are taken up more rapidly by host phagocytic cells and are cleared more rapidly. This increase in intracellular localization enhances the effectiveness of plasmid delivery and gene knockdown by RNA interference. The increased clearance rate from tumors/tissues increases the tolerability of the therapy, and the lack of biofilm formation prevents colonization of prosthetics and gall bladders in patients. In another example, a live attenuated strain of *Salmonella typhimurium* that contains a

*purI* deletion, an *msbB* deletion, an *asd* gene deletion and is engineered to deliver plasmids encoding interfering RNA, is further modified to delete *csgD*. This gene is responsible for activation of *adrA*, and also induces expression of the curli fimbriae, a TLR2 agonist. Loss of *csgD* also prevents biofilm formation, with the added benefit of inhibiting TLR2 activation, thereby further reducing the bacterial virulence and enhancing delivery of RNAi.

***pagP* deletion**

In this example a live attenuated strain of *S. typhimurium* that contains a *purI* deletion, an *msbB* deletion, and an *asd* gene deletion, and is engineered to deliver plasmids encoding interfering RNA, is further modified to delete *pagP*. The *pagP* gene is induced during the infectious life cycle of *S. typhimurium* and encodes an enzyme that palmitylates lipid A. In wild type *S. typhimurium,* expression of *pagP* results in a lipidA that is hepta-acylated. In an *msbB*- mutant in which the terminal acyl chain of the lipid A cannot be added, the expression of *pagP* results in a hexa-acylated LPS. Hexa-acylated LPS has been shown to be the most pro-inflammatory. In this example, a strain deleted of *pagP* and *msbB* can produce only penta-acylated LPS, allowing for lower pro-inflammatory cytokines, enhanced tolerability, and increased adaptive immunity when the bacteria are engineered to deliver interfering RNAs.

***hilA* deletion**

In this example a live attenuated strain of *Salmonella typhimurium* that contains a *purI* deletion, an *msbB* deletion, an *asd* gene deletion and is engineered to deliver plasmids encoding interfering RNA, is further modified to delete *hilA*. *hilA* is a regulatory gene that is required for expression of the salmonella pathogenicity island -1 (SPI-1)-associated type 3 secretion system (T3SS). This secretion system is responsible for injecting effector proteins into the cytosol of non-phagocytic host cells, such as epithelial cells, that cause the uptake of modified *S. typhimurium*. The SPI-1 T3SS has been shown to be essential for crossing the gut epithelial layer, but is dispensable for infection when bacteria are injected parenterally. The injection of some proteins and the needle complex itself can also induce inflammasome activation and pyroptosis of phagocytic cells. This pro-inflammatory cell death can limit the initiation of a robust adaptive immune response by directly inducing the death of

antigen-presenting cells (APCs), as well as modifying the cytokine milieu to prevent the generation of memory T-cells. In this example, the additional deletion of the *hilA* gene from a therapeutic *Salmonella typhimurium* strain that is administered either intravenously or intratumorally focuses the *Salmonella typhimurium* infection towards phagocytic cells that do not require the SPI-1 T3SS for uptake, and then prolongs the longevity of these phagocytic cells. The *hilA* mutation reduces the quantity of pro-inflammatory cytokines, increasing the tolerability of the therapy, as well as the quality of the adaptive immune response.

**Example 15**

**TREX1 Expression is Upregulated in Multiple Human Tumor Types**

In order to evaluate whether TREX1 is found upregulated in tumor tissue as compared to normal human tissue, an analysis was performed to assess the relative gene expression of the TREX1 gene using the cancer genome atlas (TCGA) database. As shown in **Fig. 52**, a broad array of tumor types demonstrated significant upregulation of TREX1 compared to normal tissue, including breast, prostate, uterine, bladder and cervical (*p* values: BRCA: 7.7e-16; PRAD: 9.4e-12; UCEC: 2.5e-05; BLCA: 3.7e-03; CESC: 7.7e-03). In addition, TREX1 was found upregulated in multiple forms of kidney cancer (*p* values: KIPAN: 8.9e-39; KIRC: 9.6e-35; KIRP: 5.8e-14; KICH: 4.9e-08). These data validate the phenomenon of TREX1 upregulation broadly correlating with tumor progression, and support its targeting as a promising cancer therapeutic strategy, as provided herein.

**Example 16**

**The modified *Salmonella typhimurium* pEQU6 Strains Containing shRNA to Multiple Immune Targets Demonstrate Potent Anti-tumor Growth Inhibition**

To compare the efficacy of a set of shRNA immune targets in a murine colon tumor flank model, 6-8 week-old female BALB/c mice (10 mice per group) were inoculated SC in the right and left flanks with CT26 (2 x$10^5$ cells in 100 µL PBS). Mice bearing established flank tumors were intratumorally (IT) injected twice, four days apart, on days 10 and 14 post tumor implantation into the right flank tumor with 5x$10^6$ CFUs each of YS1646, YS1646 (pEQU6-shVISTA), YS1646 (pEQU6-shBeta-catenin), or YS1646 (pEQU6-shTGF-beta), and compared to PBS control.

IT injection of AST-121 (YS1646 carrying pEQU6-shVISTA) induced significant tumor growth inhibition in the injected and distal tumors compared to the PBS control (injected tumor = 75% TGI, $p = 0.01$; distal tumor TGI = 57% TGI, $p = 0.04$), including one complete response, demonstrating the *in vivo* potency of inhibiting this immune checkpoint using this therapeutic modality. AST-122, (YS1646 carrying pEQU6-shTGF-beta) also demonstrated potent tumor inhibition of both the injected and distal lesions (injected tumor = 52%; distal TGI = 48.4%). AST-123 (YS1646 carrying pEQU6-shBeta-catenin) demonstrated tumor growth inhibition (injected TGI = 33.1%, distal TGI = 17% TGI), including one complete response. These strains were prepared in stationary phase instead of log-phase. In log-phase, SPI-1 would be expected to be maximally upregulated, which would have enhanced tumor cell targeting and improved the efficacy of targeting beta-catenin.

**Example 17**

**Radiotherapy Enhances Tumor Colonization of Immunostimulatory Bacteria Containing a Plasmid Encoding a microRNA to TREX1 and Enhances Efficacy in Combination with Immune Checkpoint Blockade**

Radiation therapy has been shown to synergize with *S. typhimurium* to promote tumor growth inhibition. A previous study demonstrated enhanced tumor growth inhibition with the combination of a single IV administration of $5 \times 10^5$ CFU of *S. typhimurium* (YS1646) followed by 15 Gy radiation by in a murine B16.F10 melanoma flank model (Bermudes *et al.* (2001) *Biotechnol Genet Eng Rev.* 18:1).

To determine the effect of radiation on bacterial tumor colonization, 6-8 week-old female BALB/c mice were inoculated subcutaneously in the right flank with $1 \times 10^5$ mouse TSA breast carcinoma cells (in 100 µL PBS). Mice bearing established tumors were administered the following: 1) PBS IV followed by 0 Gy radiation (1 mouse); 2) IV injection of $5 \times 10^6$ CFUs of AST-106 (YS1646 transformed with pEQU6-miTREX1, ARI-203), followed 4 hours later with 0 Gy (3 mice); 3) $5 \times 10^6$ CFUs of AST-106, followed 4 hours later with 20 Gy (3 mice); 4) 20 Gy, followed 4 hours later with $5 \times 10^6$ CFUs of AST-106 (3 mice). Radiotherapy was administered using an XStrahl SARRP as described in Vanpouille-Box *et al.* (2017) *Nat Commun.* 8:15618. Mice were sacrificed 24 hours later, and tumors were harvested and weighed. Tumors were homogenized in 10 mL sterile PBS (M tubes, GentleMACs™,

Miltenyi Biotec), then 10-fold serial dilutions were performed and plated on LB (Luria Broth) agar plates containing kanamycin. The following day, colony forming units (CFUs) were counted and CFU per gram of tumor tissue was calculated.

As shown in **Fig. 53**, administration of 20 Gy of radiation prior to IV administration of AST-106 resulted in fewer CFU/g than administering AST-106 IV alone, with no radiation. Administration of 20 Gy of radiation after administration of AST-106 IV demonstrated significantly enhanced tumor colonization, compared to the opposite regimen ($p < 0.05$).

Experiments are performed to determine whether IV administration of *S. typhimurium* containing shTREX1, prior to administering 20 Gy of radiation, would inhibit the activity of TREX1 and potentiate the abscopal activity of the radiation therapy. As discussed in the detailed description, TREX1 has been shown to suppress the abscopal anti-tumor efficacy of radiation, even with the addition of the checkpoint inhibitor anti-CTLA4. The potentiating effects of adminstration of the *S. typhimurium* containing shTREX1 prior to administration of the radiation therapy is further enhanced in the presence of anti-CTLA4 or anti-PD-1 therapy.

To demonstrate this, administration of the modified *S. typhimurium* shTREX1 is combined with 20 Gy of radiotherapy in the presence or absence of anti-CTLA4 or anti-PD-1 immune checkpoint blockade in a dual flank TSA murine mammary carcinoma model. For these studies, 6-8 week-old female BALB/c mice are inoculated subcutaneously in the right and left flanks with $1x10^5$ mouse TSA breast carcinoma cells (in 100 μL PBS). Mice bearing established tumors are administered radiotherapy to the right flank tumor on concurrent days using an XStrahl SARRP as described in Vanpouille-Box *et al.* ((2017) *Nat Commun.* 8:15618), in two doses of 20 Gy, or 3 fractions of 8 Gy on consecutive days. Mice are administered IV injections beginning 4 hours post the initial radiation treatment and repeated 4 and 7 days later with $1\text{-}5x10^6$ CFUs of the modified *Salmonella typhimurium* shTREX1, or the modified *Salmonella typhimurium* containing a scrambled shRNA control (modified *Salmonella typhimurium* scr). Some groups of mice are concurrently administered the checkpoint therapy anti-CTLA4 or anti-PD-1 (100 μg) or isotype control IP twice weekly. Mice are bled seven days following the last IV modified *Salmonella typhimurium* injection and PBMCs assessed for the ability to produce IFN-γ in

response to the immunodominant $CD8^+$ T cell epitope AH1 [SPSYVYHQF]-specific tetramer by flow cytometry. Separate groups of mice are harvested for spleen, tumor and tumor-draining lymph nodes 48 hours and 7 days post modified *Salmonella typhimurium* IV treatment and assessed for lymphoid and myeloid populations by flow cytometry, and tissue is assessed for CFUs by homogenization and plating on LB agar plates. Remaining mice are assessed for tumor growth in the primary irradiated tumor and the distal (abscopal) tumor by caliper measurements, and mice that demonstrate complete tumor regression are re-challenged with autologous tumors and compared to age-matched, tumor-naïve mice. Separate groups of mice are depleted of $CD4^+$ and/or $CD8^+$ T cells prior to re-challenge, to demonstrate the requirement for adaptive immunity. These data demonstrate that inhibition of Trex1 in the context of high dose radiation therapy enhances the anti-tumor immunity of the combined immunotherapies.

## Example 18

## The Addition of Anti-PD-1 Antibody to Modified *Salmonella typhimurium* Therapy Containing Plasmid Encoding Anti-TREX1 microRNA Enhances Distal Tumor Regression in a CD8-dependent Manner in the Dual Flank Murine Colon Carcinoma Model

To demonstrate that addition of anti-PD-1 checkpoint therapy can enhance the efficacy of AST-106 (YS1646 carrying a plasmid encoding a microRNA to TREX1), 6-8 week-old female BALB/c mice (10 mice per group) were inoculated subcutaneously (SC) in the right and left flanks with CT26 ($2 \times 10^5$ cells in 100 µL PBS) to establish tumors. Mice bearing established flank tumors were intratumorally (IT) injected on days 10 and 14 post tumor implantation into the right flank tumor with $5 \times 10^6$ CFUs of AST-106 (YS1646 transformed with pEQU6-miTREX1, ARI-203), or AST-103 (YS1646 transformed with pEQU6-scrambled shRNA), and compared to PBS control, either alone or in combination with weekly IP injections of anti-PD-1 (4 mg/kg, clone RMP1-14, BioXCell). To determine whether the primary and distal tumor efficacy was dependent on $CD8\alpha^+$ T cells and DCs, groups were administered anti-CD8α depleting antibody IP on days 5 and 7, prior to IT injection, and then on days 10, 14 and 17 (4 mg/kg, clone 2.43, BioXCell).

IT injection of AST-106, the YS1646 strain containing a plasmid encoding a miTREX1, induced significant tumor growth inhibition in the injected tumor and distal tumors, compared to PBS control (injected TGI: 67.5%, distal TGI: 67.2%; $p$ = 0.027). This anti-tumor activity was completely abrogated with depletion of $CD8\alpha^+$ cells (injected TGI: 14.6%, distal TGI: 0%), demonstrating the requirement for cytolytic $CD8^+$ T cells and $CD8\alpha^+$ DCs for AST-106 anti-tumor activity. The administration of anti-PD-1 antibody with AST-106 further enhances the activity of the AST-106, resulting in 2/10 complete remissions. This effect also was completely reversed upon $CD8\alpha^+$ cell depletion. No other groups of mice, other than those treated with the combination of AST-106 miTREX1 with anti-PD1 mAb, resulted in complete dual flank remissions, including the scramble control (AST-103) with anti-PD-1 antibody, or the anti-PD-1 antibody alone. These data demonstrate that engineered *S. typhimurium* containing a plasmid encoding an anti-TREX1 inhibitory microRNA induces a potent, CD8α-dependent adaptive immune response. This activity is synergistic with anti-PD-1 checkpoint therapy.

**Example 19**

**Examples of Additional Therapeutic Bacteria and Combination Therapy**

The table below sets forth, in the first column, targets of the RNA; the second column sets forth combinations of targets encoded by RNA in the plasmid; the third column sets forth the types (format) of the encoded RNA in the plasmids; and the fourth column sets forth exemplary additional therapeutic agents that can be used in combination therapy with the immunostimulatory bacteria in the table, or herein. The next column lists modifications to the genome of the bacterial strain, and the last column describes features of plasmids that can be used. Each of the listed elements in the columns can be matched with any other elements/features listed in the table and provided throughout the disclosure herein. The bacterium can be any therapeutic bacterium, particularly any listed throughout the disclosure herein, such as, but not limited to, *Salmonella, Shigella, E. coli, Bifidobacteriae, Rickettsia, Vibrio, Listeria, Klebsiella, Bordetella, Neisseria, Aeromonas, Franciesella, Cholera, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Bacillus,* and *Erysipelothrix.* Exemplary of such bacteria are *Salmonella* strains, such as *S.*

*typhimurium*. Among the *Salmonella typhimurium* strains are the well known strains designated VNP20009 (ATCC #202165), RE88, SL7207, χ 8429, χ 8431, and χ 8468.

| Target | RNAi + RNAi Combinations | RNAi format | Therapeutic Combinations | Therapeutic Strains | Plasmid features |
|---|---|---|---|---|---|
| TREX1 | TREX1+PD-L1 | shRNA | anti-PD-1 mAb | *asd* knockout | encodes asd gene |
| PD-L1 | TREX1+VISTA | microRNA | anti-CTLA4 mAb | *purI* (*purM*) knockout | low copy origin |
| VISTA | TREX1+SIRP-alpha | shRNA with RIG-I binding element | anti-VEGF mAb | *msbB* knockout | medium copy origin |
| TGF-beta | PD-L1+ TGF-beta | micro RNA with RIG-I binding element (polyA) | Radiation Therapy | cytoLLO knock-in | U6 Promoter |
| beta-catenin | PD-L1 + beta-catenin | | Immunogenic chemotherapy: nimustine, carmustine, fotemustine, topotecan, cisplatin, irinotecan, doxorubicin and etoposide | *purD* knockout | H1 Promoter |
| SIRP-alpha | PD-L1 + VISTA | | | flagellin (*fliC*/*FljB*) knockout | CMV Promoter for RNAi expression |
| VEGF | TGF-beta + VISTA | | | *pagP* knockout | removable Kan Cassette |
| Rnase H2 | SIRP-alpha + VISTA | | | *adrA* knockout | SV40 DNA nuclear targeting sequence |
| Dnase II | TREX1 + Rnase H2 | | | *hilA* knockout | CpG sequences |
| CLEVER-1/Stabilin-1 | | | | | |

Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

**WHAT IS CLAIMED:**

1. An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA, wherein:

the RNA inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increase the anti-tumor immune response in a subject;

the RNA is encoded on a plasmid in the bacterium;

the immunostimulatory bacterium is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$).

2. The immunostimulatory bacterium of claim 1, wherein the plasmid is present in low to medium copy number.

3. An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA, wherein:

the RNA inhibits, suppresses or disrupts expression of an immune checkpoint;

the RNA is encoded on a plasmid in the bacterium; and

the plasmid comprises one or more of a sequence of nucleic acid(s) encoding listeriolysin O (LLO) protein lacking the signal sequence (cytoLLO), a CpG motif, a DNA nuclear targeting sequence (DTS), a deletion of the gene encoding a flagellin subunit(s), and a retinoic acid-inducible gene-I (RIG-I) binding element.

4. The immunostimulatory bacterium of claim 3, wherein the immunostimulatory bacterium is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$).

5. The immunostimulatory bacterium of any of claims 1-4, wherein 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 the endogenous *asd* is not expressed.

6. The immunostimulatory bacterium of any of claims 1-5, wherein the plasmid comprises a medium-to-low copy number origin of replication.

7. The immunostimulatory bacterium of any of claims 1-6, wherein the plasmid comprises a low copy number origin of replication.

8. The immunostimulatory bacterium of any of claims 1-7, wherein the plasmid is present in low copy number.

9. The immunostimulatory bacterium of any of claims 1-6, wherein 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.

10. The immunostimulatory bacterium of any of claims 1-9, wherein low copy number is less than 25 or less than 20 or less than about 25 or less than about 20 copies.

11. The immunostimulatory bacterium of any of claims 1-10 that is auxotrophic for adenosine.

12. The immunostimulatory bacterium of any of claims 1-11 that is auxotrophic for adenosine and adenine.

13. The immunostimulatory bacterium of any of claims 1-12 that comprises a plasmid that encodes *asd* for expression in the bacterium.

14. The immunostimulatory bacterium of any of claims 1-12 that comprises a plasmid that encodes *asd* expressed by a bacterial promoter.

15. The immunostimulatory bacterium of any of claims 1-14 that comprises nucleic acid that includes a CpG motif, wherein the motif is recognized by toll-like receptor 9 (TLR9).

16. The immunostimulatory bacterium of claim 15, wherein the nucleic acid comprising a CpG motif is encoded on the plasmid.

17. The immunostimulatory bacterium of any of claims 1-16, that comprises a nucleic acid encoding a CpG motif, wherein the nucleic acid encoding the CpG motif is included in or is part of a bacterial gene that is encoded in the plasmid.

18. The immunostimulatory bacterium of claim 17, wherein the gene that comprises CpGs is *asd*.

19. The immunostimulatory bacterium of any of claims 1-18 that is flagellin deficient, wherein the wild-type bacterium comprises flagella.

20. The immunostimulatory bacterium of claim 19 that has a deletion in a gene(s) encoding the flagella.

21. The immunostimulatory bacterium of any of claims 1-20 that comprises deletions in the genes encoding one or both of flagellin subunits *fliC* and *fljB*, whereby the bacterium is flagella deficient, and wherein the wild-type bacterium expresses flagella.

22. The immunostimulatory bacterium of any of claims 1-21 that comprises nucleic acid encoding cytoLLO, which is listeriolysin O (LLO) protein lacking the signal sequence.

23. The immunostimulatory bacterium of claim 22, wherein the RNA inhibits, suppresses or disrupts expression of one or both of TREX1 and PD-L1 or one or both of TREX1 and PD-1.

24. The immunostimulatory bacterium of any of claims 1-23 that comprises a DNA nuclear targeting sequence (DTS) encoded on the plasmid.

25. The immunostimulatory bacterium of claim 24, wherein the DTS is an SV40 DTS.

26. The immunostimulatory bacterium of any of claims 1-25 that has a deletion or modification in the gene encoding endonuclease A (*endA*), whereby *endA* activity is inhibited or eliminated.

27. The immunostimulatory bacterium of any of claims 1-26 that comprises one or more of a CpG motif, an *asd* gene selectable marker for plasmid maintenance and a DNA nuclear targeting sequence.

28. The immunostimulatory bacterium of any of claims 1-27 that comprises nucleic acid on the plasmid encoding two or more different RNA molecules that inhibit, suppress or disrupt expression of an immune checkpoint or an RNA molecule that encodes an inhibitor of a metabolite that is immunosuppressive or in an immunosuppressive pathway.

29. The immunostimulatory bacterium of claim 28, wherein the plasmid comprises a transcriptional terminator following the RNA-encoding nucleic acid.

30. The immunostimulatory bacterium of any of claims 1-29, wherein the RNA is short hairpin RNA (shRNA) or micro-RNA (miRNA).

31. The immunostimulatory bacterium of any of claims 1-30, wherein the RNA inhibits, suppresses or disrupts expression of one or more of three prime repair exonuclease 1 (TREX1), PD-1, PD-L1 (B7-H1), VEGF, TGF-beta isoform 1, Beta-catenin, CTLA-4, PD-L2, PD-2, IDO1, IDO2, SIRPα, CD47, VISTA (B7-H5), LIGHT, HVEM, CD28, LAG3, TIM3, TIGIT, Galectin-9, CEACAM1, CD155, CD112, CD226, CD244 (2B4), B7-H2, B7-H3, ICOS, GITR, B7-H4, B7-H6, CD27, CD40/CD40L, CD48, CD70, CD80, CD86, CD137( 4-1BB),

CD200, CD272 (BTLA), CD160, CD39, CD73, A2a receptor, A2b receptor, HHLA2, ILT-2, ILT-4, gp49B, PIR-B, HLA-G, ILT-2/4, OX40/OX-40L, KIR, TIM1, TIM4, STAT3, Stabilin-1 (CLEVER-1), DNASE II and RNASE H2.

32. The immunostimulatory bacterium of any of claims 1-30, wherein the RNA inhibits, suppresses or disrupts expression of one or a combination of TREX1, PD-L1, VISTA, TGF-beta isoform 1, beta-catenin, SIRP-alpha, VEGF, RNase H2, DNase II, and CLEVER-1/Stabilin-1.

33. The immunostimulatory bacterium of any of claims 1-32, wherein:

the plasmid comprises a sequence of nucleotides that encodes RNA that inhibits, suppresses or disrupts expression of at least two targets; and

each RNA is expressed from a different promoter.

34. The immunostimulatory bacterium of claim 33, where the targets for inhibition, suppression or disruption comprise combinations of at least two that are selected from among TREX1 and PD-L1, TREX1 and PD-1, TREX1 and VISTA, TREX1 and SIRP-alpha, PD-L1 and TGF-beta isoform 1, PD-L1 and beta-catenin, PD-L1 and VISTA, TGF-beta isoform 1 and VISTA, SIRP-alpha and VISTA and TREX1 and RNASE H2.

35. The immunostimulatory bacterium of any of claims 1-34, wherein:

the plasmid encodes an shRNA or micro RNA that specifically inhibits, suppresses or disrupts expression of TGF-beta isoform 1 but not TGF-beta isoform 2 or TGF-beta isoform 3; or

the plasmid encodes an shRNA or micro RNA that specifically inhibits, suppresses or disrupts expression of TGF-beta isoforms 1 and 3, but not isoform 2.

36. The immunostimulatory bacterium of any of claims 1-35, wherein the plasmid encodes a sequence of nucleotides that is an agonist of retinoic acid-inducible gene I (RIG-I).

37. The immunostimulatory bacterium of any of claims 1-36 that is *msbB*$^-$.

38. The immunostimulatory bacterium of any of claims 1-37 that is one or more of a *purI*$^-$ *(purM*$^-$*)*, *msbB*$^-$, *purD*$^-$, flagellin$^-$ *(fliC*$^-$*/fljB*$^-$*)*, *pagP*$^-$, *adrA*$^-$, *Csg*D$^-$ and *hilA*$^-$.

39. The immunostimulatory bacterium of any of claims 1-38 that comprises a *purI* deletion, an *msbB* deletion, an *asd* deletion, and an *adrA* deletion.

40. The immunostimulatory bacterium of any of claims 1-39 that comprises:

one or more of a mutation in a gene that alters the biosynthesis of lipopolysaccharide selected from among one or more of *rfaL, rfaG, rfaH, rfaD, rfaP, rFb, rfa, msbB, htrB, firA, pagL, pagP, lpxR, arnT, eptA,* and *lpxT*; and/or

one or more of a mutation that introduces a suicide gene and is selected from one or more of *sacB, nuk, hok, gef, kil* and *phlA*; and/or

one or more of a mutation that introduces a bacterial lysis gene and is selected from one or both of *hly* and *cly*; and/or

a mutation in one or more virulence factor(s) selected from among *IsyA, pag, prg, iscA, virG, plc* and *act*; and/or

one or more mutations that modify the stress response selected from among *recA, htrA, htpR, hsp* and *groEL*; and/or

a mutation in *min* that disrupts the cell cycle; and/or

one or more mutations that disrupt or inactivate regulatory functions selected from among *cya, crp, phoP/phoQ* and *ompR*.

41. The immunostimulatory bacterium of any of claims 1-40, wherein the RNA is a short hairpin RNA (shRNA) or a micro-RNA (miRNA).

42. The immunostimulatory bacterium of claim 41, wherein:

the RNA is miRNA; and

the miRNA backbone into which the RNA that encodes the target or complement thereof is designated miR-16-2 (SEQ ID NO:248), or is the miRNA backbone of SEQ ID NO:249.

43. The immunostimulatory bacterium of any of claims 1-42, wherein the RNA is expressed under control of an RNA polymerase III (RNAP III) or RNA polymerase II (RNAP II) promoter.

44. The immunostimulatory bacterium of claim 41 or claim 43, wherein the RNA is a shRNA.

45. The immunostimulatory bacterium of any of claims 41-43, wherein the RNA is miRNA.

46. The immunostimulatory bacterium of any of claims 41, 43 and 44, wherein the promoter is RNAP III and the RNA is shRNA.

47. The immunostimulatory bacterium of any of claims 41-43 and 45, wherein the promoter is RNAP II and the RNA is miRNA.

48. The immunostimulatory bacterium of any of claims 41-46, wherein the RNA is expressed under the control of an RNAP III promoter selected from among U3, H1, U6, 7SK and 7SL.

49. The immunostimulatory bacterium of any of claims 41-45 and 47, wherein the RNA is expressed under the control of an RNAP II promoter that is a viral promoter.

50. The immunostimulatory bacterium of claim 49, wherein the viral promoter is selected from among a cytomegalovirus (CMV) promoter, an SV40 promoter, and an adenovirus promoter.

51. The immunostimulatory bacterium of any of claims 1-50, wherein the bacterium is a strain of *Salmonella, Shigella, E. coli, Bifidobacteriae, Rickettsia, Vibrio, Listeria, Klebsiella, Bordetella, Neisseria, Aeromonas, Francisella, Cholera, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Bacillus,* and *Erysipelothrix*, or an attenuated strain thereof or modified strain thereof of any of the preceding list of bacterial strains.

52. The immunostimulatory bacterium of any of claims 1-51 that is an attenuated bacterium.

53. The immunostimulatory bacterium of any of claims 1-52 that is a strain of *Salmonella.*

54. The immunostimulatory bacterium of claim 53 that is a *Salmonella typhimurium* strain.

55. An immunostimulatory bacterium that is a *Salmonella typhimurium* strain selected among strains AST-104, AST-105, AST-106, AST-108, AST-110, AST-112, AST-113, AST-115, AST-117, AST-118, AST-119, AST-120, AST-121, AST-122, and AST-123.

56. An immunostimulatory bacterium that comprises a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of three prime repair exonuclease 1 (TREX1), and is auxotrophic for adenosine.

57. An immunostimulatory bacterium that comprises a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of VISTA, and is auxotrophic for adenosine.

58. An immunostimulatory bacterium that comprises a sequence of nucleotides encoding RNA that inhibits, suppresses, disrupts expression of programmed death-ligand 1 (PD-L1).

59. An immunostimulatory *Salmonella* bacterium, comprising nucleic acid that encodes an RNA that inhibits, suppresses, disrupts or silences expression of three prime repair exonuclease 1 (TREX1).

60. An immunostimulatory *Salmonella* bacterium, comprising nucleic acid that encodes an RNA that inhibits, suppresses, disrupts or silences expression of VISTA.

61. An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of three prime repair exonuclease 1 (TREX1), and a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of PD-L1.

62. An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of VISTA, and a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of PD-L1.

63. The immunostimulatory bacterium of any of claims 1-62 that comprises a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of one or a combination of TREX1, PD-L1, VISTA, TGF-beta isoform 1, beta-catenin, SIRP-alpha, VEGF, RNase H2, DNase II, and CLEVER-1/Stabilin-1.

64. The immunostimulatory bacterium of claim 63, where the target for inhibition, suppression or disruption is a combination of at least two selected from among TREX1 and PD-L1, TREX1 and PD-1, TREX1 and VISTA, TREX1 and SIRP-alpha, PD-L1 and TGF-beta isoform 1, PD-L1 and beta-catenin, PD-L1 and

VISTA, TGF-beta isoform 1 and VISTA, SIRP-alpha and VISTA, TREX1 and RNASE H2, VISTA and RNASE H2, and VISTA and DNASE II.

65. An immunostimulatory bacterium that comprises a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of TREX1 and SIRPα, or TREX1 and VISTA, or TREX1 and VEGF, or PD-L1 and β-catenin, or PD-L1 and TGF-beta isoform 1, or PD-L1 and VEGF or TREX and PD-1.

66. The immunostimulatory bacterium of any of claims 1-65, wherein the bacterium further comprises nucleic acid encoding RNA that inhibits, suppresses or disrupts expression of another different immune checkpoint or target to be inhibited, suppressed or disrupted selected from among any of CTLA-4, PD-L1 (B7-H1), PD-L2, PD-1, PD-2, IDO1, IDO2, SIRPα, CD47, VISTA (B7-H5), LIGHT, HVEM, CD28, LAG3, TIM3, TIGIT, Galectin-9, CEACAM1, CD155, CD112, CD226, CD244 (2B4), B7-H2, B7-H3, ICOS, GITR, B7-H4, B7-H6, CD27, CD40/CD40L, CD48, CD70, CD80, CD86, CD137( 4-1BB), CD200, CD272 (BTLA), CD160, CD39, CD73, A2a receptor, A2b receptor, HHLA2, ILT-2, ILT-4, gp49B, PIR-B, HLA-G, ILT-2/4, OX40/OX-40L, KIR, TIM1, TIM4, STAT3, CLEVER-1, DNASE II and RNASE-H2.

67. The immunostimulatory bacterium of claim 66, wherein the other different immune checkpoint to be inhibited, suppressed or disrupted is selected from among human PD-L1 (SEQ ID NO:31), human Beta-catenin (SEQ ID NO:32), human SIRPα (SEQ ID NO:33), human TREX1 (SEQ ID NO:34), human VISTA (SEQ ID NO:35), human TGF-beta isoform 1 (SEQ ID NO:193) and human VEGF (SEQ ID NO:194).

68. The immunostimulatory bacterium of any of claims 1-67, wherein the RNA targets a sequence in the immune checkpoint nucleic acid set forth in any of SEQ ID NOs.: 1-30, 36-40, and 195-209.

69. The immunostimulatory bacterium of any of claims 1-68, wherein the nucleic acid encoding RNA that inhibits, suppresses or disrupts expression encodes an shRNA, or micro RNA (miRNA).

70. The immunostimulatory bacterium of any of claims 1-69, wherein the RNA is a miRNA that is miR-103, where mature miR-103 comprises the sequence:

5'-AGCAGCAUUGUACAGGGCUAUGA-3'.

71. The immunostimulatory bacterium of any of claims 1-70, wherein:

the RNA is encoded on a plasmid in the bacterium; and

the plasmid comprises a transcriptional terminator following the RNA-encoding nucleic acid.

72. The immunostimulatory bacterium of any of claims 1-71 or 56-71, wherein the plasmid comprises one or more of a sequence of nucleic acids encoding listeriolysin O (LLO) protein lacking the signal sequence (cytoLLO), a CpG motif, a DNA nuclear targeting sequence (DTS), a deletion of the gene encoding a flagellin subunit(s), and a retinoic acid-inducible gene-I (RIG-I) binding element.

73. The immunostimulatory bacterium of any of claims 1-72 or 56-72 that comprises a nucleic acid that includes a CpG motif, wherein the motif is recognized by toll-like receptor 9 (TLR9).

74. The immunostimulatory bacterium of any of claims 1-73 that comprises a *purI* deletion, an *msbB* deletion, an *asd* deletion, and *adrA* deletion.

75. The immunostimulatory bacterium of any of claims 1-74, wherein the immunostimulatory bacterium is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$).

76. The immunostimulatory bacterium of any of claims 1-75 or 56-75 that is one or more of *purI*$^-$ (*purM*$^-$), *msbB*$^-$, *purD*$^-$, flagellin$^-$ (*fliC*$^-$/*FljB*$^-$), *pagP*$^-$, *adrA*$^-$, and *hilA*$^-$.

77. The immunostimulatory bacterium of any of claims 1-76 or 56-76 that has a modification in the gene encoding endonuclease-1 (*endA*), whereby endA activity is inhibited or eliminated, wherein a modification is an insertion or deletion.

78. The immunostimulatory bacterium of any of claims 56-77 that comprises:

one or more of a mutation in a gene that alter the biosynthesis of lipopolysaccharide selected from among one or more of *rfaL, rfaG, rfaH, rfaD, rfaP, rFb, rfa, msbB, htrB, firA, pagL, pagP, lpxR, arnT, eptA, and lpxT*; and/or

one or more of a mutation that introduces a suicide gene and is selected from one or more of *sacB, nuk, hok, gef, kil* or *phlA*; and/or

one or more of a mutation that introduces a bacterial lysis gene and is selected from one or both of *hly* and *cly*; and/or

a mutation in one or more virulence factor(s) selected from among *IsyA, pag, prg, iscA, virG, plc* and *act*; and/or

one or more mutations that modify the stress response selected from among *recA, htrA, htpR, hsp* and *groEL*; and/or

a mutation in *min* that disrupts the cell cycle; and/or

one or more mutations that disrupt or inactivate regulatory functions selected from among *cya, crp, phoP/phoQ*, and *ompR*.

79. The immunostimulatory bacterium of any of claims 56-78, wherein:

the RNA is encoded on a plasmid in the bacterium; and

the plasmid comprises a transcriptional terminator following the RNA-encoding nucleic acid.

80. The immunostimulatory bacterium of any of claims 56-79, wherein the plasmid comprises a medium-to-low copy number origin of replication.

81. The immunostimulatory bacterium of any of claims 56-80, wherein the plasmid comprises a low copy number origin of replication.

82. The immunostimulatory bacterium of any of claims 56-81, wherein the plasmid is present in low copy number.

83. The immunostimulatory bacterium of any of claims 56-80, comprising a medium copy number plasmid, wherein 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.

84. The immunostimulatory bacterium of any of claims 56-82, comprising a low copy number plasmid, wherein low copy number is less than 25 or less than 20 or less than about 25 or less than about 20 copies.

85. The immunostimulatory bacterium of any of claims 1-84, wherein the origin of replication is selected from among the origins derived from pBR322, p15A, pSC101, pMB1, colE1, colE2, pPS10, R6K, R1, RK2, and pUC.

86. The immunostimulatory bacterium of any of claims 1-85, wherein the nucleic acid encoding RNA that inhibits, suppresses or disrupts expression is under the control of a eukaryotic promoter that is recognized by the subject for whom the bacterium is administered or to be administered.

87. The immunostimulatory bacterium of claim 86, wherein the promoter is selected from among U3, H1, U6, 7SK and 7SL.

88. The immunostimulatory bacterium of claim 86 or 87, wherein the subject is human.

89. The immunostimulatory bacterium of any of claims 56-88, wherein the nucleic acid encoding RNA that inhibits, suppresses or disrupts expression is encoded on a plasmid in the bacterium.

90. The immunostimulatory bacterium of any of claims 1-89, wherein each nucleic acid encoding RNA that inhibits, suppresses or disrupts expression encodes an shRNA that is under the control of a different promoter, wherein the promoter is a eukaryotic promoter.

91. The immunostimulatory bacterium of any of claims 1-90, wherein the nucleic acids encoding RNA that inhibits, suppresses or disrupts expression are each separated by at least about 75 nucleotides, or at least 75 nucleotides.

92. The immunostimulatory bacterium of any of claims 1-91, wherein, when there is more than one nucleic acid encoding RNA that inhibits, suppresses or disrupts expression each is separated by at least about 75 nucleotides, or at least 75 nucleotides, up to about or at least 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500 nucleotides (or base pairs), up to about 1600 or 1600 nucleotides (or base pairs), or between 75-1500 or 1600 nucleotides (or base pairs).

93. The immunostimulatory bacterium of any of claims 86-92, wherein the promoter is an RNA polymerase III promoter.

94. The immunostimulatory bacterium of any of claims 86-93, where the promoter is/are selected from among U3, H1, U6, 7SK and 7SL.

95. The immunostimulatory bacterium of any of claims 1-94, wherein the nucleic acid encoding the RNA that inhibits, suppresses or disrupts expression is contained on a plasmid in the bacterium.

96. The immunostimulatory bacterium of any of claims 56-95, wherein the bacterial strain is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$).

97. The immunostimulatory bacterium of any of claims 1-96, wherein the bacterial strain expresses *asd* on a plasmid; and its expression is under the control of a promoter expressed in a tumor microenvironment (TME).

98. The immunostimulatory bacterium of claim 97, wherein the promoter is expressed in hypoxic conditions, or in conditions where the pH is less than 7.

99. The bacterium of any of claims 56-98, wherein the bacterium is a strain of *Salmonella, Shigella, E. coli, Bifidobacteriae, Rickettsia, Vibrio, Listeria,*

*Klebsiella, Bordetella, Neisseria, Aeromonas, Francisella, Cholera, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Bacillus,* and *Erysipelothrix*, or an attenuated strain thereof or modified strain thereof of any of the preceding list of bacterial strains.

100. The immunostimulatory bacterium of any of claims 56-99, wherein the bacterium is attenuated.

101. The immunostimulatory bacterium of any of claims 1-100 that is derived from AST100 (VNP20009 or YS1646).

102. The immunostimulatory bacterium of any of claims 1-101, wherein the bacterium is attenuated when it is *in vivo* in a eukaryotic subject.

103. The immunostimulatory bacterium of any of claims 1-102, wherein the bacterium is attenuated *in vivo* in a subject, wherein it replicates in conditions in a tumor microenvironment (TME).

104. An immunostimulatory bacterium that is auxotrophic for adenosine, and comprises:

a deletion in its nucleic acid encoding the flagella;

a deletion in *endA*; and

a plasmid that encodes CytoLLO, contains a nuclear localization sequence, an *asd* plasmid complementation system, and encodes RNA that inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increases the anti-tumor immune response in a subject.

105. The immunostimulatory bacterium of any of claims 1-104, wherein the bacterium is a strain of *Salmonella*.

106. The immunostimulatory bacterium of any of claims 1-105, wherein the bacterium is a *Salmonella typhimurium* strain.

107. The immunostimulatory bacterium of any of claims 1-105 or 56-106, wherein the bacterium is an attenuated *Salmonella typhimurium* strain selected from among strains designated as AST100, VNP20009, or strain YS1646 (ATCC #202165), RE88, SL7207, χ 8429, χ 8431, and χ 8468.

108. The immunostimulatory bacterium of any of claims 1-107, wherein the bacterium comprises a plasmid encoding shRNA that targets TREX1 and encoding shRNA that targets PD-L1.

109. The immunostimulatory bacterium of any of claims 1-108, wherein the bacterium is auxotrophic for adenosine.

110. The immunostimulatory bacterium of any of claims 1-109, wherein the bacterium comprises a plasmid encoding an shRNA, wherein the shRNA is encoded by the sequence of nucleotides set forth in any of SEQ ID NOs.: 36-40 and 75-78.

111. The immunostimulatory bacterium of any of claims 1-110 or 56-110, wherein the bacterium comprises a plasmid encoding a miRNA, wherein the miRNA is encoded by the sequence of nucleotides set forth in any of SEQ ID NOs: 214-217.

112. The immunostimulatory bacterium of any of claims 1-111, wherein the bacteria is harvested at stationary phase.

113. The immunostimulatory bacterium of any of claims 1-111, wherein:

the plasmid encoding the RNA does not comprise a sequence of nucleotides encoding aspartate-semialdehyde dehydrogenase (*asd*), wherein the resulting strain is *asd*$^-$, and the resulting strain has limited replication *in vivo*.

114. A pharmaceutical composition, comprising an immunostimulatory bacterium of any of claims 1-113 in a pharmaceutically acceptable vehicle.

115. A pharmaceutical composition, comprising an immunostimulatory bacterium that comprises a sequence of nucleotides encoding RNA that inhibits or disrupts or suppresses expression of the gene encoding the enzyme three prime repair exonuclease 1 (TREX1) in a pharmaceutically acceptable vehicle.

116. A pharmaceutical composition, comprising an immunostimulatory bacterium that is auxotrophic for adenosine but not for adenine in a pharmaceutically acceptable vehicle.

117. A pharmaceutical composition, comprising an immunostimulatory bacterium that is auxotrophic for adenosine and encodes RNA that inhibits, suppresses or disrupts expression of three prime repair exonuclease 1 (TREX1) in a pharmaceutically acceptable vehicle.

118. A pharmaceutical composition, comprising an immunostimulatory bacterium that is auxotrophic for adenosine, and is not *purI*, in a pharmaceutically acceptable vehicle.

119. A pharmaceutical composition, comprising the bacterium of any of claims 1, 59 and 61 in a pharmaceutically acceptable vehicle.

120. The immunostimulatory bacterium of any of claims 1-113 or pharmaceutical composition of any of claims 114-119 for use for treating cancer.

121. Use of the immunostimulatory bacterium of any of claims 1-113 or pharmaceutical composition of any of claims 114-119 for treating cancer.

122. The immunostimulatory bacterium or pharmaceutical composition of claim 120 or use of claim 121, wherein the cancer is a solid tumor or a hematologic malignancy.

123. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 120-122, wherein the cancer is selected from among cancer of the breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head and neck, ovary, prostate, brain, pancreas, skin, bone, liver, bone marrow, blood, thymus, uterus, testicles, cervix or liver.

124. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-123, wherein the bacterium is a species of *Salmonella, Shigella, E. coli, Bifidobacteriae, Rickettsia, Vibrio, Listeria, Klebsiella, Bordetella, Neisseria, Aeromonas, Francisella, Cholera, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Bacillus,* and *Erysipelothrix*, or an attenuated strain thereof or modified strain thereof of any of the preceding list of bacterial strains.

125. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-124, wherein the bacterium comprises nucleic acid encoding RNA that inhibits, suppresses or disrupts expression or TREX1 and another different immune checkpoint selected from among any of CTLA-4, PD-L1 (B7-H1), PD-L2, PD-1, PD-2, IDO1, IDO2, SIRPα, CD47, VISTA (B7-H5), LIGHT, HVEM, CD28, LAG3, TIM3, TIGIT, Galectin-9, CEACAM1, CD155, CD112, CD226, CD244 (2B4), B7-H2, B7-H3, ICOS, GITR, B7-H4, B7-H6, CD27,

CD40/CD40L, CD48, CD70, CD80, CD86, CD137( 4-1BB), CD200, CD272 (BTLA), CD160, CD39, CD73, A2a receptor, A2b receptor, HHLA2, ILT-2, ILT-4, gp49B, PIR-B, HLA-G, ILT-2/4, OX40/OX-40L, KIR, TIM1, TIM4, STAT3, CLEVER-1, DNASE II and RNASE-H2.

126. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-124, wherein the immune checkpoint to be inhibited is selected from among one or more of human PD-L1, SIRPα, TREX1, VISTA, and CLEVER-1.

127. The immunostimulatory bacterium or pharmaceutical composition or use of claim 125 or claim 126, wherein the immune checkpoint to be inhibited is selected from among human PD-L1 (SEQ ID NO:31), human Beta-catenin (SEQ ID NO:32), human SIRPα (SEQ ID NO:33), human TREX1 (SEQ ID NO:34), human VISTA (SEQ ID NO:35), human TGF-beta isoform 1 (SEQ ID NO:193) and human VEGF (SEQ ID NO:194).

128. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 114-127, wherein the RNA targets a sequence in the immune checkpoint encoding nucleic acid set forth in any of SEQ ID NOs.: 1-30, 36-40 and 195-209.

129. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-41, 43, 44, 46, 48-69, 71-110 and 112-127, wherein the nucleic acid encoding RNA that inhibits, suppresses or disrupts expression encodes an shRNA.

130. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-129, wherein the nucleic acid encoding RNA that inhibits, suppresses or disrupts expression is under the control of a eukaryotic promoter that is recognized by the subject for whom the bacterium or composition is intended.

131. The immunostimulatory bacterium or pharmaceutical composition or use of claim 130, wherein the promoter is from a species orthologous to the subject.

132. The immunostimulatory bacterium or pharmaceutical composition or use of claim 130 or claim 131, wherein the nucleic acid encoding RNA that inhibits, suppresses or disrupts expression is encoded on a plasmid in the bacterium.

133. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-132, wherein each nucleic acid encoding RNA that inhibits,

suppresses or disrupts expression encodes an shRNA or microRNA that is under the control of a different promoter, wherein the promoter is a eukaryotic promoter.

134. The immunostimulatory bacterium, pharmaceutical composition or use of any of claims 1-133, wherein:

the RNA is encoded on a plasmid in the bacterium;

the plasmid encodes two or more RNA molecules that inhibit, suppress or disrupt expression of a target; and

each sequence of nucleic acids encoding each RNA is separated by at least about 75 nucleotides, or at least 75 nucleotides.

135. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-133, wherein:

the RNA is encoded on a plasmid in the bacterium;

the plasmid encodes two or more RNA molecules that inhibit, suppress or disrupt expression of a target; and

each of the nucleic acids encoding RNA that inhibits, suppresses or disrupts expression is separated by at least about 75 nucleotides, or at least 75 nucleotides, up to about or at least 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500 nucleotides (or base pairs), up to about 1600 or 1600 nucleotides (or base pairs), or between 75- 1500 or 1600 nucleotides (or base pairs).

136. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 133-135, wherein the promoter is an RNA polymerase III promoter.

137. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 133-136, wherein the promoter is/are selected from among U3, H1, U6, 7SK and 7SL.

138. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-137, wherein the bacterial strain is aspartate-semialdehyde dehydrogenase$^-$ (*asd*$^-$).

139. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-138, wherein the bacterial strain expresses *asd* under the control of a promoter expressed in a tumor microenvironment (TME).

140. The immunostimulatory bacterium or pharmaceutical composition or use of claim 139, wherein the promoter is expressed in hypoxic conditions, or in conditions where the pH is less than 7.

141. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-140, wherein the bacterium is a strain of *Salmonella, Shigella, E. coli, Bifidobacteriae, Rickettsia, Vibrio, Listeria, Klebsiella, Bordetella, Neisseria, Aeromonas, Franciesella, Cholera, Corynebacterium, Citrobacter, Chlamydia, Haemophilus, Brucella, Mycobacterium, Mycoplasma, Legionella, Rhodococcus, Pseudomonas, Helicobacter, Bacillus,* and *Erysipelothrix*, or an attenuated strain thereof or modified strain thereof of any of the preceding list of bacterial strains.

142. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-141, wherein the bacterium is attenuated when it is *in vivo* in a eukaryotic subject.

143. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-142, wherein the bacterium is attenuated *in vivo* in a subject, wherein it replicates in conditions in a tumor microenvironment (TME).

144. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 120-143, wherein the subject is a human.

145. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-144, wherein the bacterium is a strain of *Salmonella*.

146. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-144, wherein the bacterium is a *Salmonella typhimurium* strain.

147. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-146, wherein the bacterium is an attenuated *Salmonella typhimurium* strain selected from among strains designated as VNP20009 (ATCC #202165), RE88, SL7207, χ 8429, χ 8431, and χ 8468.

148. The immunostimulatory bacterium or pharmaceutical composition of any of claims 145-147, wherein the bacterium comprises a plasmid encoding shRNA or miRNA that targets TREX1 and encoding shRNA that targets PD-L1.

149. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-148, wherein the bacterium is auxotrophic for adenosine and/or the plasmid has a low copy number origin of replication, and/or the strain is *asd*$^{-}$.

150. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 1-149, wherein the bacterium comprises a plasmid encoding an shRNA or miRNA, wherein:

the shRNA is encoded by the sequence of nucleotides set forth in any of SEQ ID NOs.: 1-30, 36-40 and 75-78; and

the miRNA is encoded by the sequence of nucleotides set forth in any of SEQ ID NOs.: 214-217.

151. A method of treating a subject who has cancer, comprising administering an immunostimulatory bacterium of any of claims 1-113 or pharmaceutical composition of any of claims 114-119 to a subject.

152. A method of treating a subject who has cancer, comprising administering an immunostimulatory bacterium of any of claims 1-113, wherein the cancer is a solid tumor.

153. The method of claim 151 or claim 152, wherein the treatment comprises combination therapy in which a second anti-cancer agent or treatment is administered.

154. The method of claim 153, wherein the second anti-cancer agent or treatment is administered before, concomitantly with, after, or intermittently with the immunostimulatory bacterium.

155. The method of claim 153 or claim 154, wherein the second anti-cancer agent is a chemotherapeutic agent that results in cytosolic DNA, or is radiotherapy.

156. The method of claim 154 or claim 155, wherein the immunostimulatory bacterium comprises nucleic acid encoding RNA that inhibits, suppresses or disrupts expression of TREX1.

157. The method of claim 156, wherein the RNA is shRNA or miRNA.

158. The method of any of claims 151-157, wherein administration of the immunostimulatory bacterium is systemic or local or topical.

159. The method of any of claims 151-157, wherein administration of the immunostimulatory bacterium is parenteral.

160. The method of any of claims 151-157, wherein administration of the immunostimulatory bacterium is oral or rectal or by aerosol into the lung or intratumoral.

161. The method of any of claims 151-157, wherein administration of the immunostimulatory bacterium is intravenously, intramuscularly, or subcutaneously.

162. The method of any of claims 151-161, wherein the cancer is a solid tumor or a hematologic malignancy.

163. The method of any of claims 151-162, wherein the cancer is selected from among cancer of the breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head and neck, ovary, prostate, brain, pancreas, skin, bone, liver, bone marrow, blood, thymus, uterus, testicles, cervix and liver.

164. The method of any of claims 151-162, comprising administering a second therapeutic that is an immune checkpoint inhibitor that is an antibody.

165. The method of claim 164, wherein the antibody is an anti-PD-1, or anti-PD-L1 or anti-CTLA4 antibody.

166. A method of treating a tumor or metastasis or a hematologic malignancy, comprising:

identifying a subject who has a tumor that is $cd73^+$; and

administering an immunostimulatory bacterium that is auxotrophic for adenosine.

167. The method of claim 166, wherein the tumor is cd73+/cd39+.

168. The method of claim 166 or claim 167, wherein the immunostimulatory bacterium is a *Salmonella, Shigella, Listeria, or E. coli* species.

169. The method of any of claims 166-168, wherein the bacterium is a *Salmonella* species.

170. The method of any of claims 166-168, wherein the bacterium is a *Salmonella typhimurium* strain.

171. The method of any of claims 151-170, wherein administration of the immunostimulatory bacterium is by intraperitoneal or intra-tumoral administration.

172. The method of any of claims 151-171, wherein the subject is a human.

173. The method of any of claims 151-172, wherein the subject has cancer or a tumor or metastases or a hematologic malignancy.

174. The method of any of claims 151-173, wherein the tumor or cancer comprises a solid tumor.

175. The method of any of claims 151-174, wherein the tumor is selected from among lung cancer, head and neck cancer, gastric cancer, liver cancer, kidney

cancer, breast cancer, colorectal cancer, prostate cancer, and chronic lymphoblastic leukemia.

176. The method of any of claims 151-175, wherein the tumor is bladder, liver, prostate, gastric, pancreatic or colorectal cancer.

177. The method of any of claims 151-176, wherein the immunostimulatory bacterium is the immunostimulatory bacterium of any of claims 1-113.

178. An immunostimulatory bacterium for use for treating a subject who has a tumor that is $cd73^+$, wherein:

the immunostimulatory bacterium is auxotrophic for adenosine; and

the subject has been identified as having a tumor that is $cd73^+$ by testing a tumor biopsy or other body tissue or fluid sample.

179. A method of increasing colonization of an immunostimulatory bacterium in a subject, comprising:

administering the immunostimulatory bacterium to the subject; and

inhibiting or suppressing expression of TREX1 and/or the activity of the encoded product of TREX1 in the subject.

180. The method of claim 179, wherein the immunostimulatory bacterium encodes an inhibitory RNA that inhibits, disrupts or silences expression of TREX1 in the subject.

181. The method of claim 179 or claim 180, wherein the immunostimulatory bacterium encodes an shRNA that inhibits, disrupts or suppresses expression of TREX1 in the subject.

182. The method of any of claims 179-181, wherein the immunostimulatory bacterium is a *Salmonella* species.

183. The method of any of claims 179-181, wherein the immunostimulatory bacterium is *S. typhimurium*.

184. The method of claim 183, wherein the unmodified *S. typhimurium* is AST-100 or Strain YS1646 (ATCC #202165).

185. The immunostimulatory bacterium or pharmaceutical composition or use or method of any of claims 1-178, wherein the unmodified *S. typhimurium* is VNP20009 (AST-100).

186. A method of improving radiotherapy or treatment with a chemotherapeutic agent for treatment of a tumor, metastasis or hematologic malignancy in a subject, comprising:

treating the subject with radiotherapy or an anti-cancer agent that results in cytosolic DNA or disrupts DNA and thereby increases expression of TREX1; and

inhibiting or suppressing expression of TREX1 and/or the activity of the encoded product of TREX1 in the subject by administering an immunostimulatory bacterium of any of claims 1-113 or an immunostimulatory bacterium that encodes a product that inhibits or suppresses expression of TREX1 and/or the activity of the encoded product of TREX1.

187. The method of claim 186, wherein an immunostimulatory bacterium is administered to inhibit or suppress expression of TREX1 and/or the activity of the encoded product of TREX1.

188. The method of claim 186 or claim 187, wherein the immunostimulatory bacterium and radiotherapy or anti-cancer agent are concomitantly administered, or administered intermittently, or the bacterium is administered after the radiotherapy or anti-cancer agent, or the bacterium is administered before the radiotherapy or anti-cancer agent.

189. The method of any of claims 186-188, wherein the immunostimulatory bacterium encodes an inhibitory RNA that inhibits, disrupts or silences expression of TREX1 in the subject.

190. The method of any of claims 186-189, wherein the immunostimulatory bacterium encodes an shRNA that inhibits, disrupts or suppresses expression of TREX1 in the subject.

191. The method of any of claims 186-190, wherein the bacterium is a *Salmonella* species.

192. The method of any of claims 186-191, wherein the immunostimulatory bacterium is *S. typhimurium*.

193. The method of claim 192, wherein the unmodified bacterium is an *S. typhimurium* strain that is VNP20009.

194. The method of any of claims 151-193, wherein the immunostimulatory bacterium is attenuated.

195. The method of claim 193, wherein the unmodified bacterium is VNP20009 or a derivative of VNP20009.

196. The method of any of claims 151-193 or immunostimulatory bacterium of any of claims 1-113 or immunostimulatory bacterium or composition or use of any

of claims 114-150, wherein the bacterium encodes an siRNA or shRNA or miRNA that inhibits, suppresses or disrupts expression of a checkpoint inhibitor or gene encoding such inhibitor or a metabolite that is immunosuppressive or is in an immunosuppressive pathway.

197. A method of treating tumors or malignancies, comprising:

administering an anti-tumor therapy that results in cytosolic DNA; and

administering an inhibitor of expression of TREX1, wherein the inhibitor of expression of TREX1 is an immunostimulatory bacterium of any of claims 1-113.

198. A method of treating cancer in a subject in need thereof, comprising:

regulating endogenous interferon-beta (IFN-β) production in the subject;

administering an immunostimulatory bacterium of any of claims 1-113, wherein the bacterium encodes a product that inhibits or reduces or disrupts expression of TREX1; and

administering a therapeutically effective amount of ionizing radiation or a chemotherapeutic agent.

199. A method of treating cancer, comprising maintaining the activation of Type I Interferon in the subject in order to maintain ionizing radiation, chemotherapy sensitization and/or sensitization to agents that induce immunogenic cell death in the subject by administering an immunostimulatory bacterium of any of claims 1-113 that encodes a product that inhibits expression of or disrupts expression of TREX1.

200. A method of treating cancer by radiotherapy, comprising administering an immunostimulatory bacterium of any of claims 1-113, wherein the bacterium encodes a product that inhibits or reduces or disrupts expression of TREX1 to restore an abscopal effect at lower radiation doses.

201. The method of any of claims 197-200, wherein the immunostimulatory bacterium is a *Salmonella* strain that encodes an shRNA or miRNA that inhibits, suppresses or disrupts expression of a checkpoint inhibitor or gene encoding such inhibitor or a metabolite that is immunosuppressive or is in an immunosuppressive pathway.

202. The method of any of claims 151-177 and 179-199 that comprises administration of a second anti-cancer agent that is an anti-VEGF agent or an anti-CTLA-4 agent.

203. The method of claim 202, wherein the anti-VEGF agent is an antibody that binds to VEGF or a VEGF receptor.

204. The method of claim 202 or claim 203, wherein the immunostimulatory bacterium comprises a sequence of nucleotides encoding RNA that inhibits, suppresses or disrupts expression of TREX1 and/or PD-L1.

205. The method of any of claims 202-204, wherein the immunostimulatory bacterium is a *Salmonella* species.

206. The method of claim 202, wherein the second anti-cancer agent is an anti-CTLA-4 agent.

207. The method of claim 206, wherein the anti-CTLA-4 agent is an antibody that inhibits or binds to CTLA-4.

208. Use of the immunostimulatory bacterium of any of claims 1-113 that encodes a product that inhibits expression or disrupts expression of TREX1 for treatment of cancer.

209. Use of the immunostimulatory bacterium of any of claims 1-113 that encodes a product that inhibits expression or disrupts expression of TREX1 to prevent or lessen the effects of radiation therapy on interferon type I activation or interferon type I levels for treating cancer.

210. The immunostimulatory bacterium or pharmaceutical composition of any of claims 1-119 for use for treatment of cancer or a tumor or metastases in a subject.

211. The immunostimulatory bacterium or pharmaceutical composition of claim 210, wherein the treatment comprises combination therapy with a second anti-cancer agent or treatment.

212. The immunostimulatory bacterium or pharmaceutical composition of claim 211, wherein the second agent or treatment is radiotherapy or a chemotherapeutic agent that results in cytosolic DNA.

213. The immunostimulatory bacterium or pharmaceutical composition or use of any of claims 208-212, wherein the subject is a human.

214. The use or immunostimulatory bacterium or pharmaceutical composition or method of any of claims 1-213, wherein the bacterium or composition containing the bacterium is formulated for systemic or local or topical administration.

215. The use or immunostimulatory bacterium or pharmaceutical composition or method of any of claims 1-207, wherein administration is parenteral, oral, intravenous, intramuscular, subcutaneous, or intraperitoneal.

216. Use of an shRNA or miRNA that inhibits, disrupts or silences expression of TREX1 or the activity of the encoded product of TREX1 in a subject for increasing colonization of an immunostimulatory bacterium that comprises nucleic acid encoding the shRNA.

217. A composition, comprising an RNA that inhibits, disrupts or silences expression of TREX1 or the activity of the encoded product of TREX1 in a subject for use for increasing colonization of an immunostimulatory bacterium that comprises nucleic acid encoding the RNA.

218. A DNA molecule, comprising the sequence of nucleic acids set forth in any of SEQ ID NOs.: 1-30, 36-40, 75-78 and 195-217.

219. A plasmid, comprising the DNA molecule of claim 218.

220. The plasmid of claim 219 that replicates in a *Salmonella* species.

221. The plasmid of claim 220, wherein the *Salmonella* is *S. typhimurium*.

222. The plasmid of claim 220, wherein the *Salmonella* is VNP20009.

223. The plasmid of any of claims 219-222 that comprises the sequence of nucleotides set forth in SEQ ID NO: 41, 42, 43, 44, 45, 46, 47, 79, 80, 218, 224, 225, 230, 231, 244, 245, or 247.

224. A *Salmonella* strain, comprising the plasmid of any of claims 219-223.

225. A method for producing an immunostimulatory bacterium of any of claims 1-113, comprising:

culturing the bacterium; and

harvesting it at stationary phase.

226. A method for producing an immunostimulatory bacterium, comprising:

culturing an immunostimulatory bacterium, wherein the bacterium comprises a plasmid encoding RNA that inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increases the anti-tumor immune response in a subject; and

harvesting the bacterium at stationary phase.

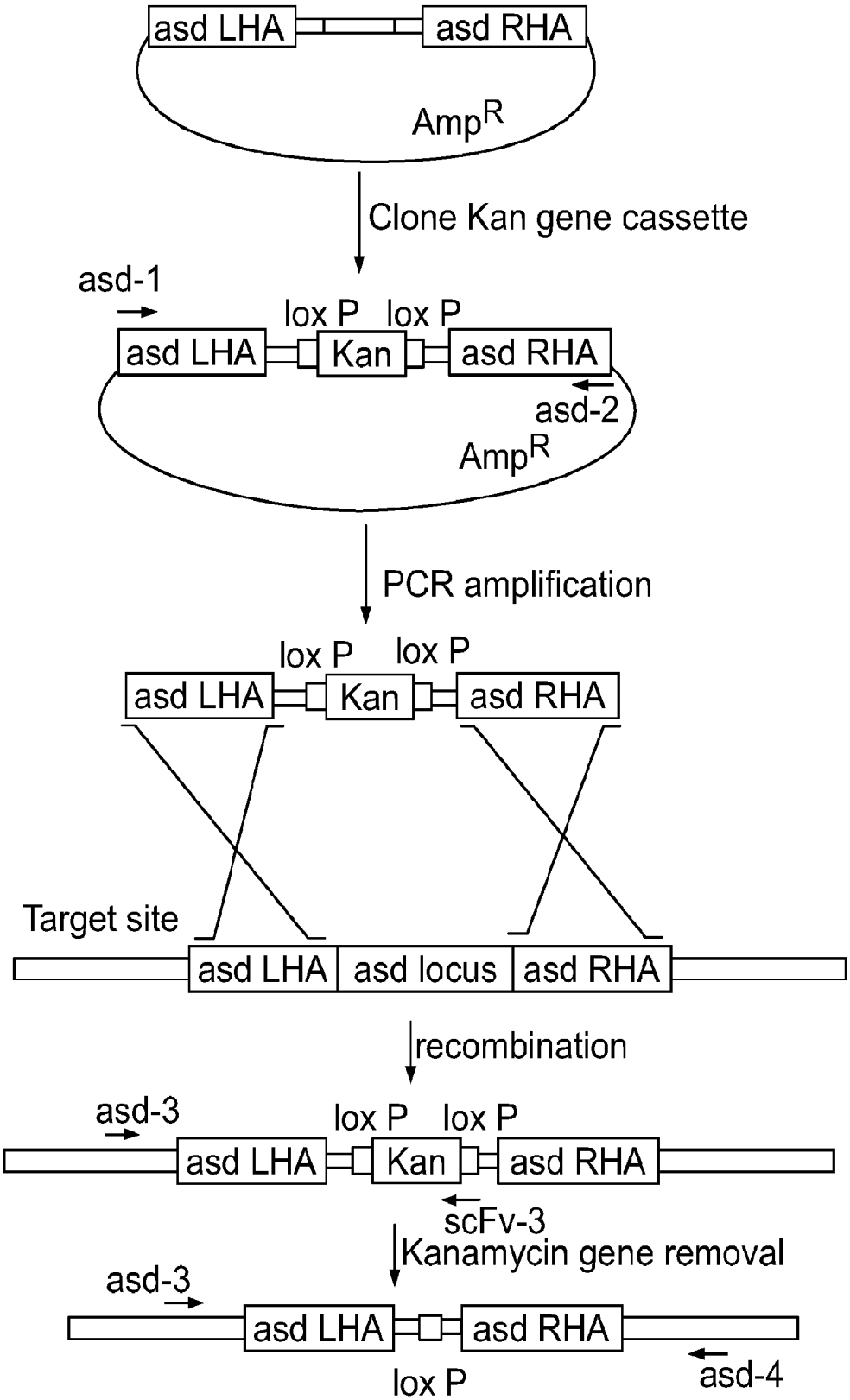

asd LHA
asd RHA
Amp^R
Clone Kan gene cassette
asd-1
lox P
lox P
asd LHA
Kan
asd RHA
asd-2
Amp^R
PCR amplification
lox P
lox P
asd LHA
Kan
asd RHA
Target site
asd LHA
asd locus
asd RHA
recombination
asd-3
lox P
lox P
asd LHA
Kan
asd RHA
scFv-3
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asd-3
asd LHA
asd RHA
lox P
asd-4

**Figure 1**

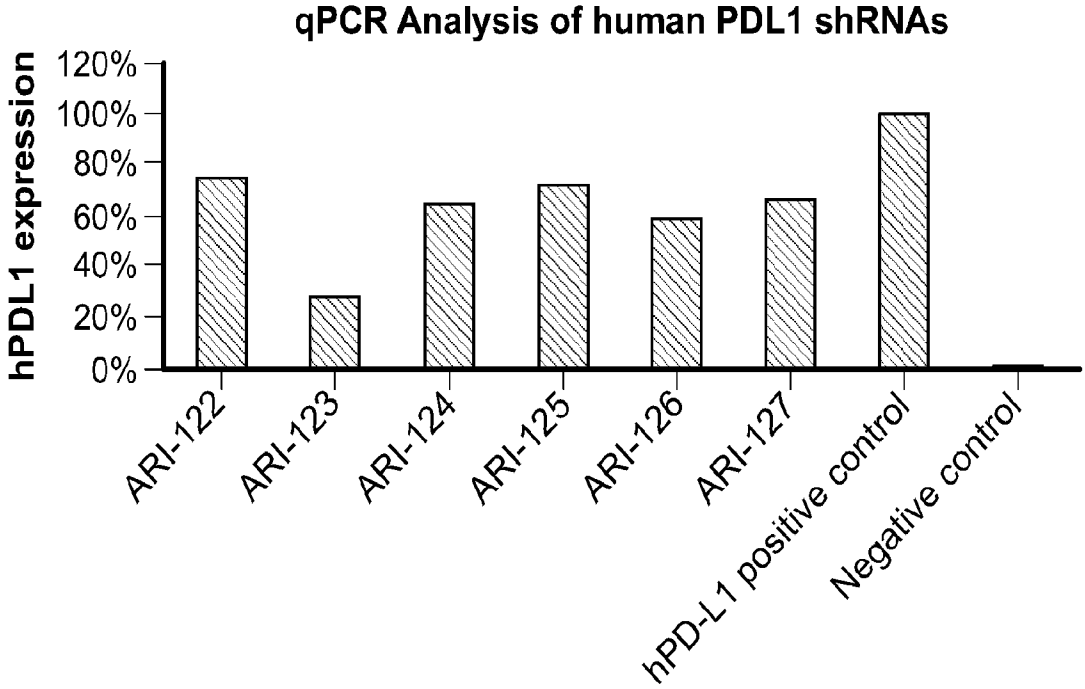



Figure 2A

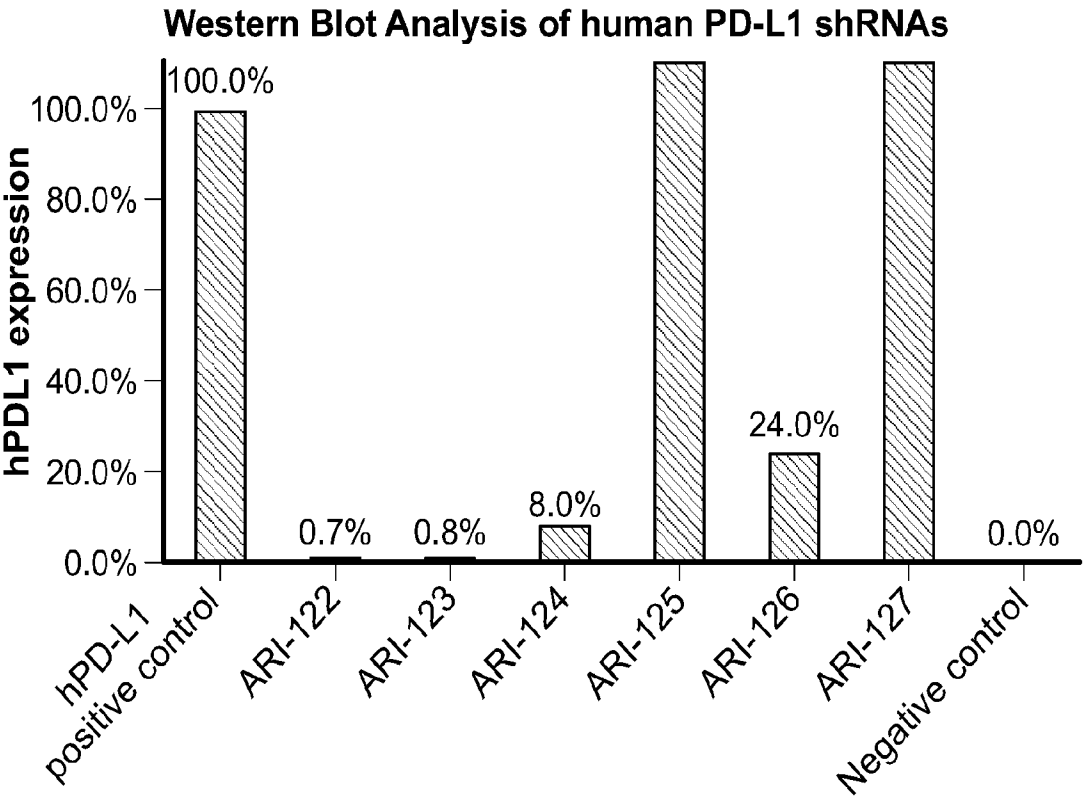



Figure 2B

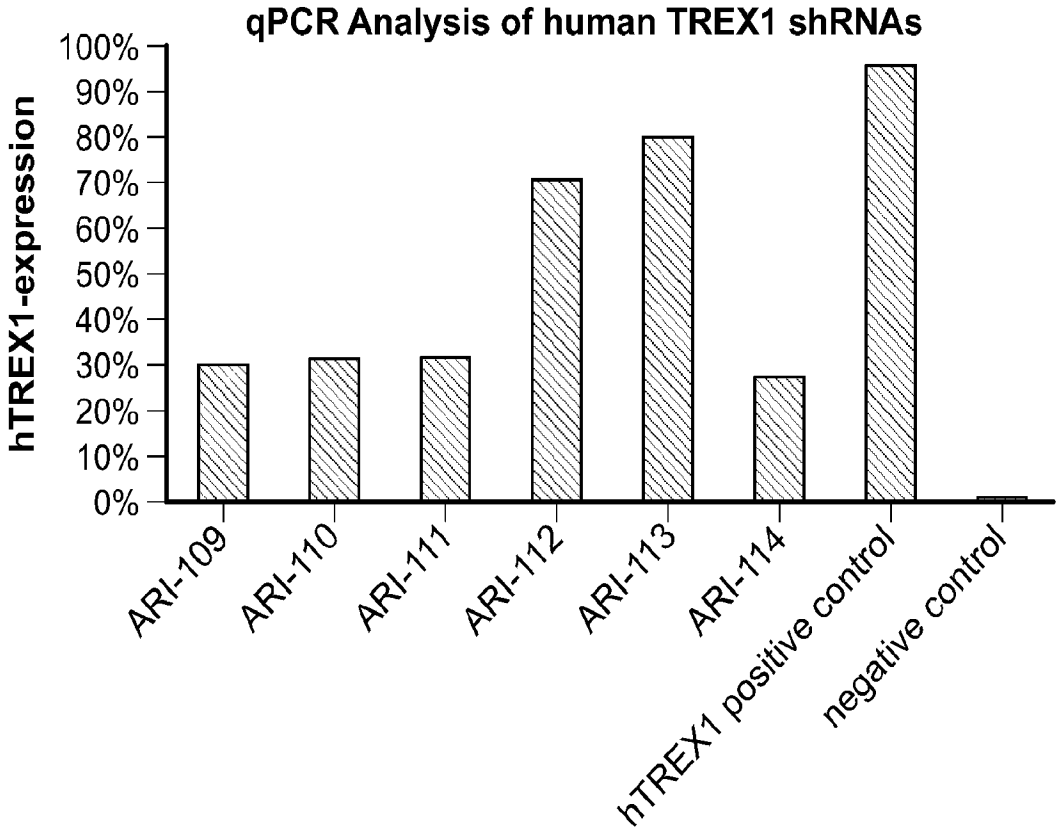



Figure 3A

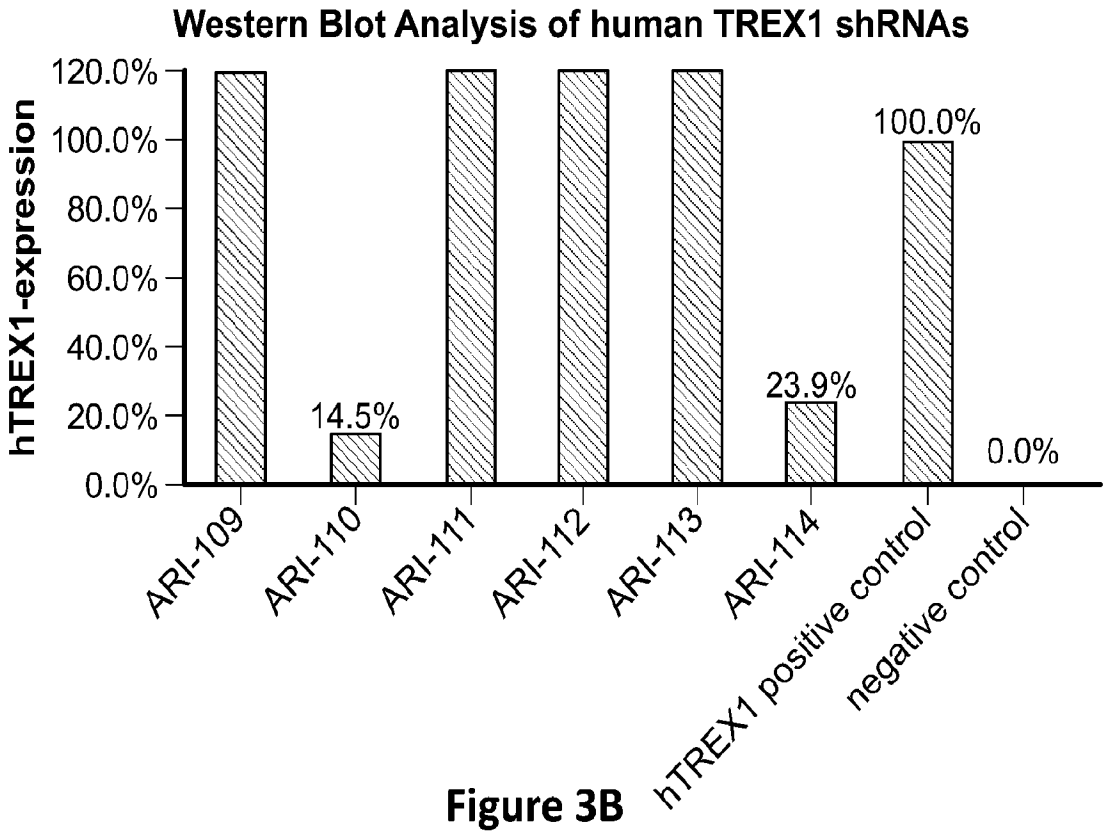



Figure 3B

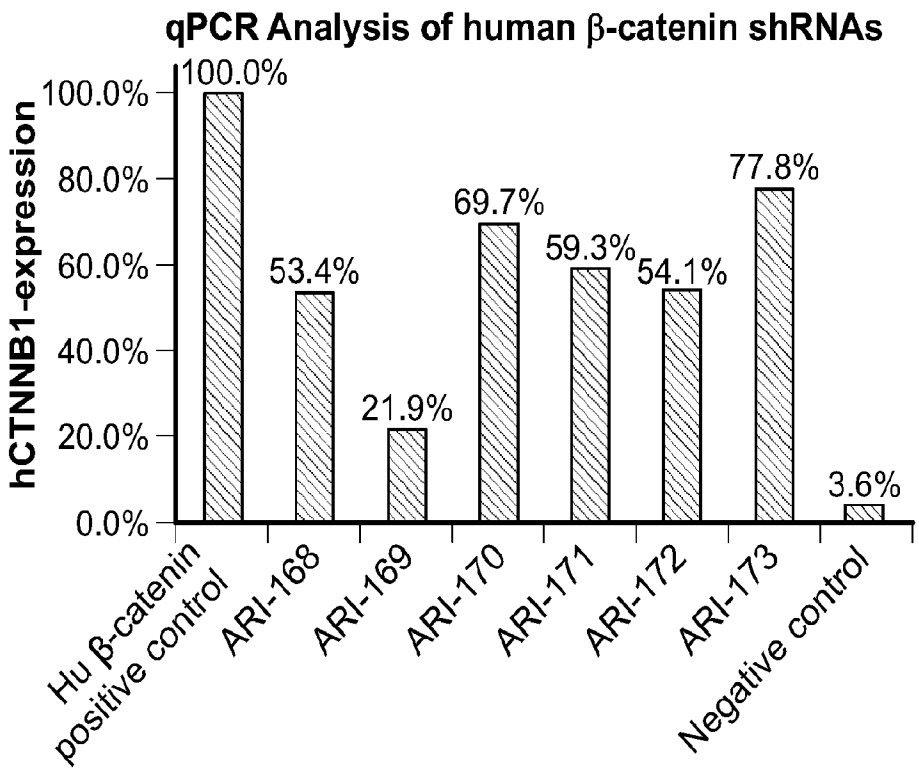



**Figure 4A**

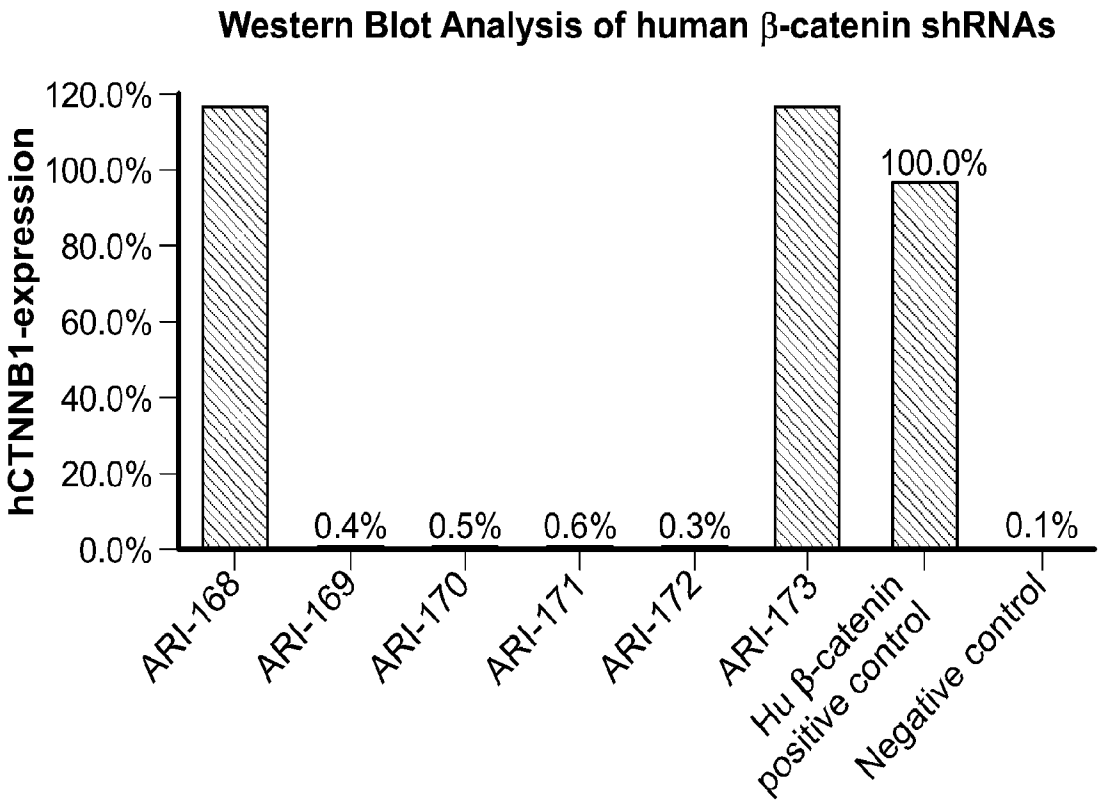



**Figure 4B**

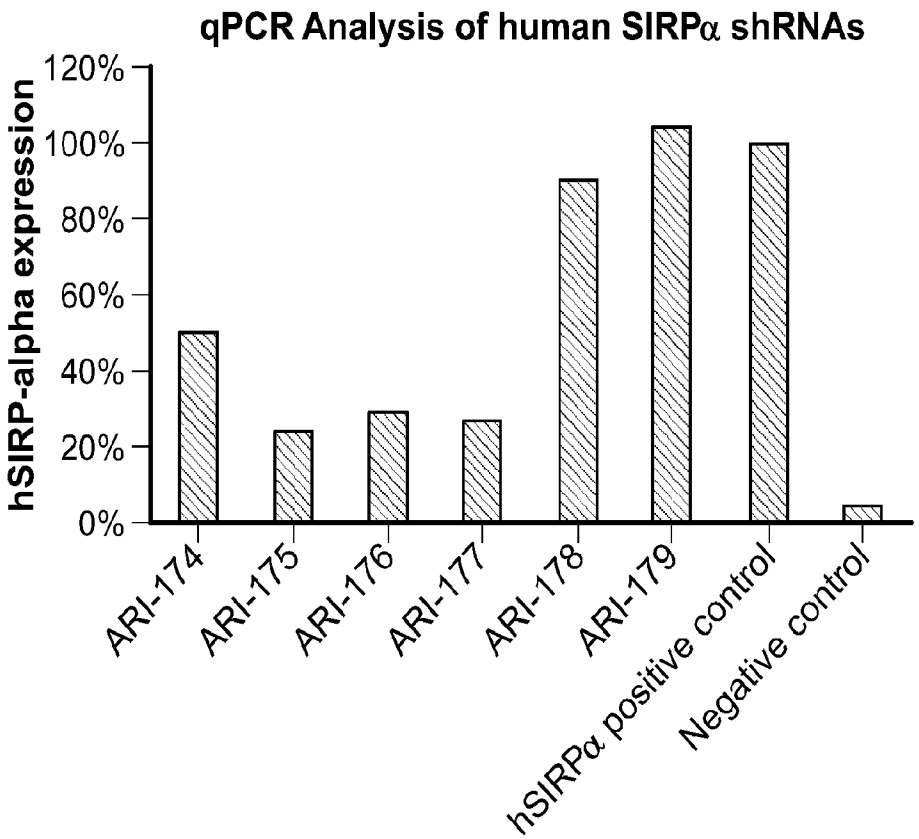



Figure 5A

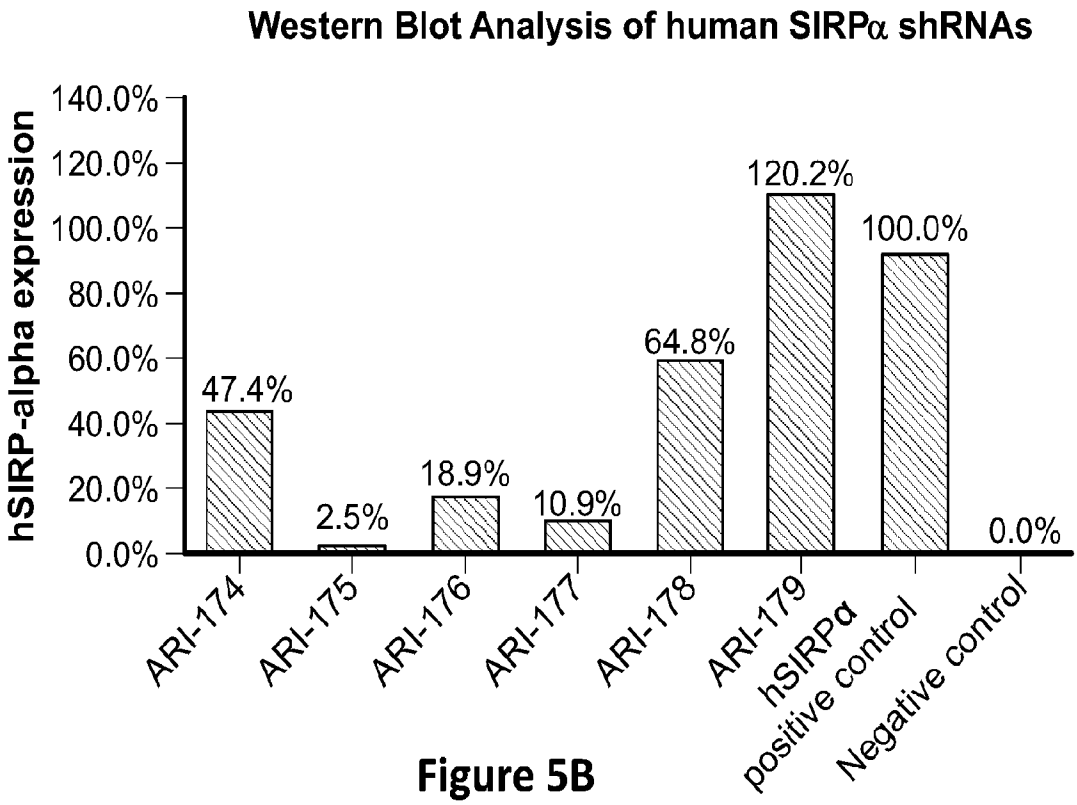



Figure 5B

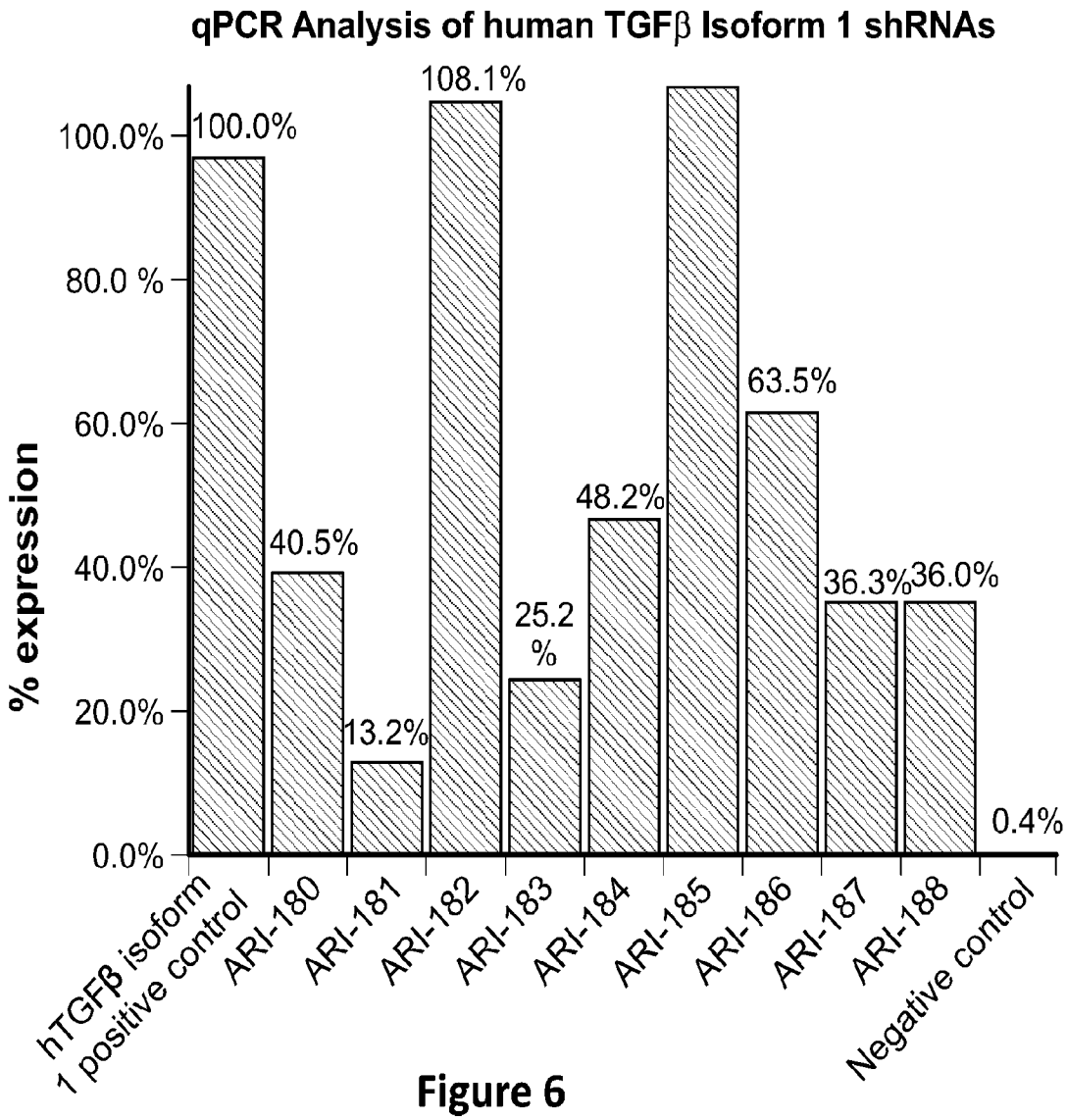

qPCR Analysis of human TGFβ Isoform 1 shRNAs
% expression
100.0%
80.0 %
60.0%
40.0%
20.0%
0.0%
100.0%
40.5%
13.2%
108.1%
25.2 %
48.2%
63.5%
36.3%
36.0%
0.4%
hTGFβ isoform 1 positive control
ARI-180
ARI-181
ARI-182
ARI-183
ARI-184
ARI-185
ARI-186
ARI-187
ARI-188
Negative control

**Figure 6**

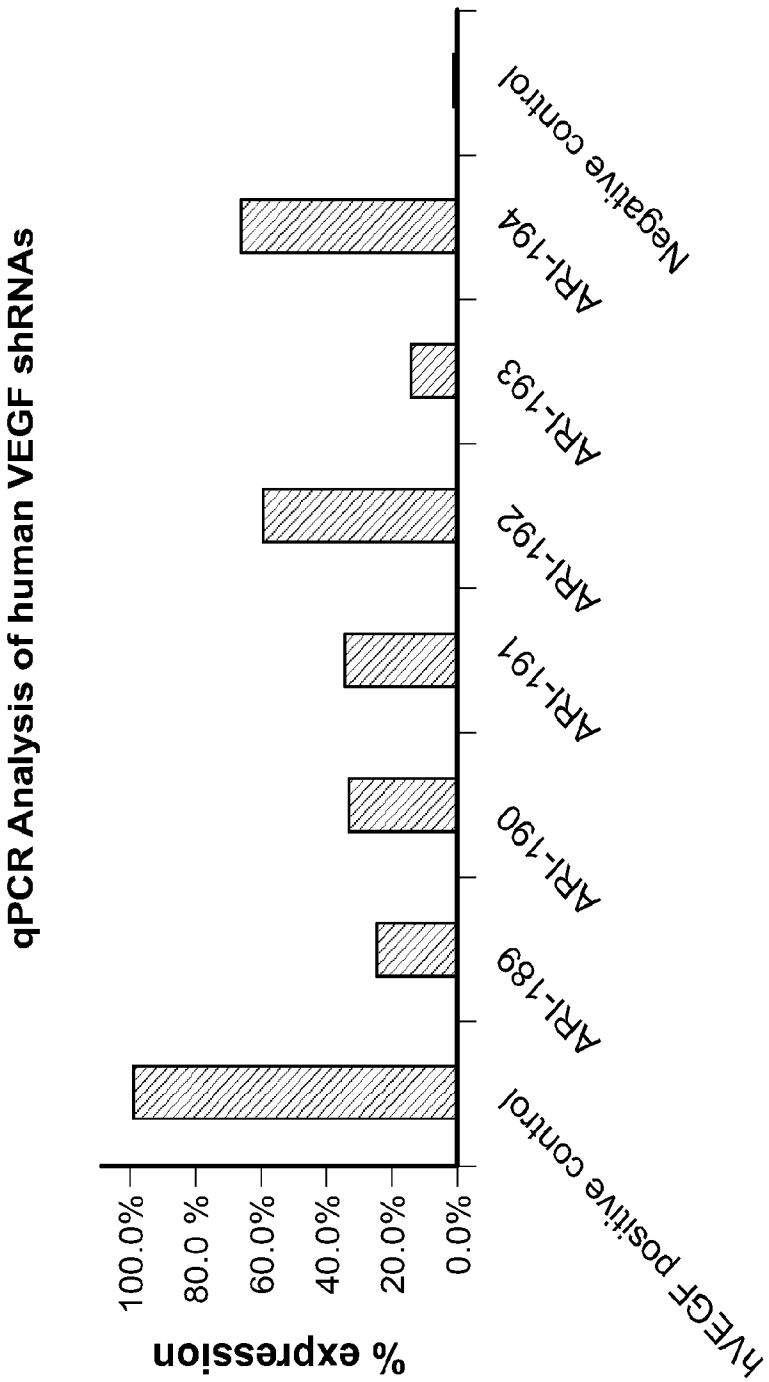

qPCR Analysis of human VEGF shRNAs
% expression
100.0%
80.0 %
60.0%
40.0%
20.0%
0.0%
hVEGF positive control
ARI-189
ARI-190
ARI-191
ARI-192
ARI-193
ARI-194
Negative control

Figure 7

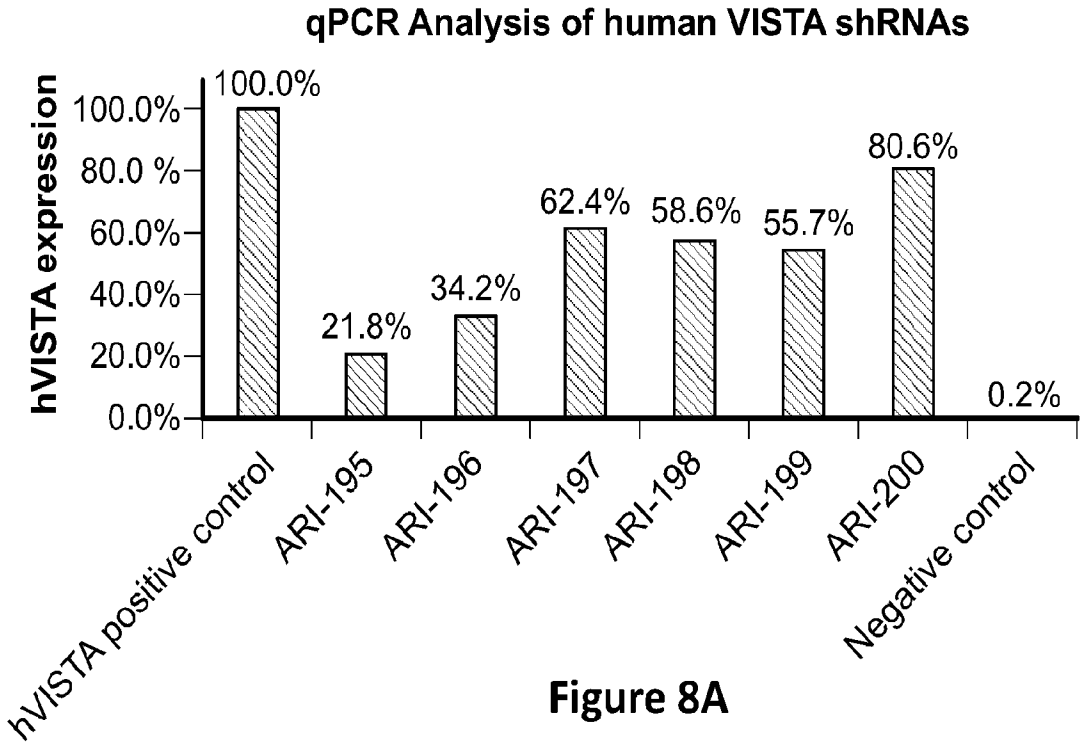



Figure 8A

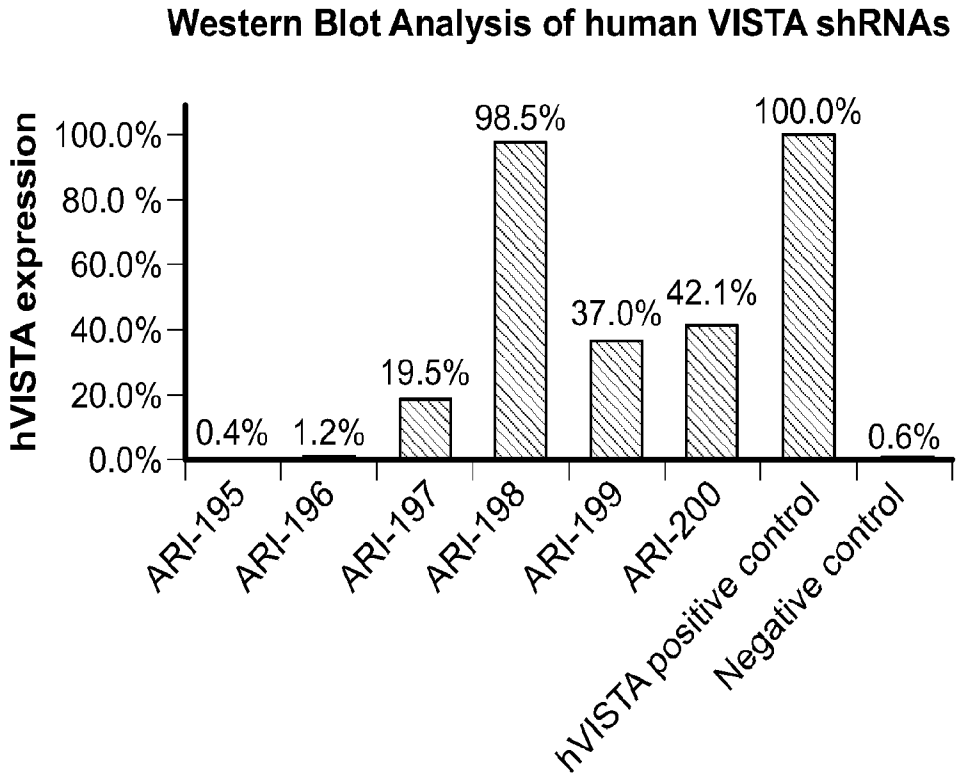



Figure 8B

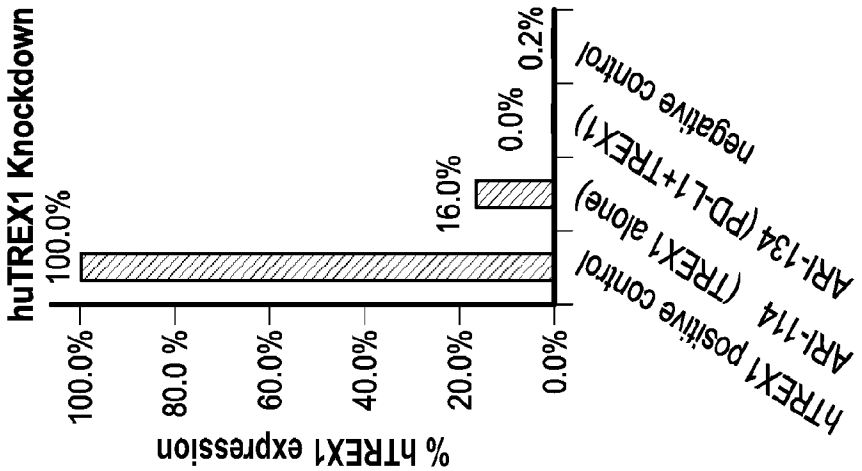



Figure 9B

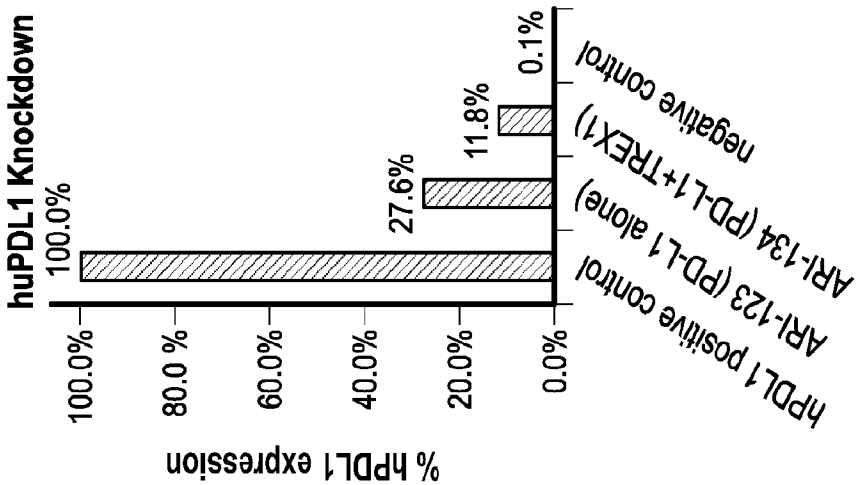



Figure 9A

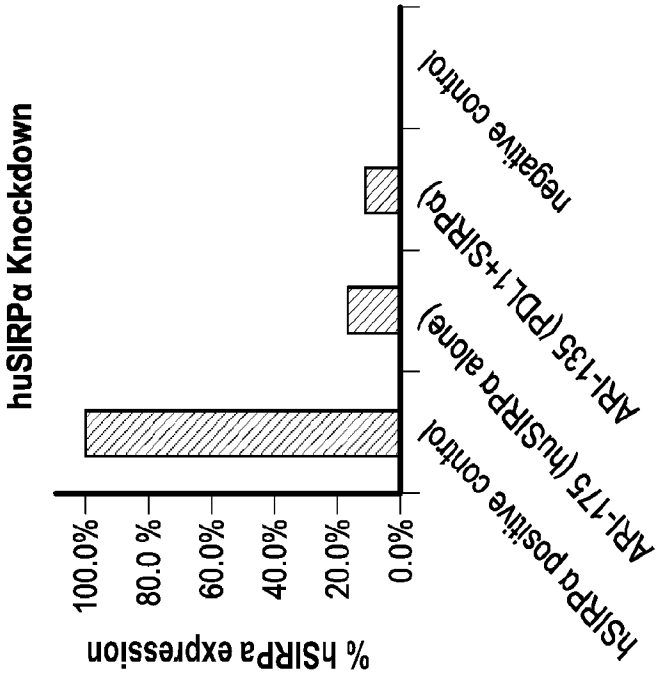



Figure 10B

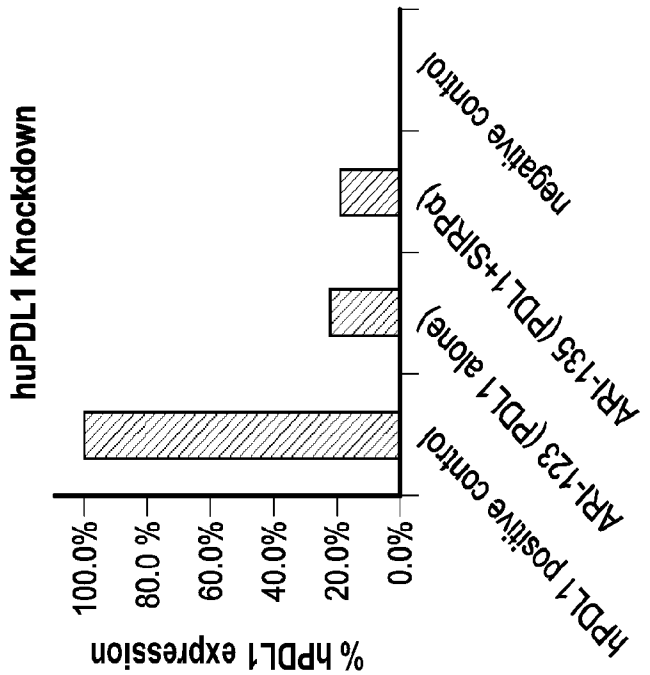



Figure 10A

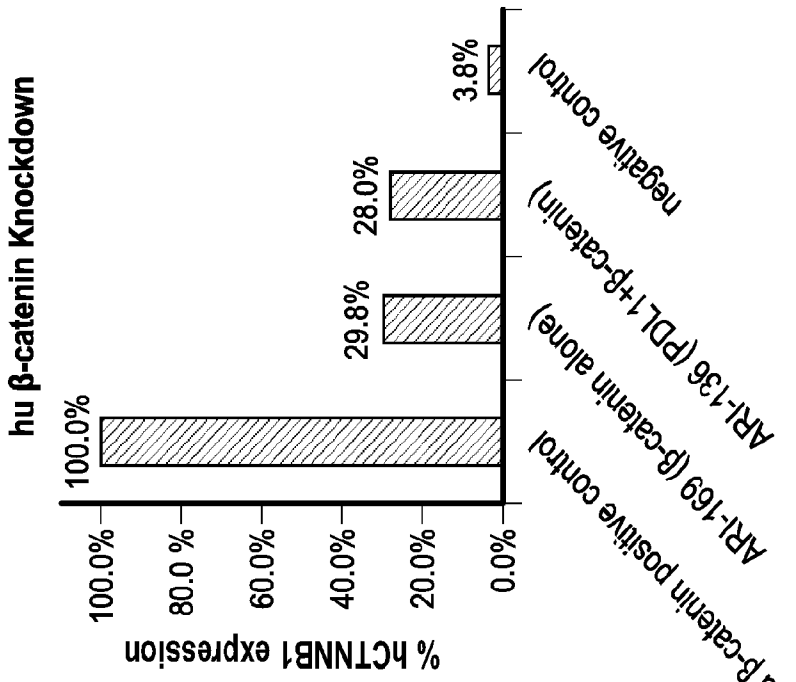



Figure 11B

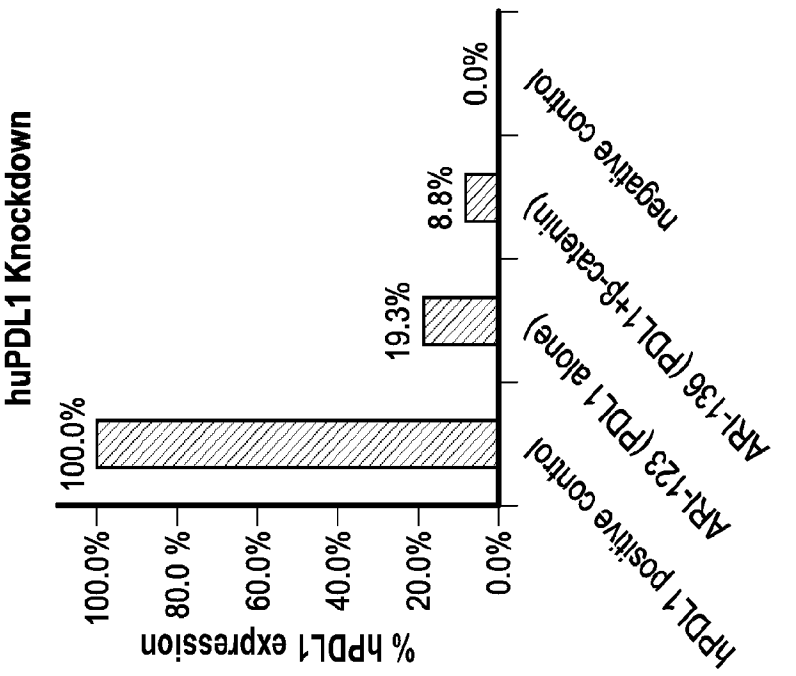



Figure 11A

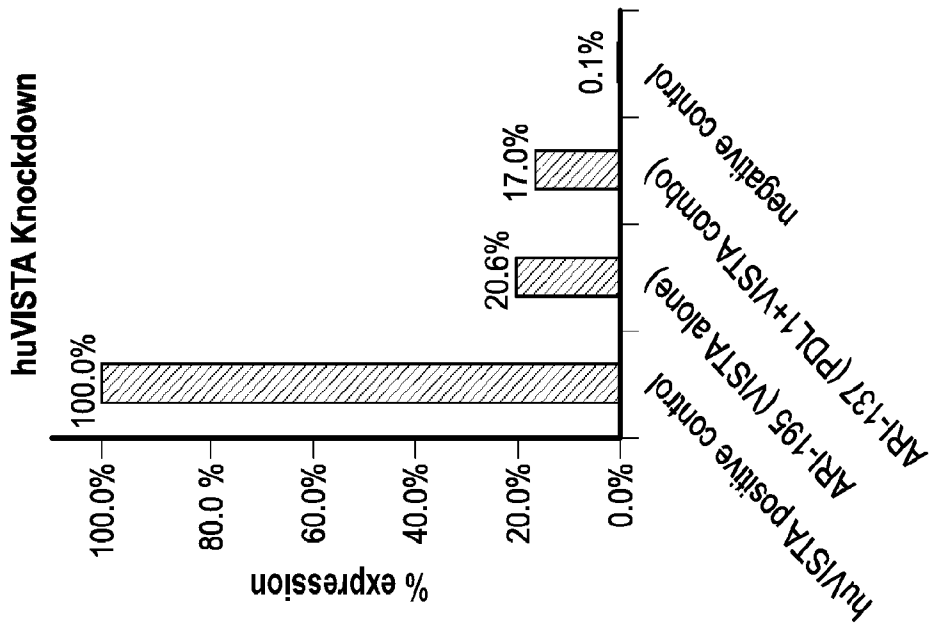



Figure 12B

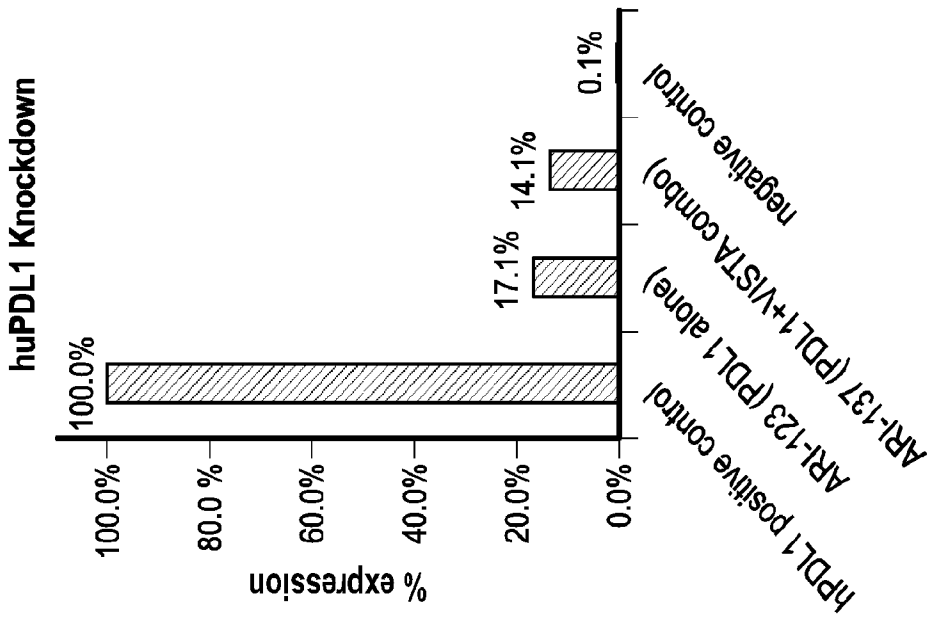



Figure 12A

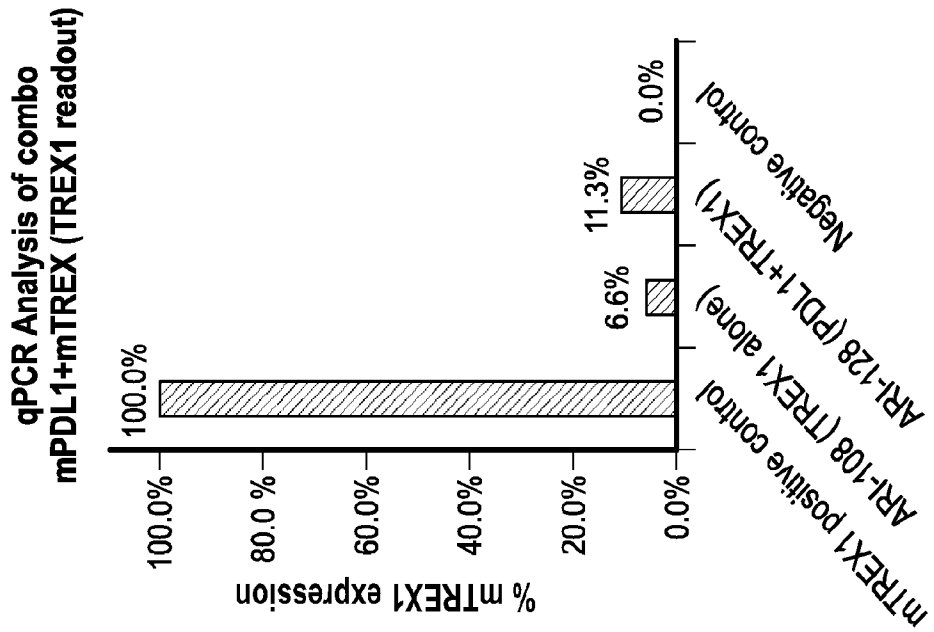



Figure 13B

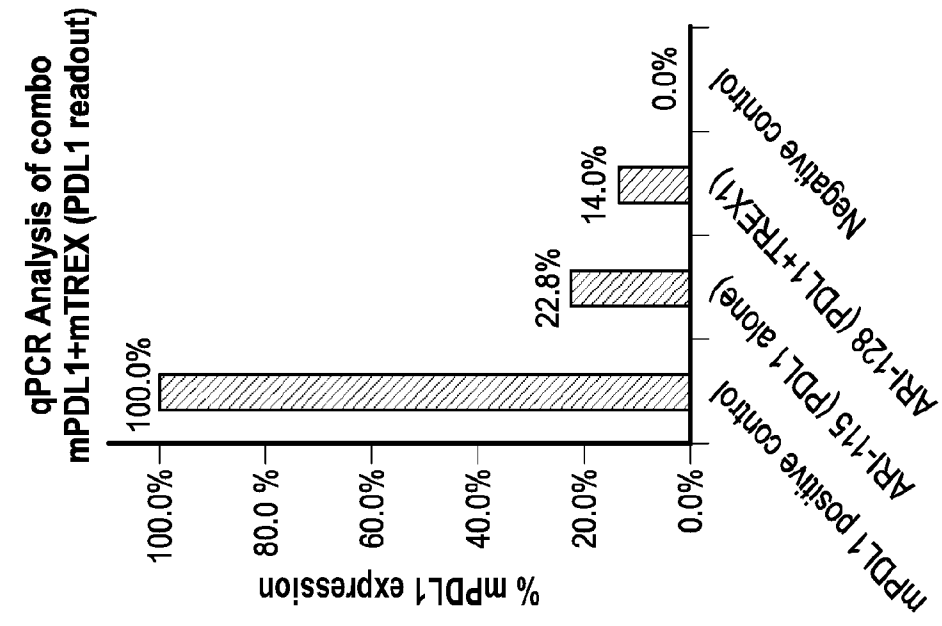



Figure 13A

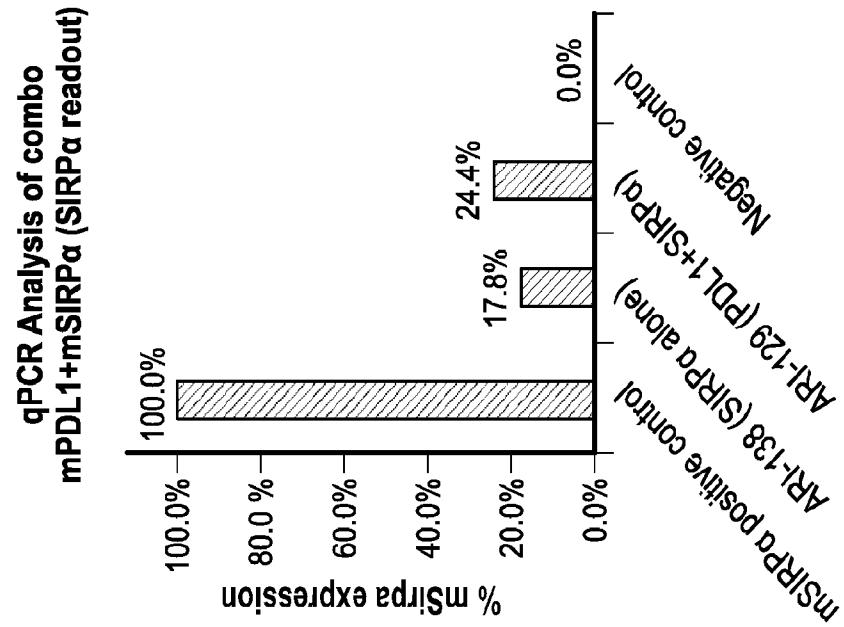



Figure 14B

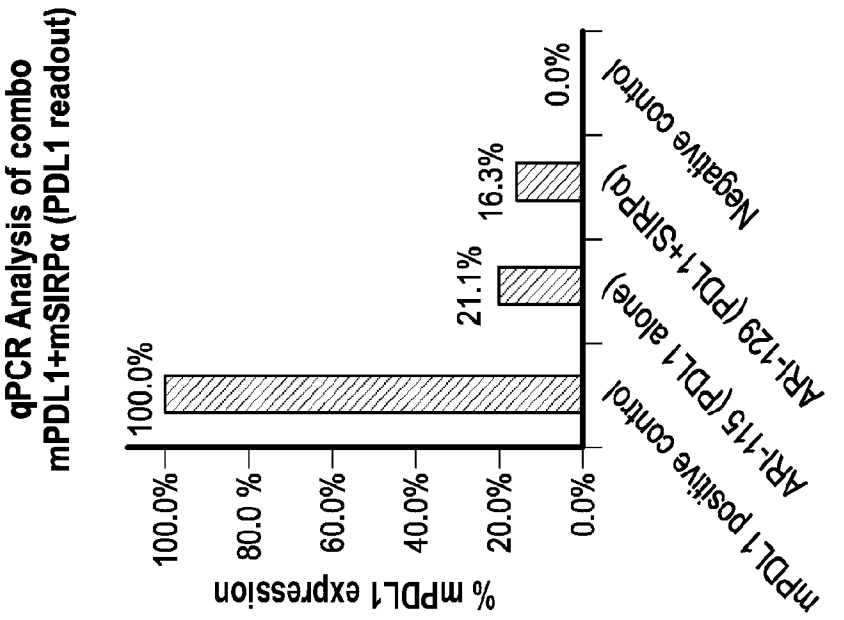



Figure 14A

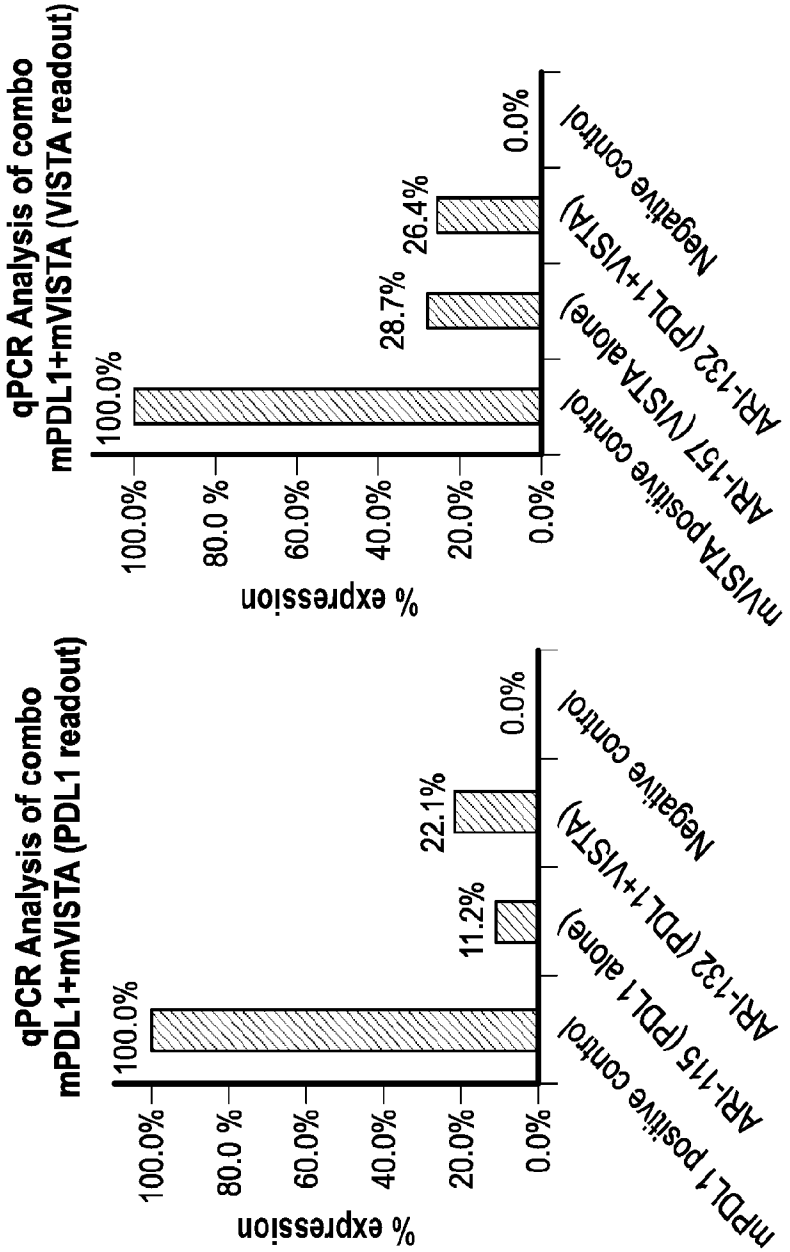



Figure 15A




Figure 15B

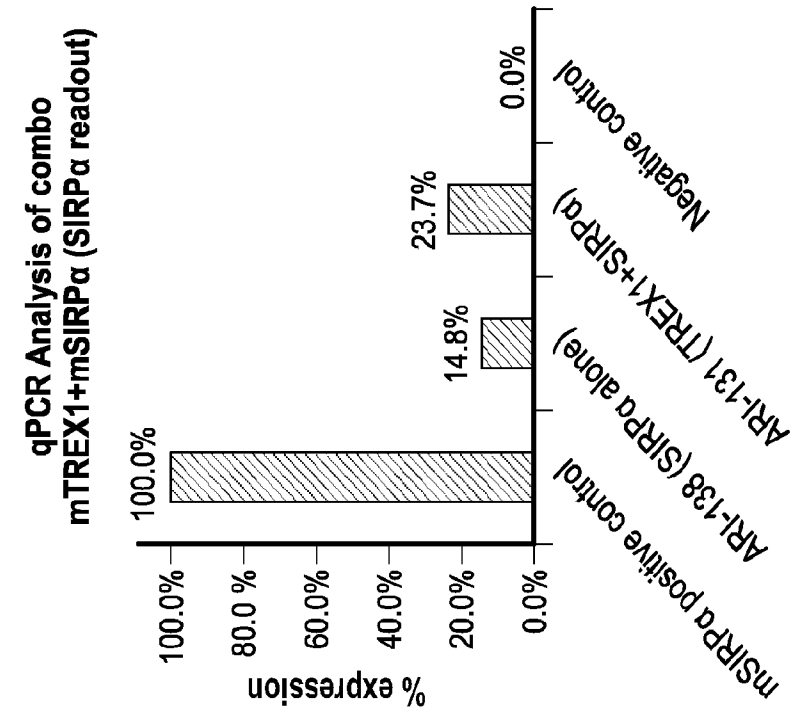



Figure 16B

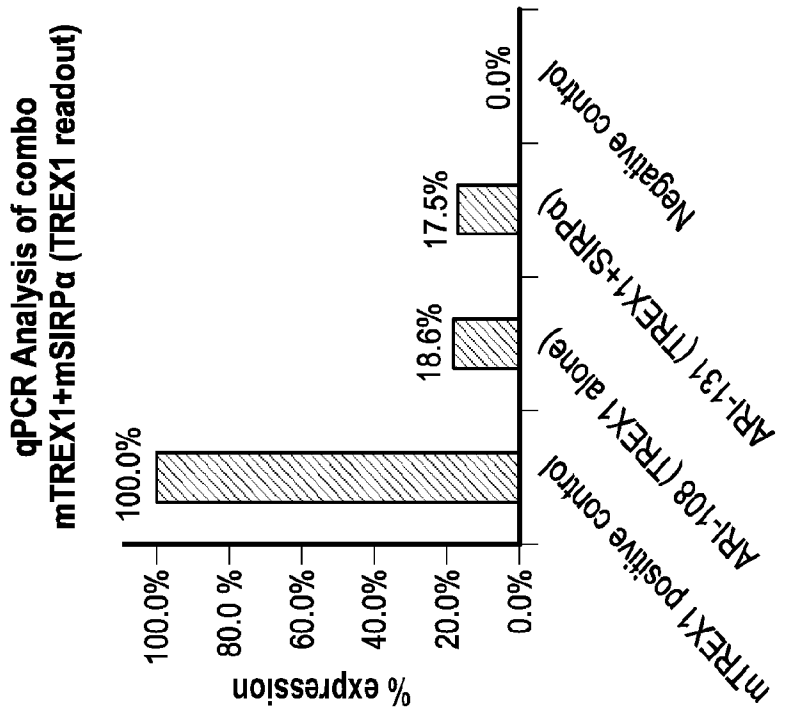



Figure 16A

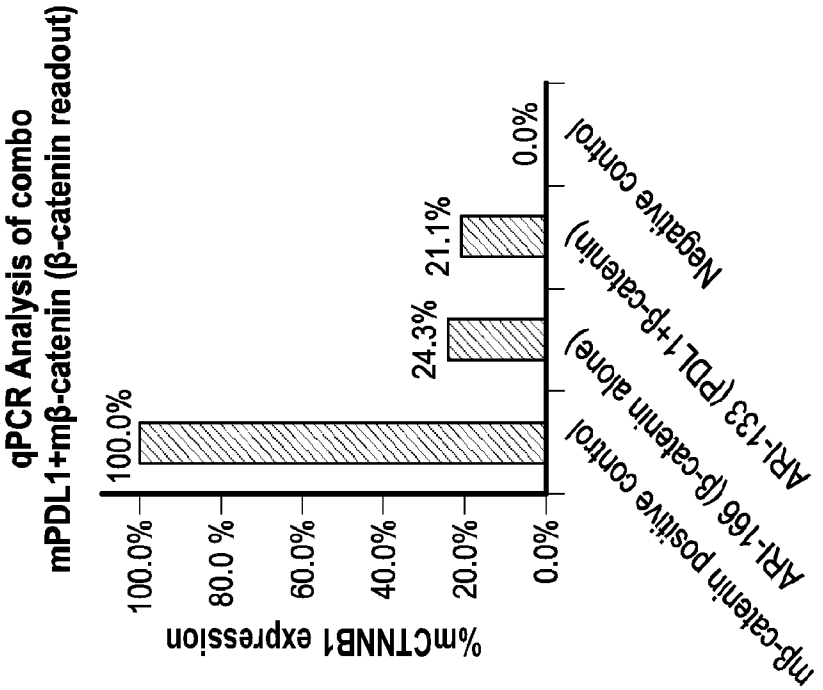



Figure 17B

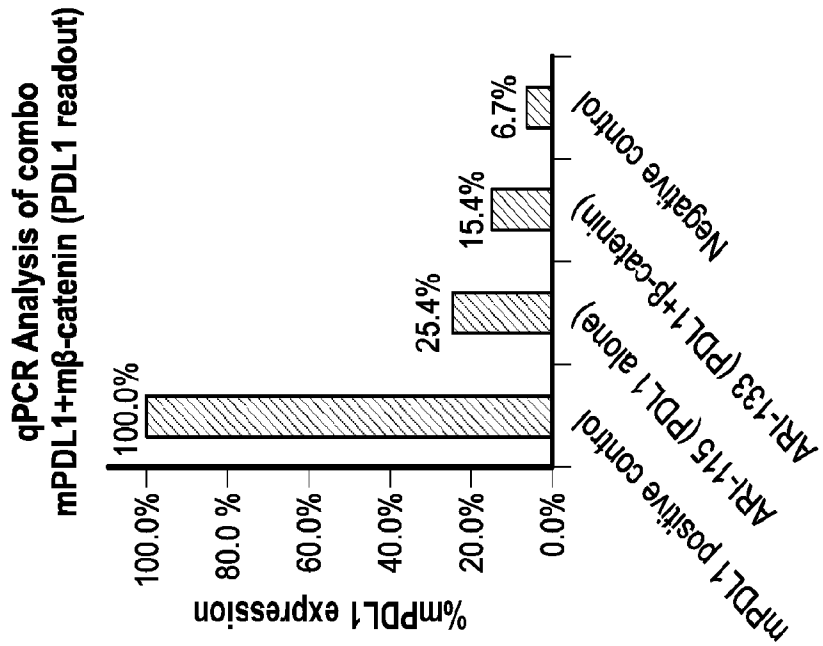



Figure 17A

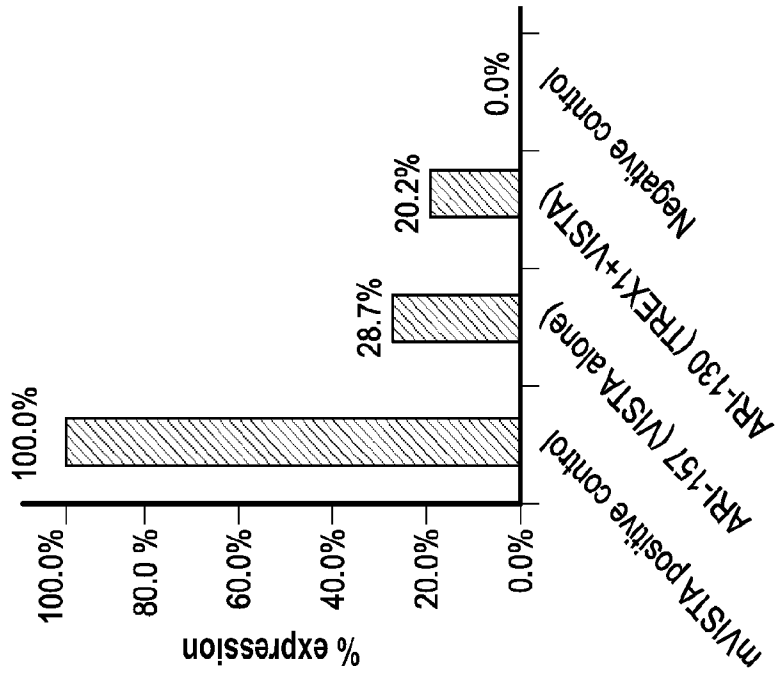



Figure 18B

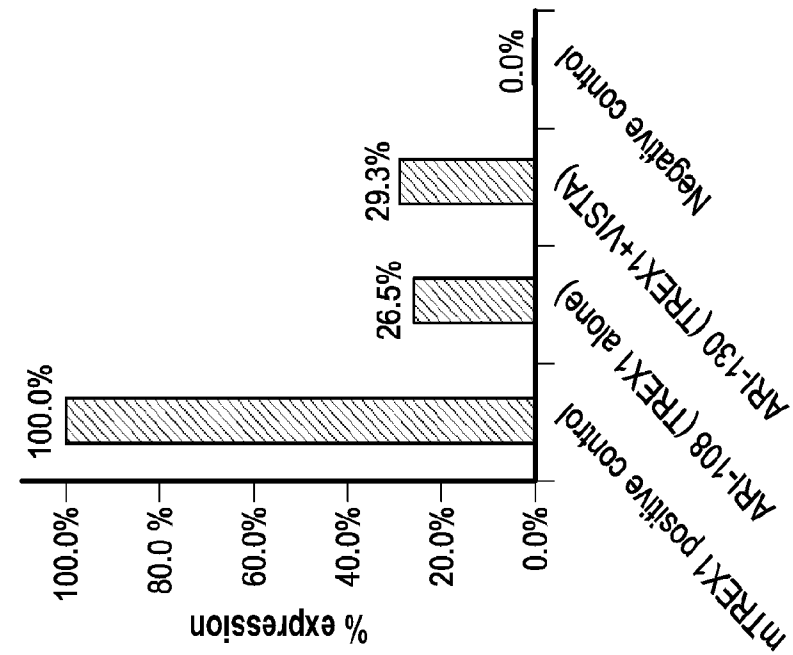



Figure 18A

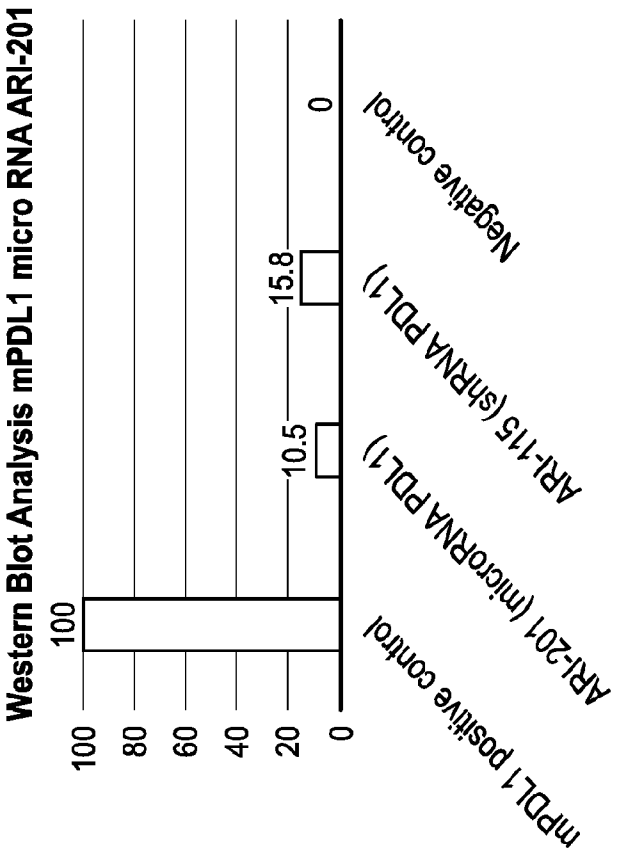



Figure 19B

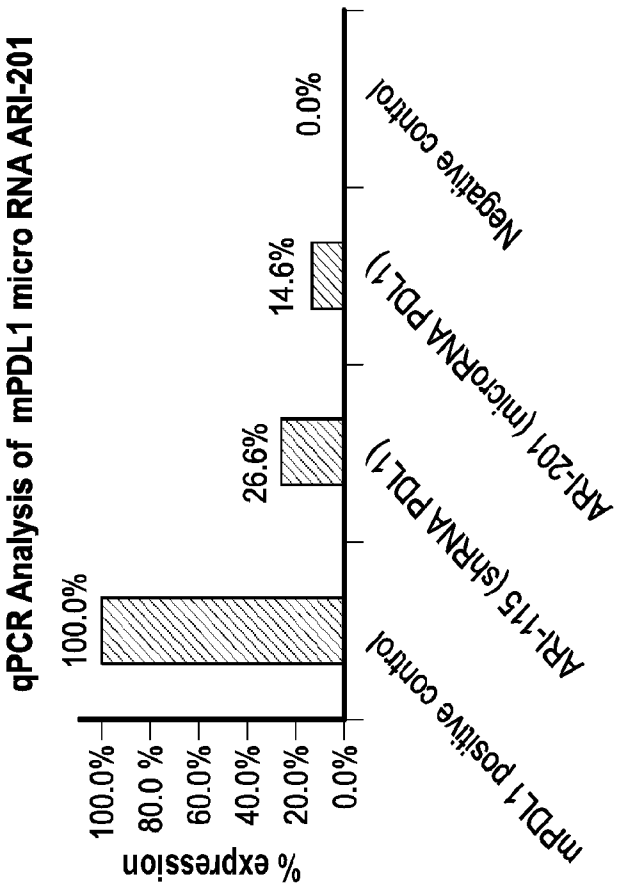



Figure 19A

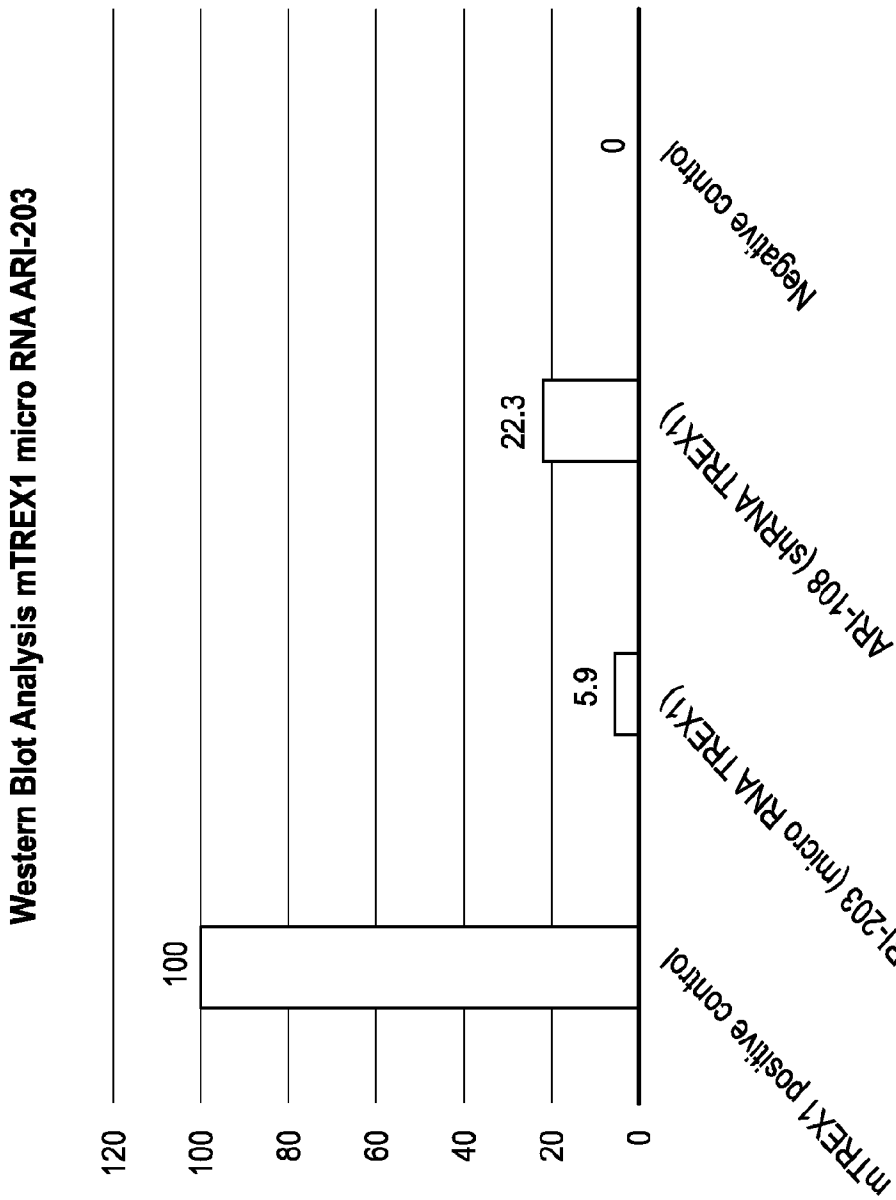

Western Blot Analysis mTREX1 micro RNA ARI-203
120
100
80
60
40
20
0
100
5.9
22.3
0
mTREX1 positive control
ARI-203 (micro RNA TREX1)
ARI-108 (shRNA TREX1)
Negative control

Figure 20

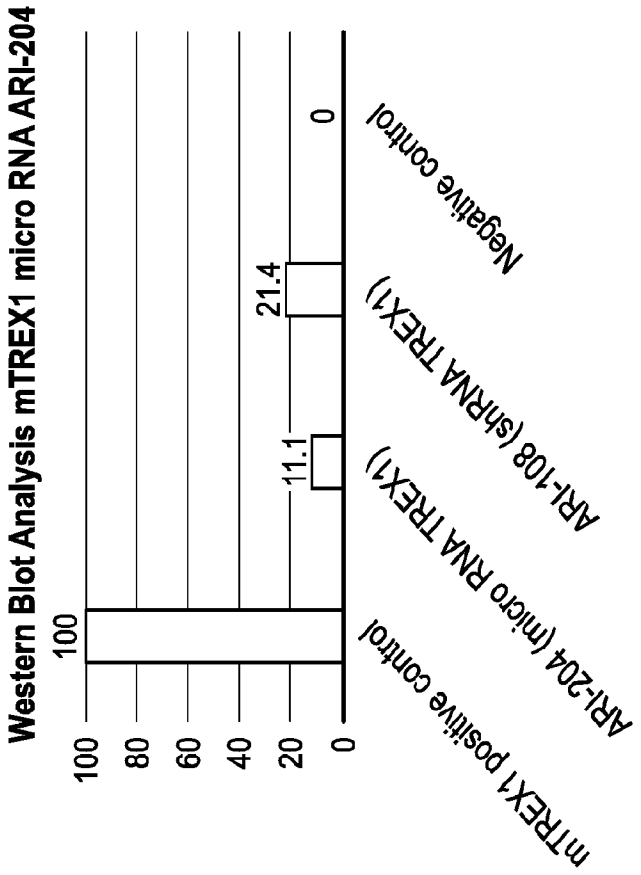



Figure 21B

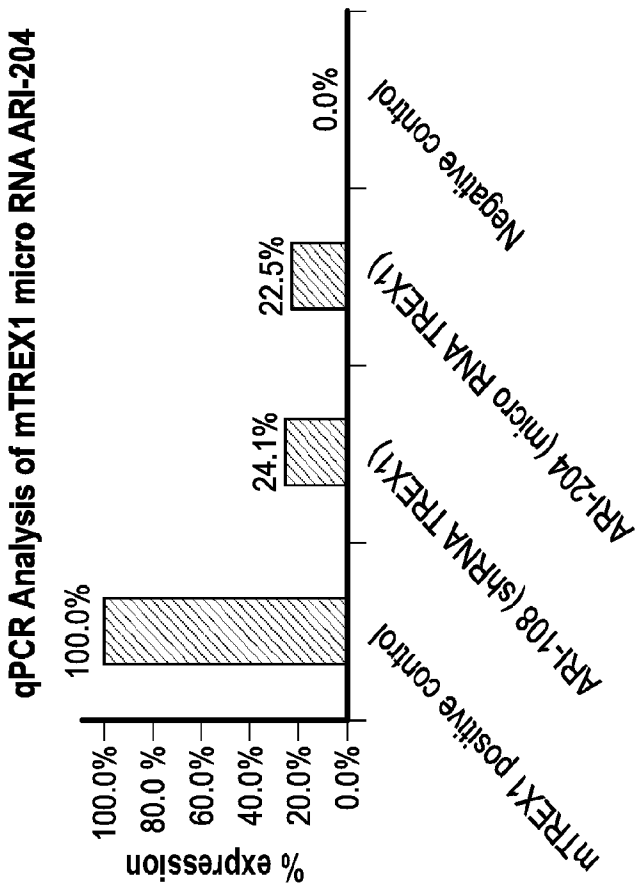



Figure 21A

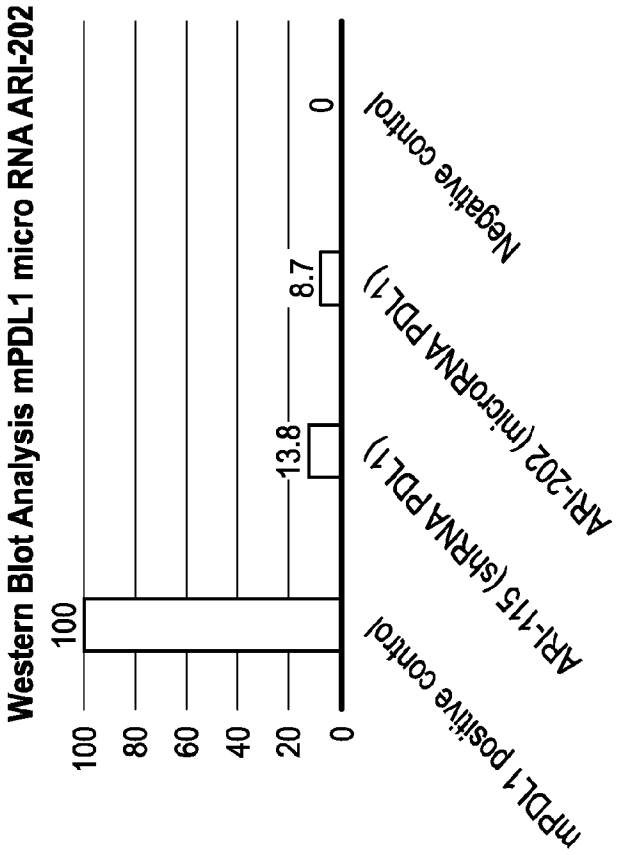



Figure 22B

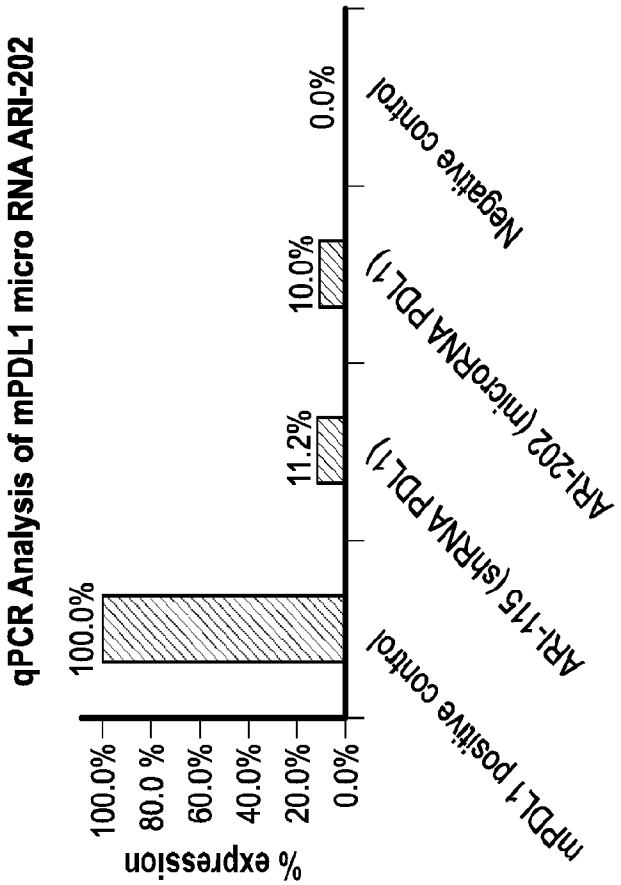



Figure 22A

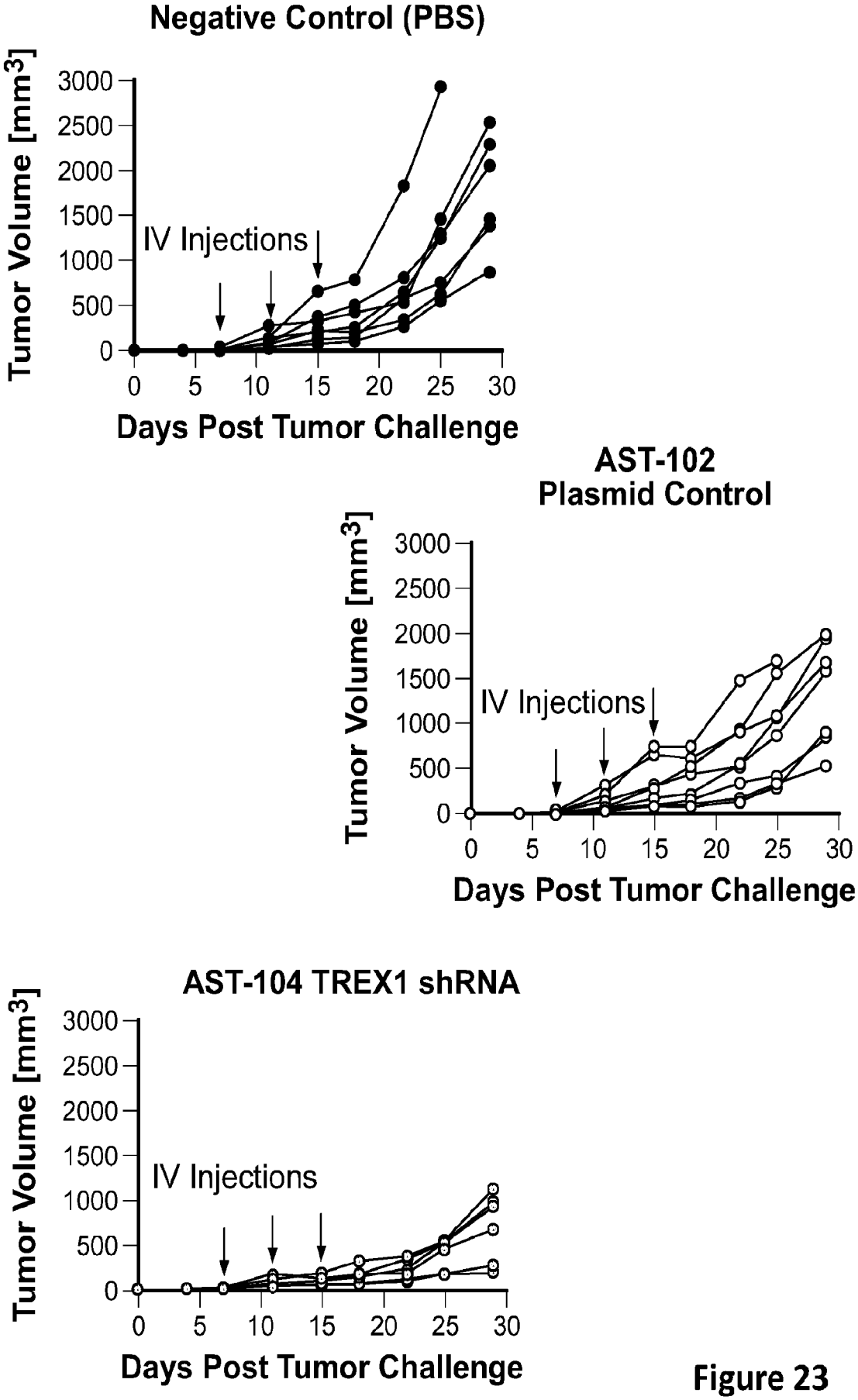

Negative Control (PBS)
Tumor Volume [mm3]
3000
2500
2000
1500
1000
500
0
IV Injections
0 5 10 15 20 25 30
Days Post Tumor Challenge
AST-102
Plasmid Control
Tumor Volume [mm3]
3000
2500
2000
1500
1000
500
0
IV Injections
0 5 10 15 20 25 30
Days Post Tumor Challenge
AST-104 TREX1 shRNA
Tumor Volume [mm3]
3000
2500
2000
1500
1000
500
0
IV Injections
0 5 10 15 20 25 30
Days Post Tumor Challenge

Figure 23

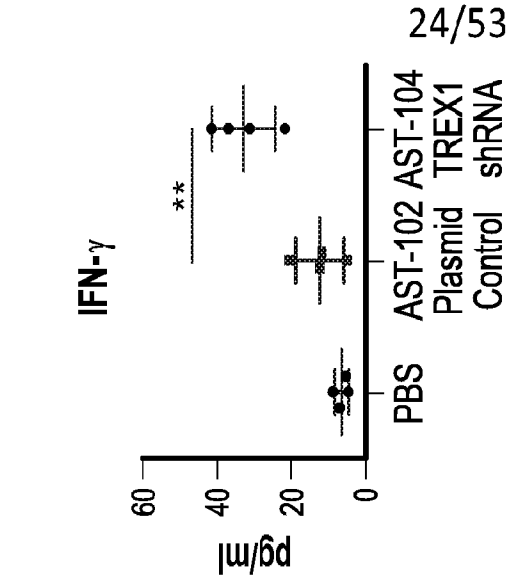
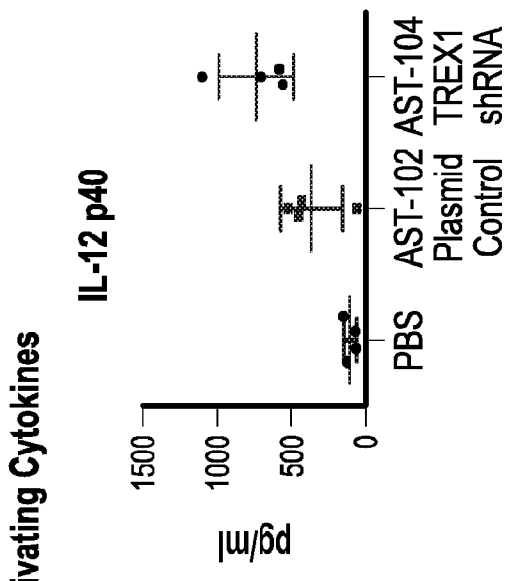



Figure 24A



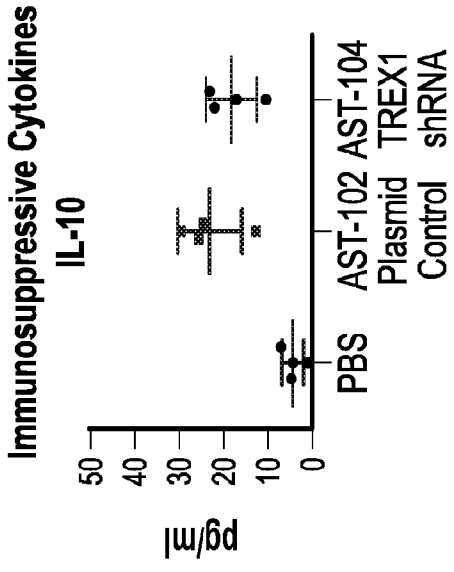




Figure 24B

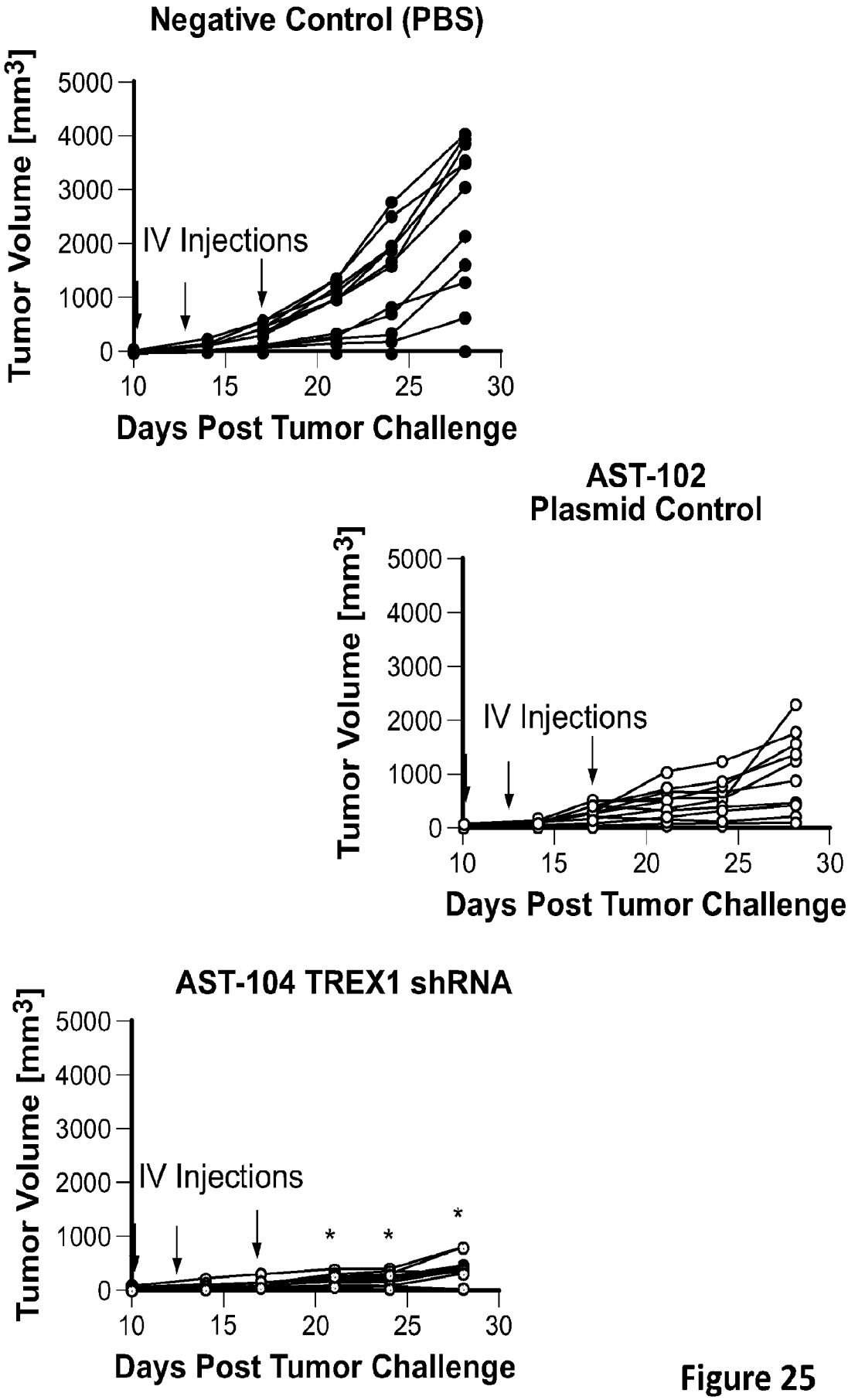

Negative Control (PBS)
Tumor Volume [mm3]
5000
4000
3000
2000
1000
0
IV Injections
10
15
20
25
30
Days Post Tumor Challenge
AST-102
Plasmid Control
Tumor Volume [mm3]
5000
4000
3000
2000
1000
0
IV Injections
10
15
20
25
30
Days Post Tumor Challenge
AST-104 TREX1 shRNA
Tumor Volume [mm3]
5000
4000
3000
2000
1000
0
IV Injections
*
*
*
10
15
20
25
30
Days Post Tumor Challenge

Figure 25

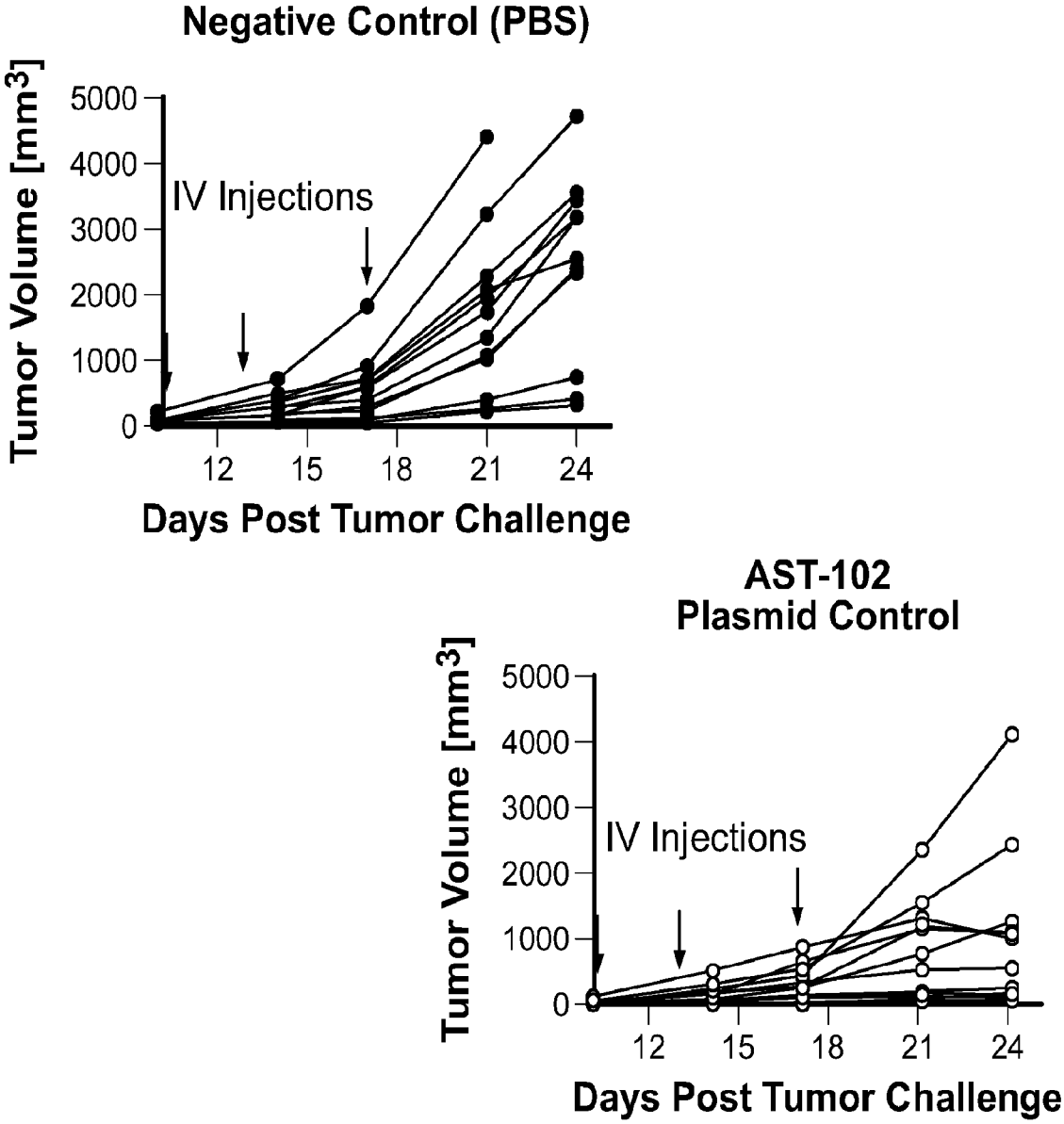

Negative Control (PBS)
Tumor Volume [mm3]
5000
4000
3000
2000
1000
0
IV Injections
12
15
18
21
24
Days Post Tumor Challenge
AST-102
Plasmid Control
Tumor Volume [mm3]
5000
4000
3000
2000
1000
0
IV Injections
12
15
18
21
24
Days Post Tumor Challenge
AST-104 TREX1 shRNA
Tumor Volume [mm3]
5000
4000
3000
2000
1000
0
IV Injections
*
12
15
18
21
24
Days Post Tumor Challenge

Figure 26

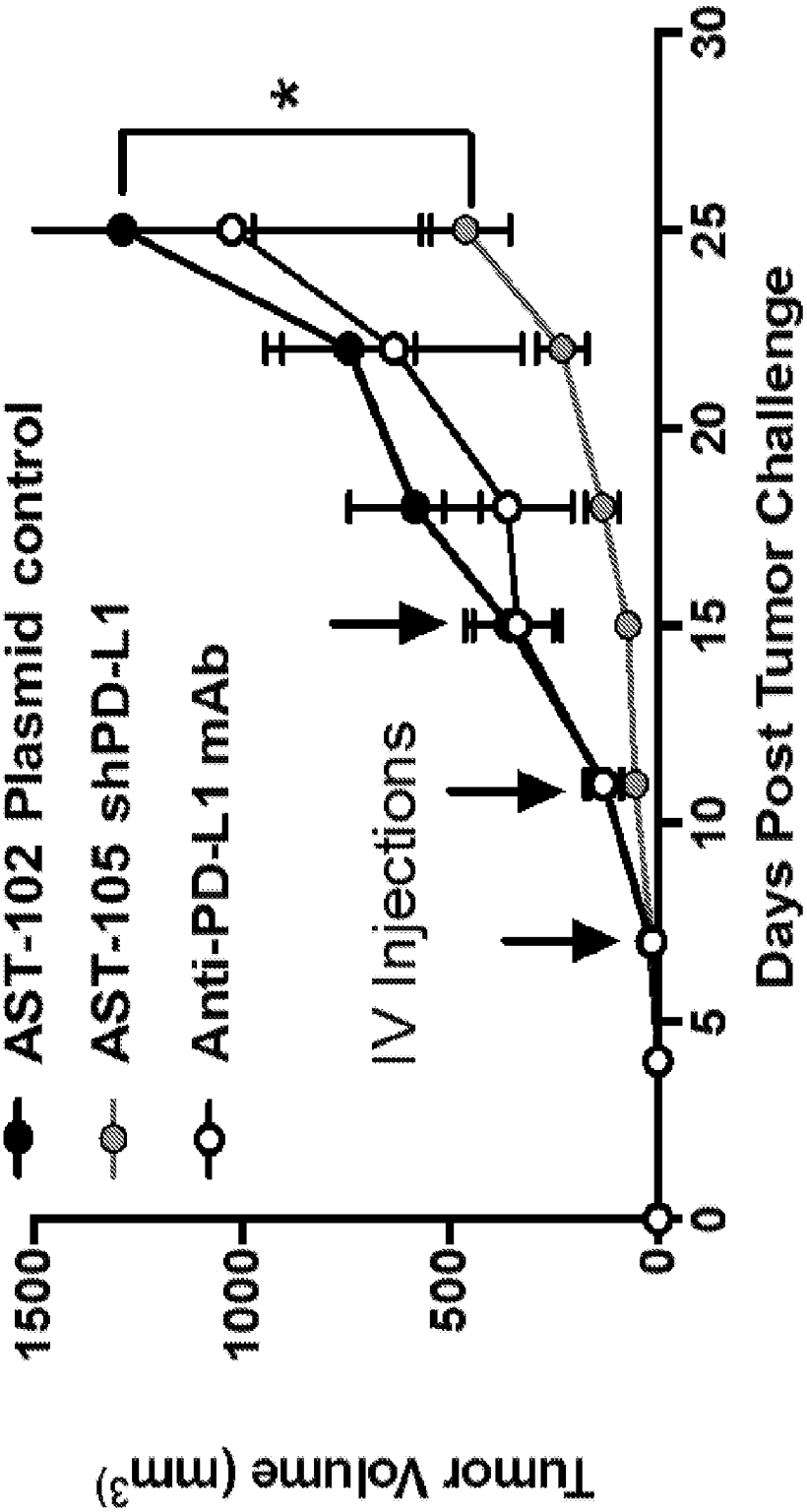

Tumor Volume (mm3)
1500
1000
500
0
0
5
10
15
20
25
30
Days Post Tumor Challenge
IV Injections
AST-102 Plasmid control
AST-105 shPD-L1
Anti-PD-L1 mAb
*

Figure 27

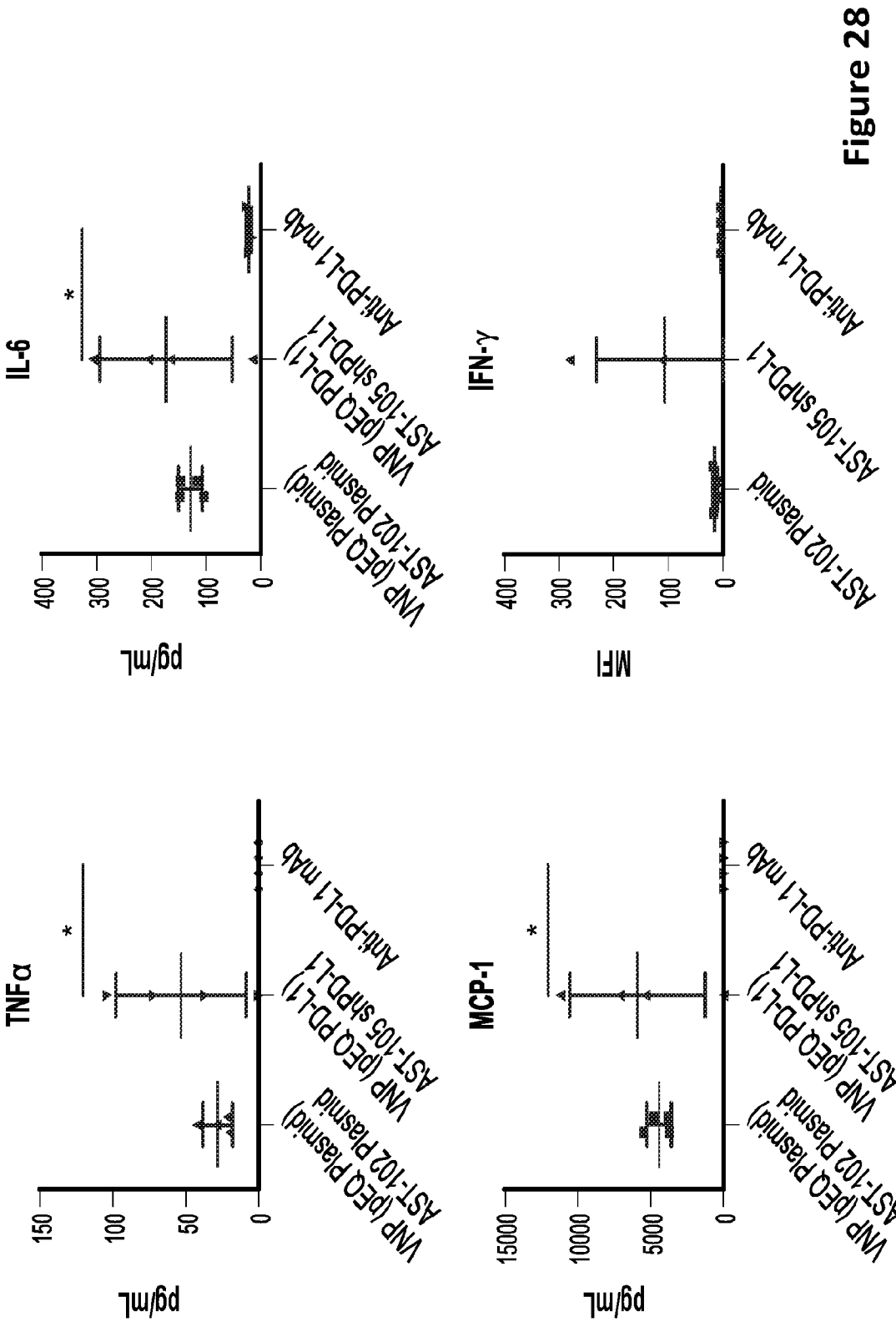

IL-6
pg/mL
400
300
200
100
0
*
AST-102 Plasmid
VNP (pEQ Plasmid)
AST-105 shPD-L1
VNP (pEQ PD-L1)
Anti-PD-L1 mAb
IFN-γ
MFI
400
300
200
100
0
AST-102 Plasmid
AST-105 shPD-L1
Anti-PD-L1 mAb
TNFα
pg/mL
150
100
50
0
*
VNP (pEQ Plasmid)
AST-102 Plasmid
VNP (pEQ PD-L1)
AST-105 shPD-L1
Anti-PD-L1 mAb
MCP-1
pg/mL
15000
10000
5000
0
*
VNP (pEQ Plasmid)
AST-102 Plasmid
VNP (pEQ PD-L1)
AST-105 shPD-L1
Anti-PD-L1 mAb

Figure 28

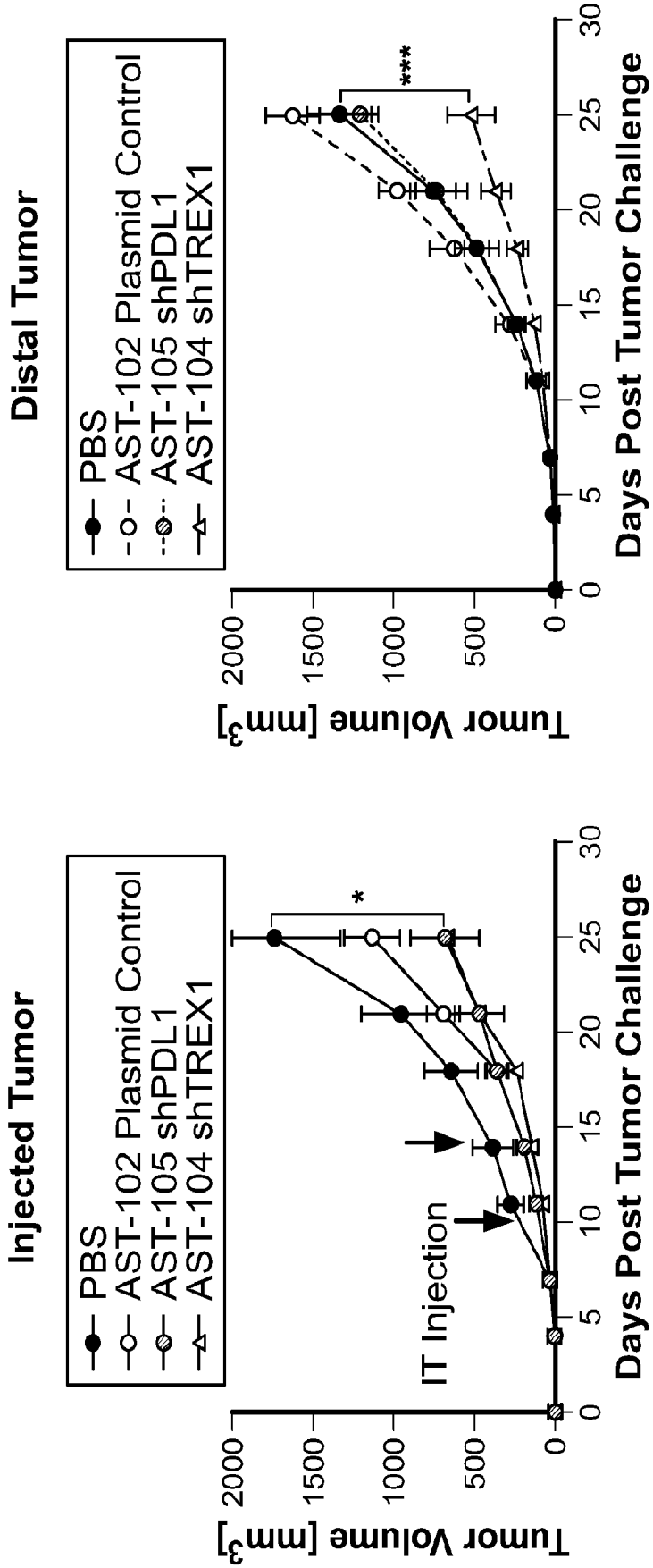

Distal Tumor
PBS
AST-102 Plasmid Control
AST-105 shPDL1
AST-104 shTREX1
***
Tumor Volume [mm3]
0
500
1000
1500
2000
0
5
10
15
20
25
30
Days Post Tumor Challenge
Injected Tumor
PBS
AST-102 Plasmid Control
AST-105 shPDL1
AST-104 shTREX1
*
IT Injection
Tumor Volume [mm3]
0
500
1000
1500
2000
0
5
10
15
20
25
30
Days Post Tumor Challenge

Figure 29

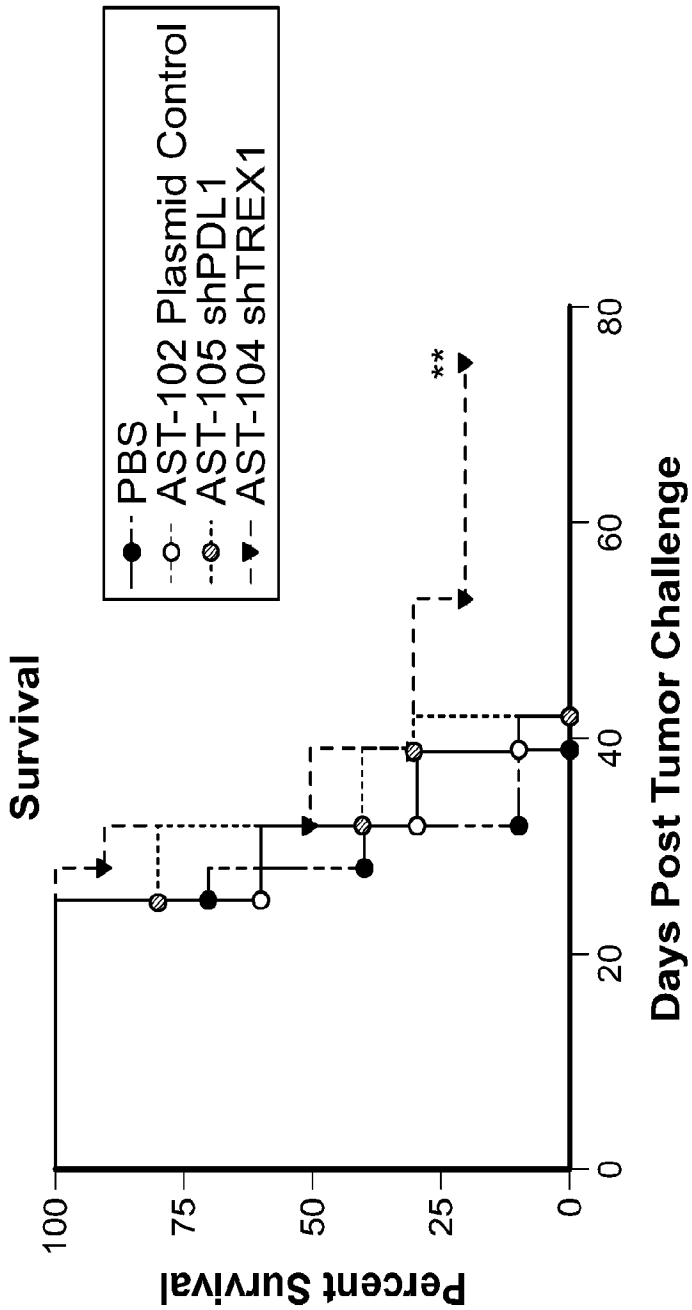

Survival
Percent Survival
100
75
50
25
0
0
20
40
60
80
Days Post Tumor Challenge
**
PBS
AST-102 Plasmid Control
AST-105 shPDL1
AST-104 shTREX1

Figure 30

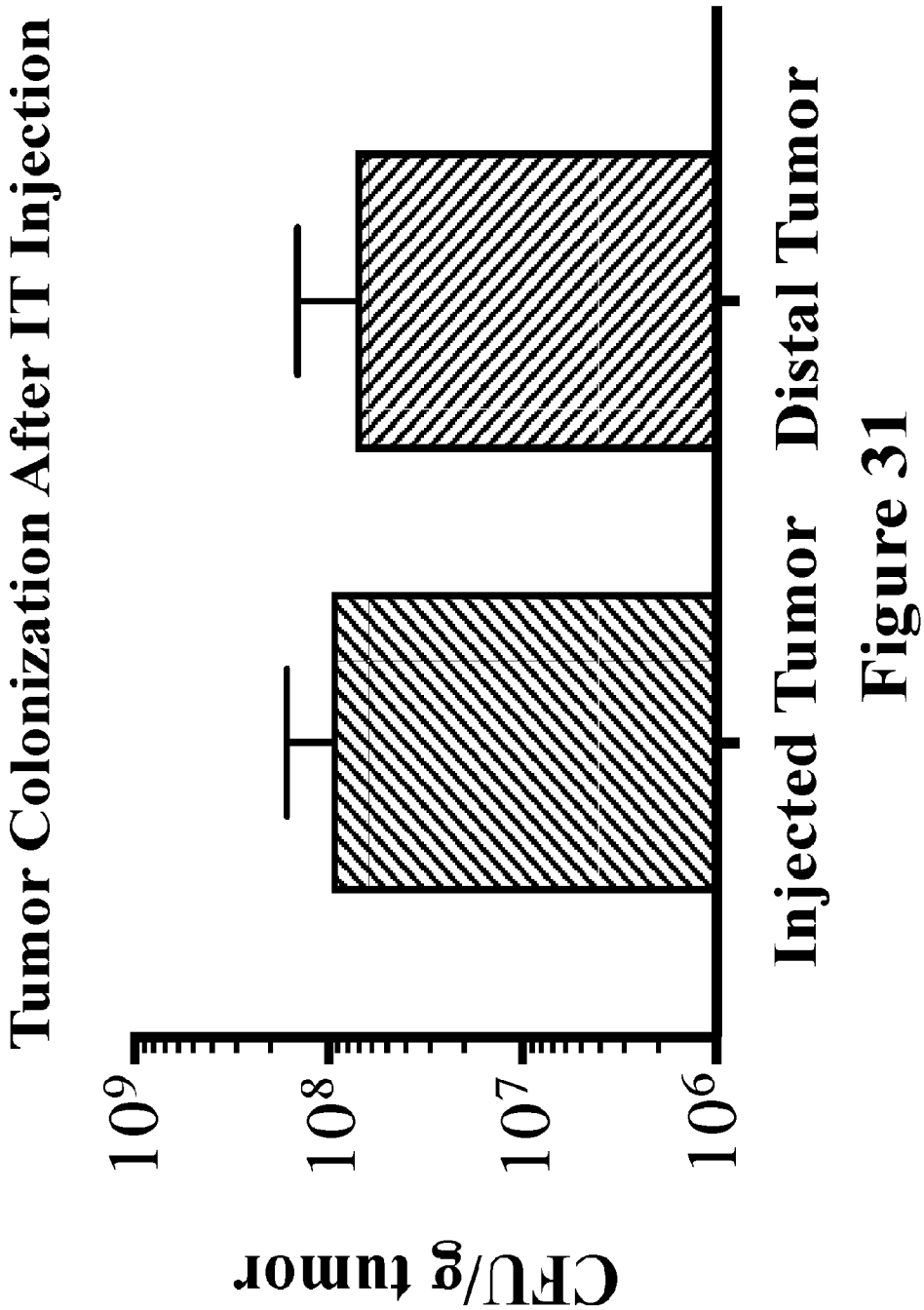

Tumor Colonization After IT Injection
CFU/g tumor
$10^9$
$10^8$
$10^7$
$10^6$
Injected Tumor
Distal Tumor

Figure 31

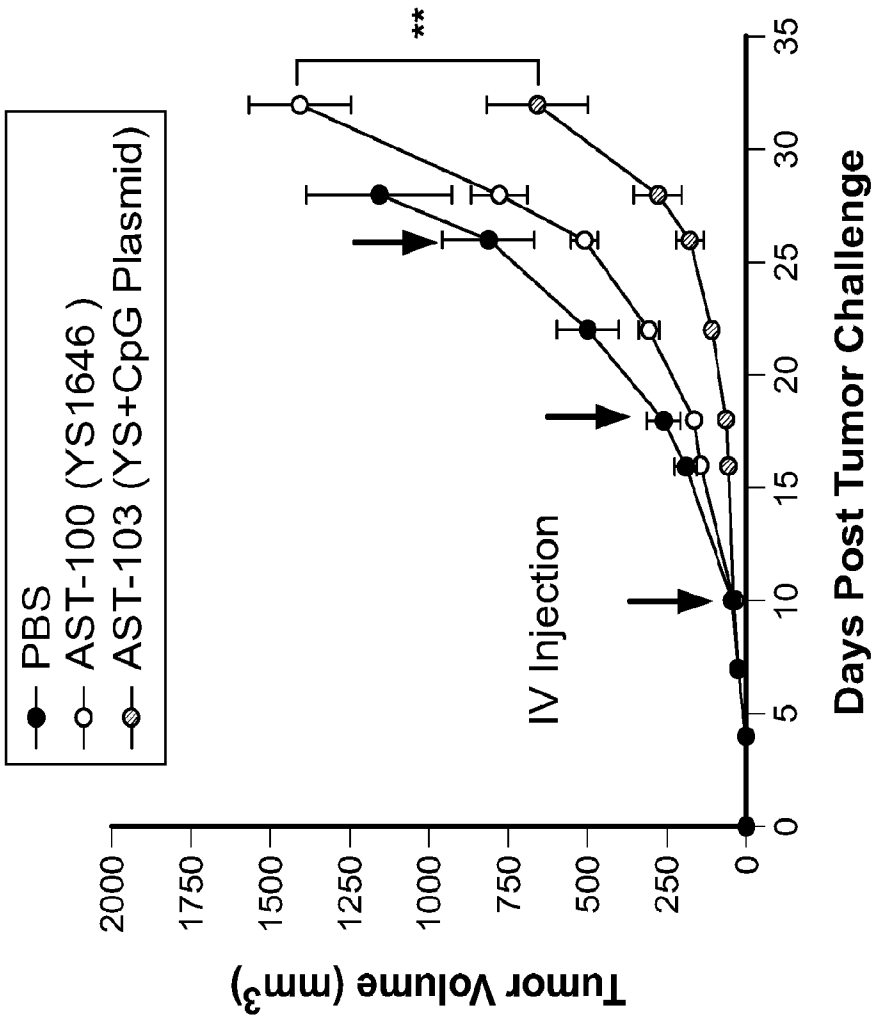

PBS
AST-100 (YS1646 )
AST-103 (YS+CpG Plasmid)
**
IV Injection
Tumor Volume (mm3)
Days Post Tumor Challenge
0
250
500
750
1000
1250
1500
1750
2000
0
5
10
15
20
25
30
35

Figure 32

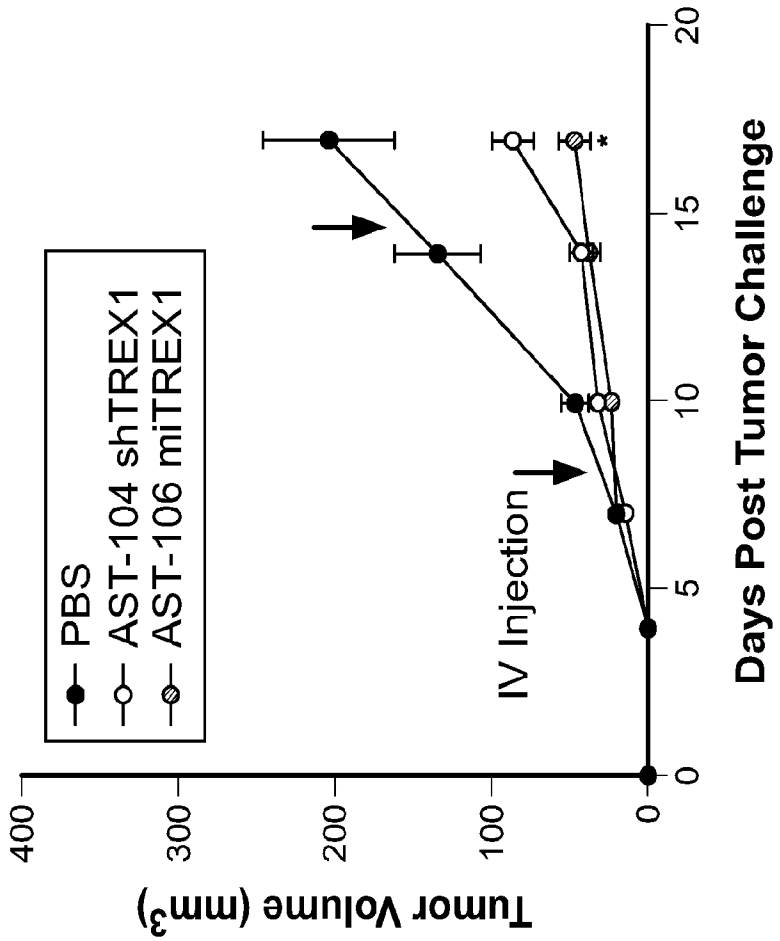

PBS
AST-104 shTREX1
AST-106 miTREX1
IV Injection
*
Tumor Volume (mm3)
Days Post Tumor Challenge
0
100
200
300
400
0
5
10
15
20

Figure 33

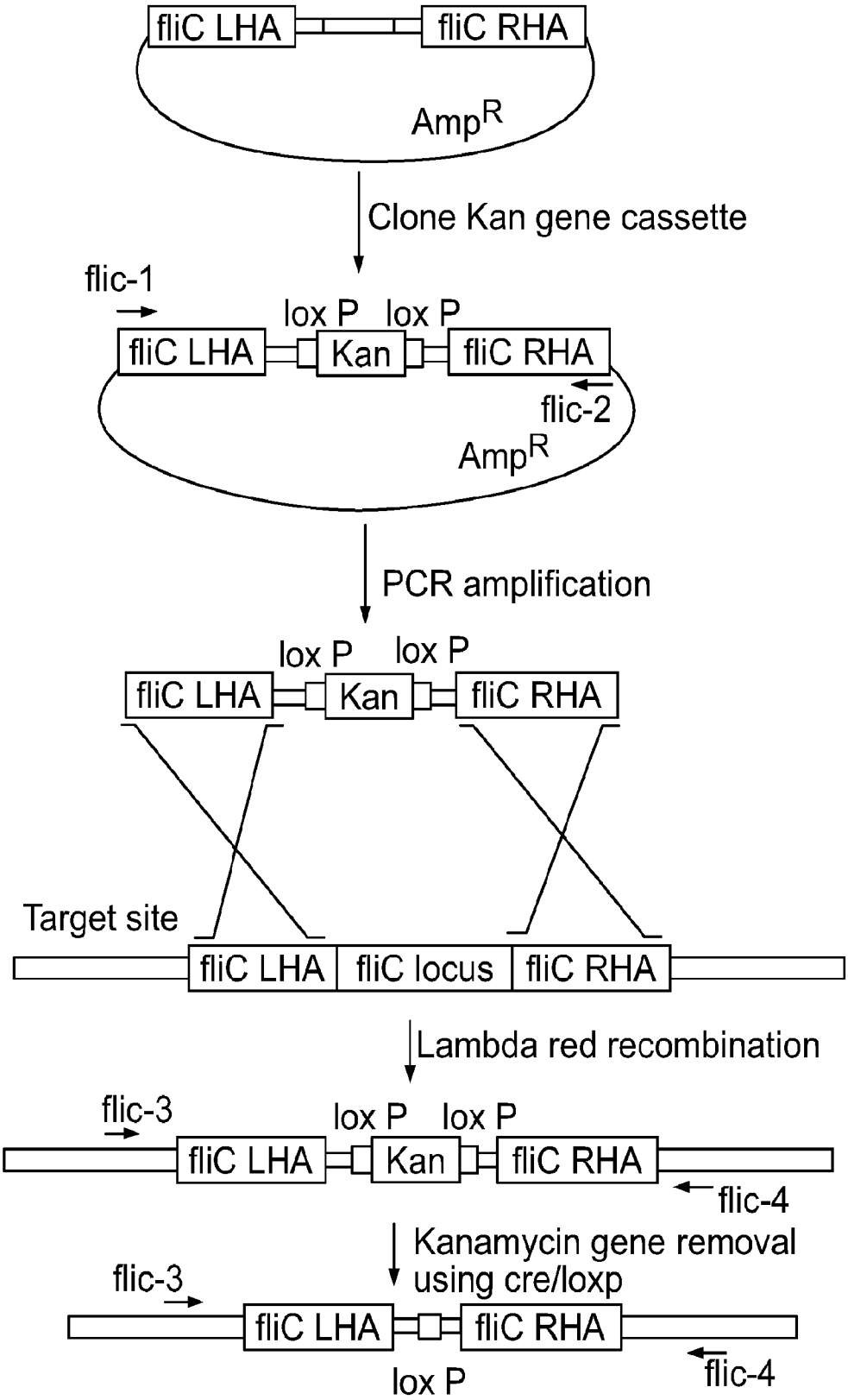

fliC LHA
fliC RHA
Amp^R
Clone Kan gene cassette
flic-1
lox P
lox P
fliC LHA
Kan
fliC RHA
flic-2
Amp^R
PCR amplification
lox P
lox P
fliC LHA
Kan
fliC RHA
Target site
fliC LHA
fliC locus
fliC RHA
Lambda red recombination
flic-3
lox P
lox P
fliC LHA
Kan
fliC RHA
flic-4
Kanamycin gene removal
using cre/loxp
flic-3
fliC LHA
fliC RHA
flic-4
lox P

**Figure 34**

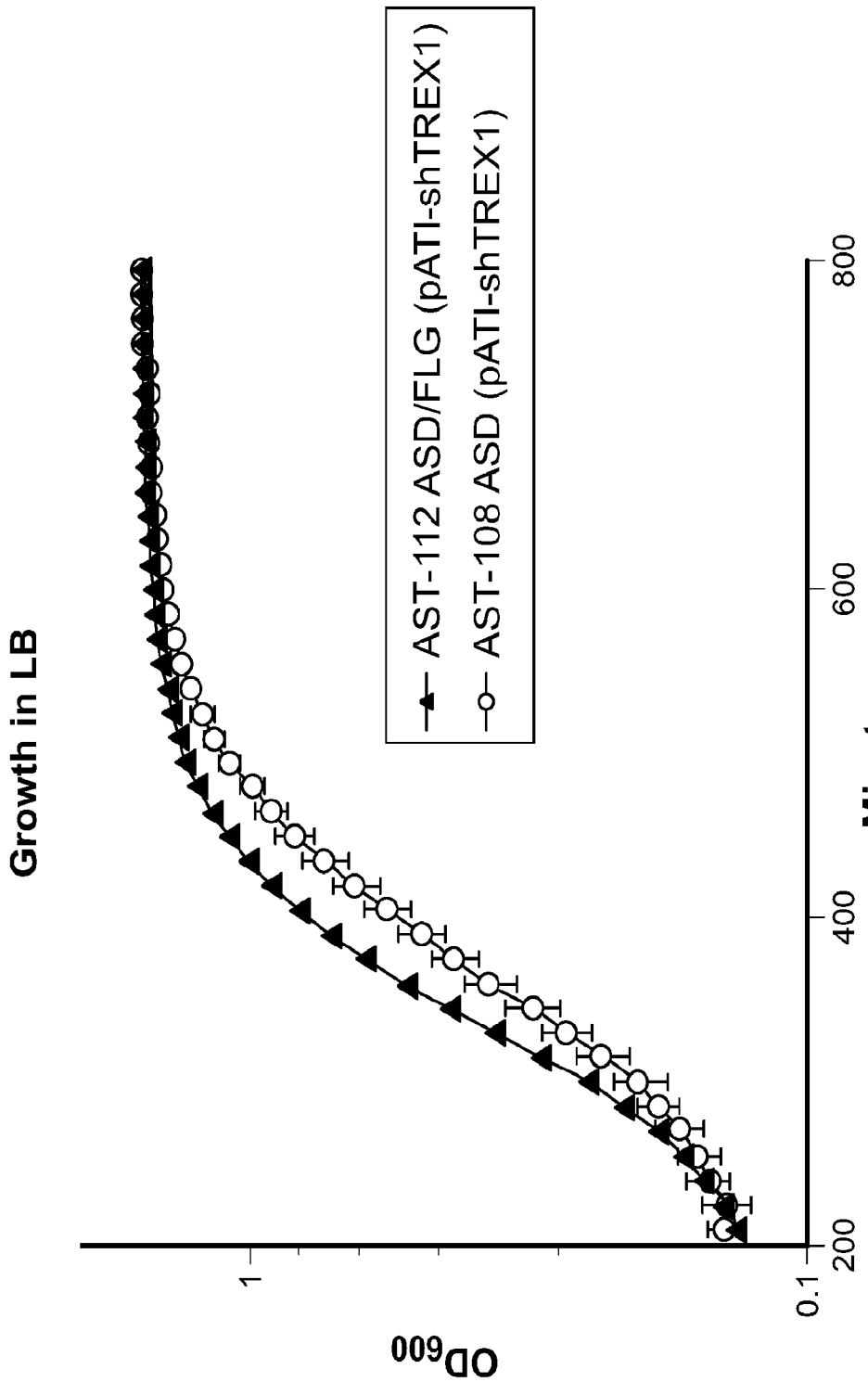

Growth in LB
AST-112 ASD/FLG (pATI-shTREX1)
AST-108 ASD (pATI-shTREX1)
OD600
1
0.1
200
400
600
800
Minutes

Figure 35

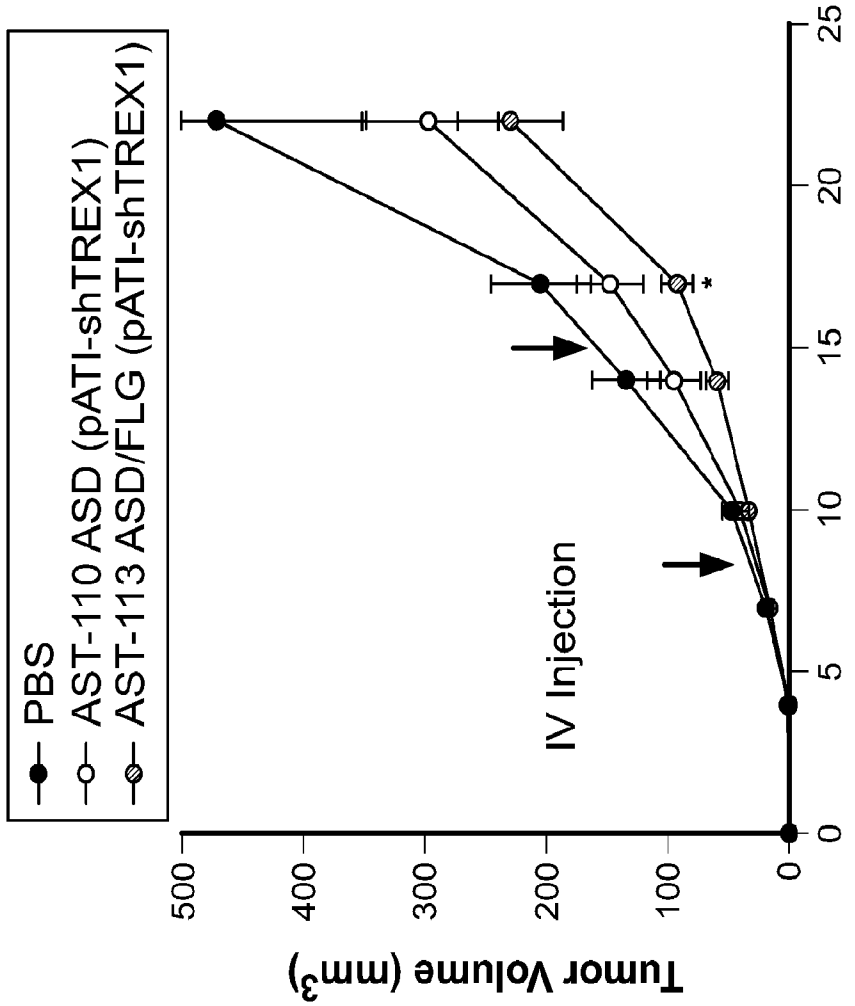

PBS
AST-110 ASD (pATI-shTREX1)
AST-113 ASD/FLG (pATI-shTREX1)
IV Injection
*
Tumor Volume (mm3)
0
100
200
300
400
500
0
5
10
15
20
25
Days Post Tumor Challenge

Figure 36

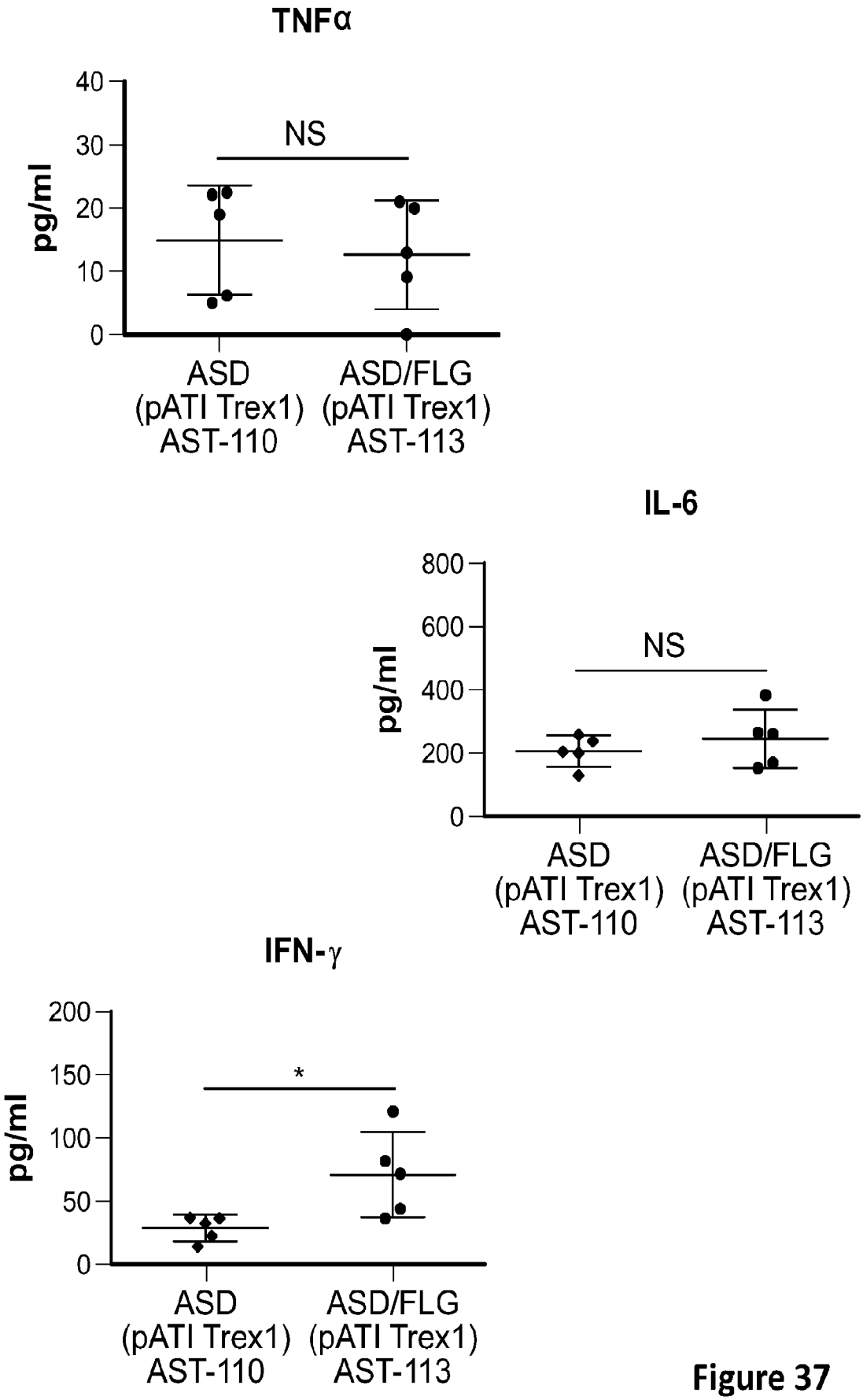

TNFα
pg/ml
40
30
20
10
0
NS
ASD (pATI Trex1) AST-110
ASD/FLG (pATI Trex1) AST-113
IL-6
pg/ml
800
600
400
200
0
NS
ASD (pATI Trex1) AST-110
ASD/FLG (pATI Trex1) AST-113
IFN-γ
pg/ml
200
150
100
50
0
*
ASD (pATI Trex1) AST-110
ASD/FLG (pATI Trex1) AST-113

Figure 37

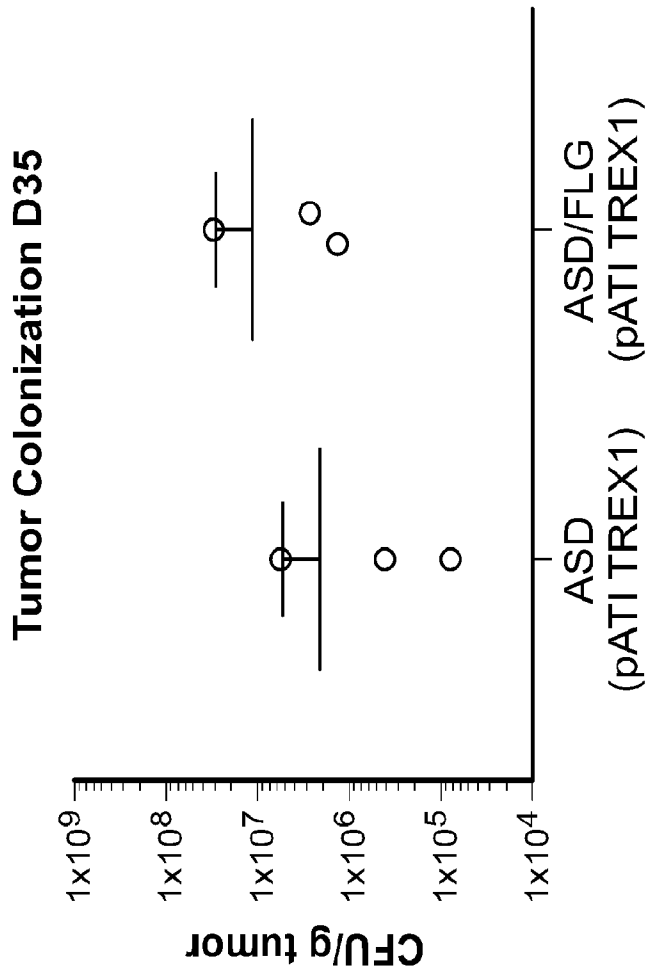

Tumor Colonization D35
CFU/g tumor
1x10^9
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
ASD (pATI TREX1) AST-110
ASD/FLG (pATI TREX1) AST-113

Figure 38

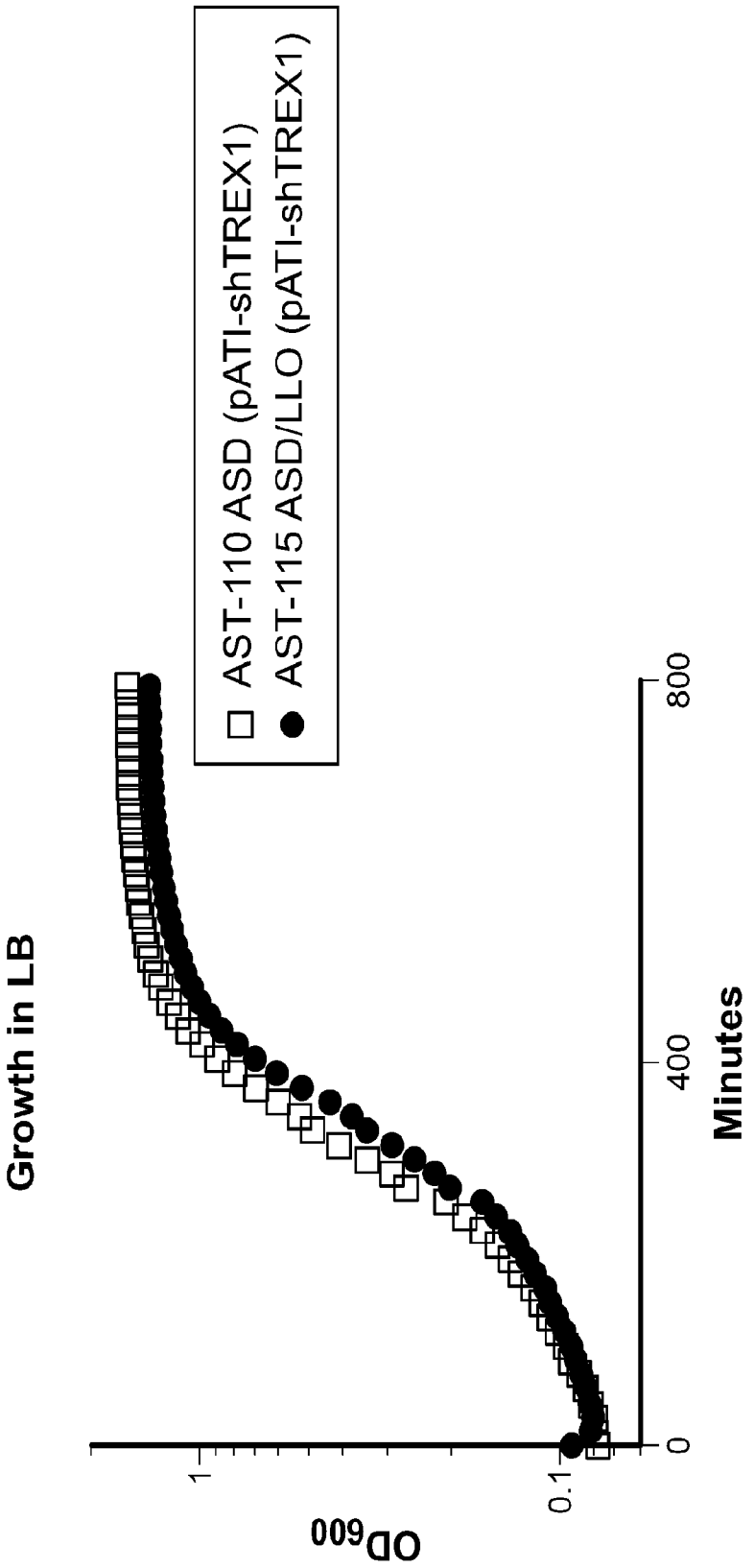

Growth in LB
AST-110 ASD (pATI-shTREX1)
AST-115 ASD/LLO (pATI-shTREX1)
OD600
1
0.1
0
400
800
Minutes

Figure 39

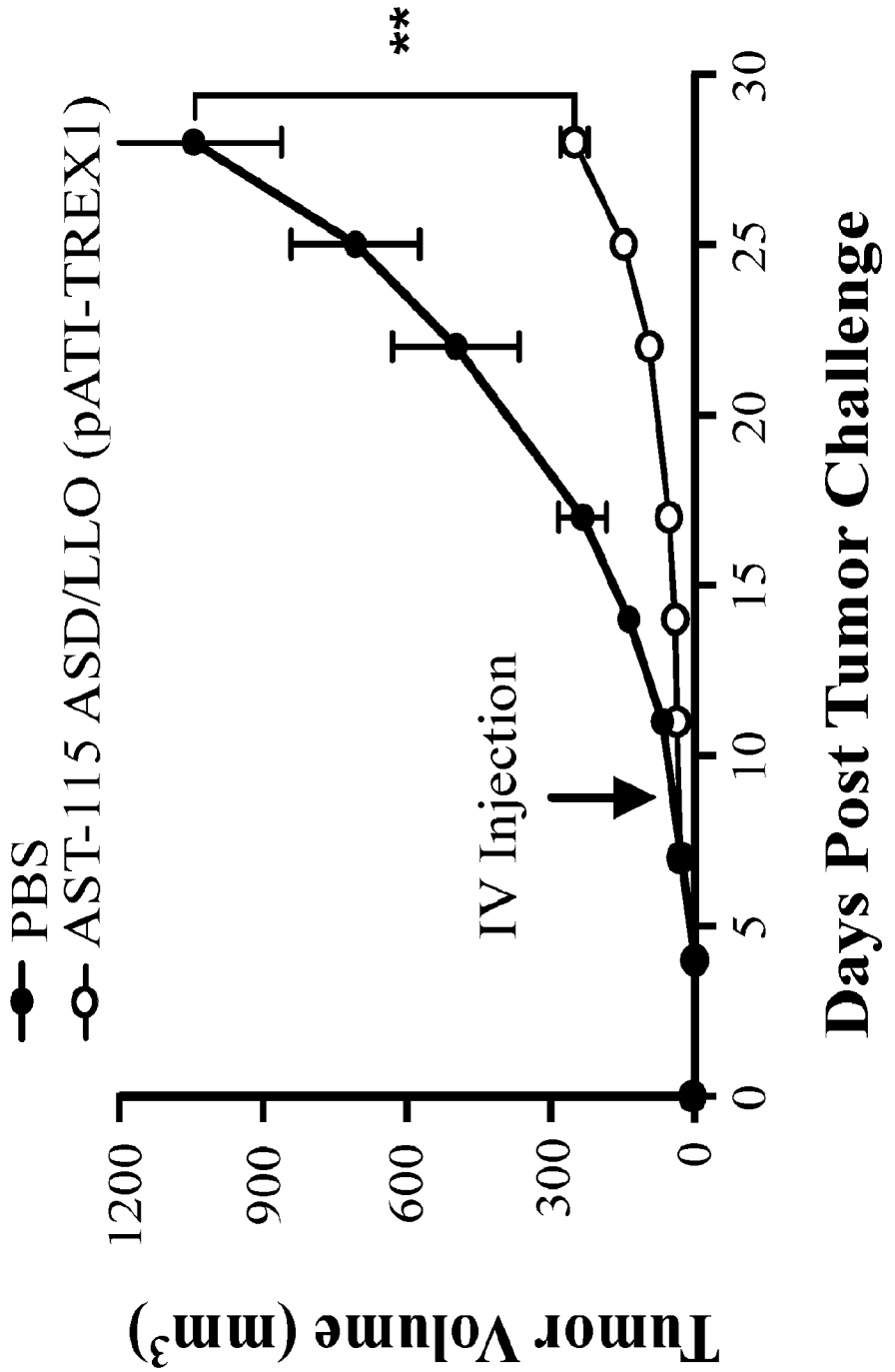

**
PBS
AST-115 ASD/LLO (pATI-TREX1)
IV Injection
Tumor Volume (mm³)
1200
900
600
300
0
0
5
10
15
20
25
30
Days Post Tumor Challenge

Figure 40

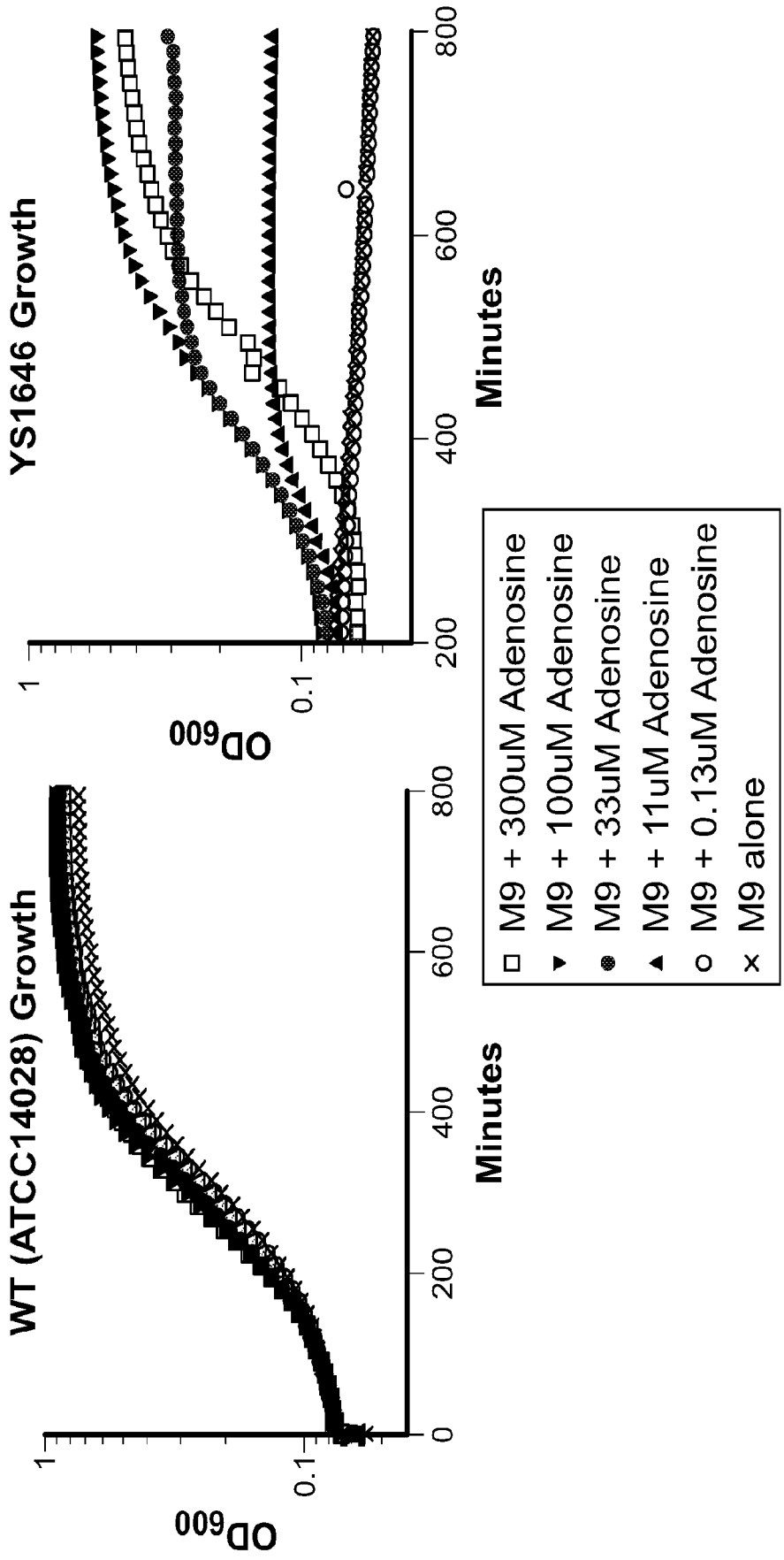

YS1646 Growth
WT (ATCC14028) Growth
OD600
Minutes
1
0.1
0
200
400
600
800
M9 + 300uM Adenosine
M9 + 100uM Adenosine
M9 + 33uM Adenosine
M9 + 11uM Adenosine
M9 + 0.13uM Adenosine
M9 alone

Figure 41

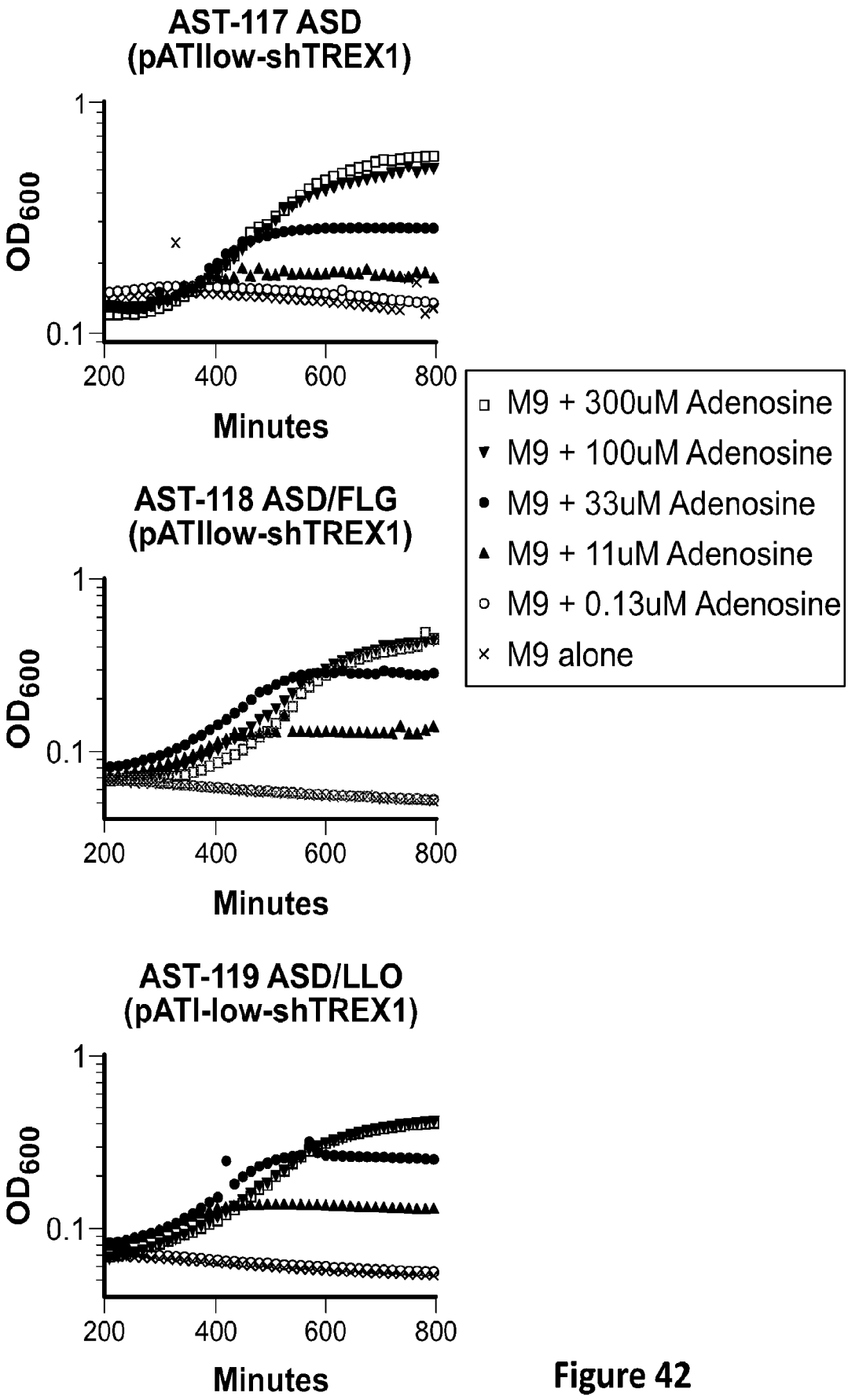

AST-117 ASD
(pATIlow-shTREX1)
OD600
1
0.1
200
400
600
800
Minutes
AST-118 ASD/FLG
(pATIlow-shTREX1)
OD600
1
0.1
200
400
600
800
Minutes
AST-119 ASD/LLO
(pATI-low-shTREX1)
OD600
1
0.1
200
400
600
800
Minutes
M9 + 300uM Adenosine
M9 + 100uM Adenosine
M9 + 33uM Adenosine
M9 + 11uM Adenosine
M9 + 0.13uM Adenosine
M9 alone

Figure 42

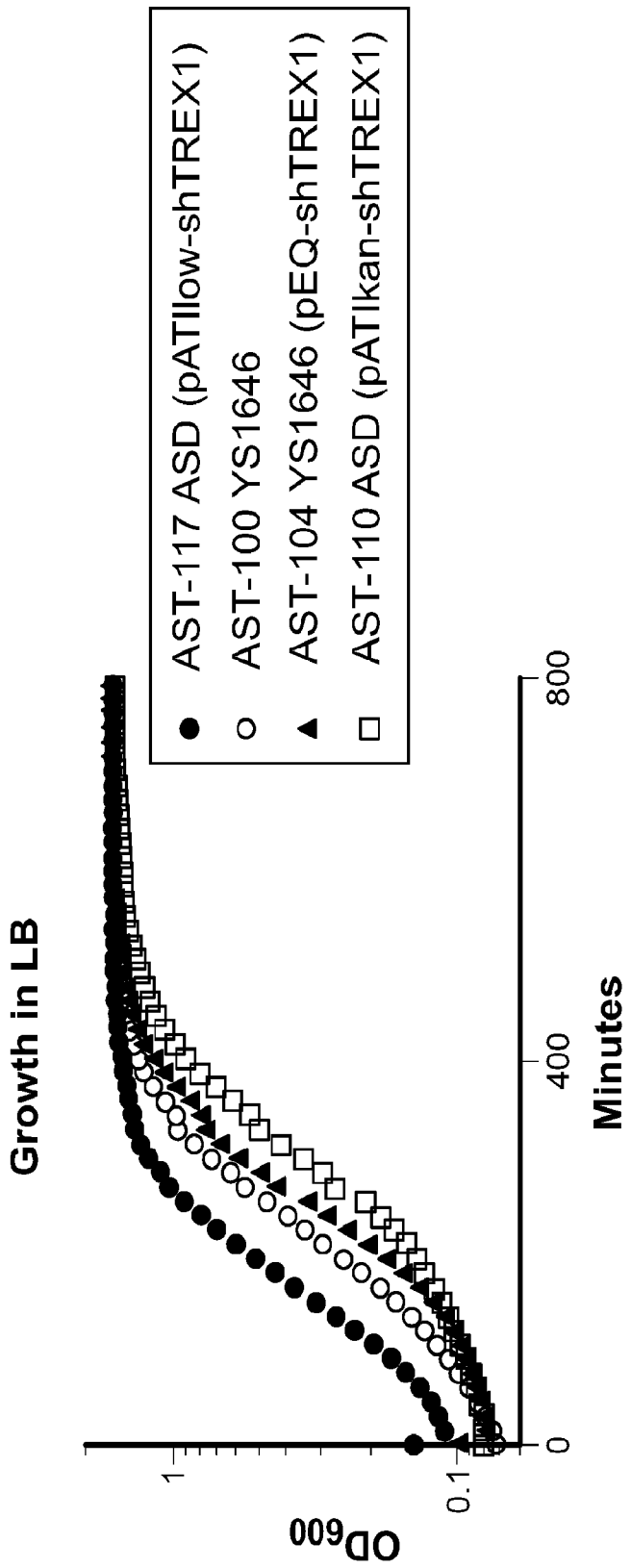

Growth in LB
OD600
1
0.1
0
400
800
Minutes
AST-117 ASD (pATIlow-shTREX1)
AST-100 YS1646
AST-104 YS1646 (pEQ-shTREX1)
AST-110 ASD (pATIkan-shTREX1)

Figure 43

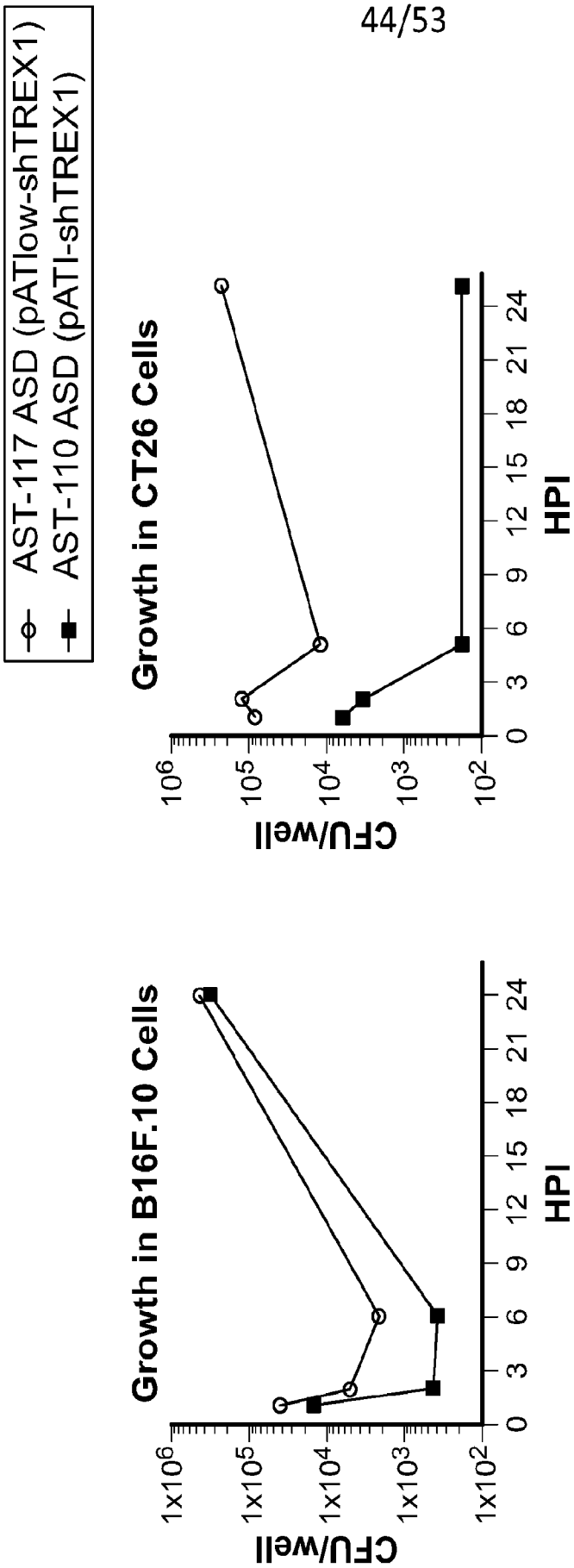

AST-117 ASD (pATlow-shTREX1)
AST-110 ASD (pATI-shTREX1)
Growth in CT26 Cells
CFU/well
HPI
Growth in B16F.10 Cells
CFU/well
HPI

Figure 44

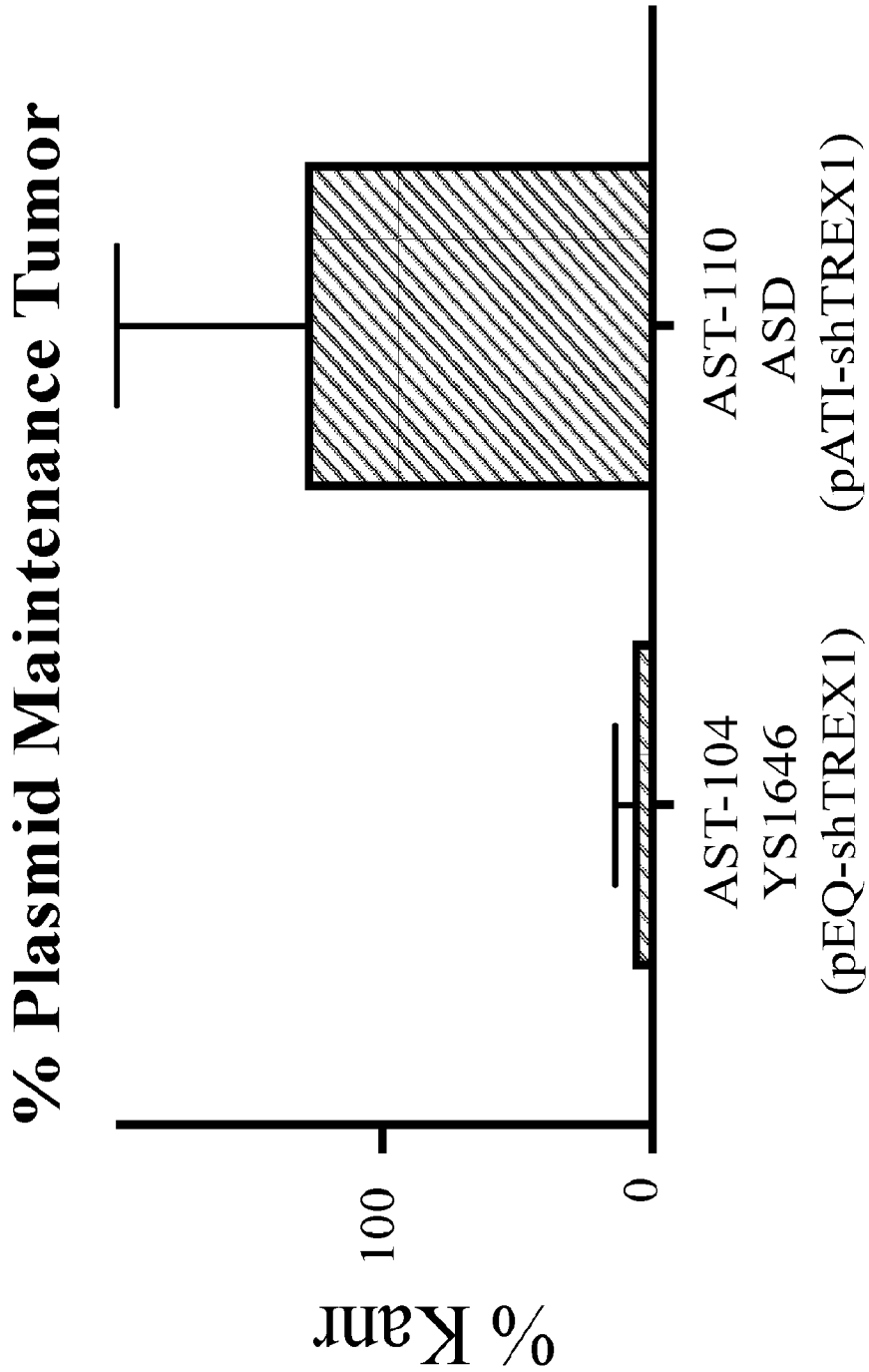

% Plasmid Maintenance Tumor
% Kanr
100
0
AST-104
YS1646
(pEQ-shTREX1)
AST-110
ASD
(pATI-shTREX1)

**Figure 45**

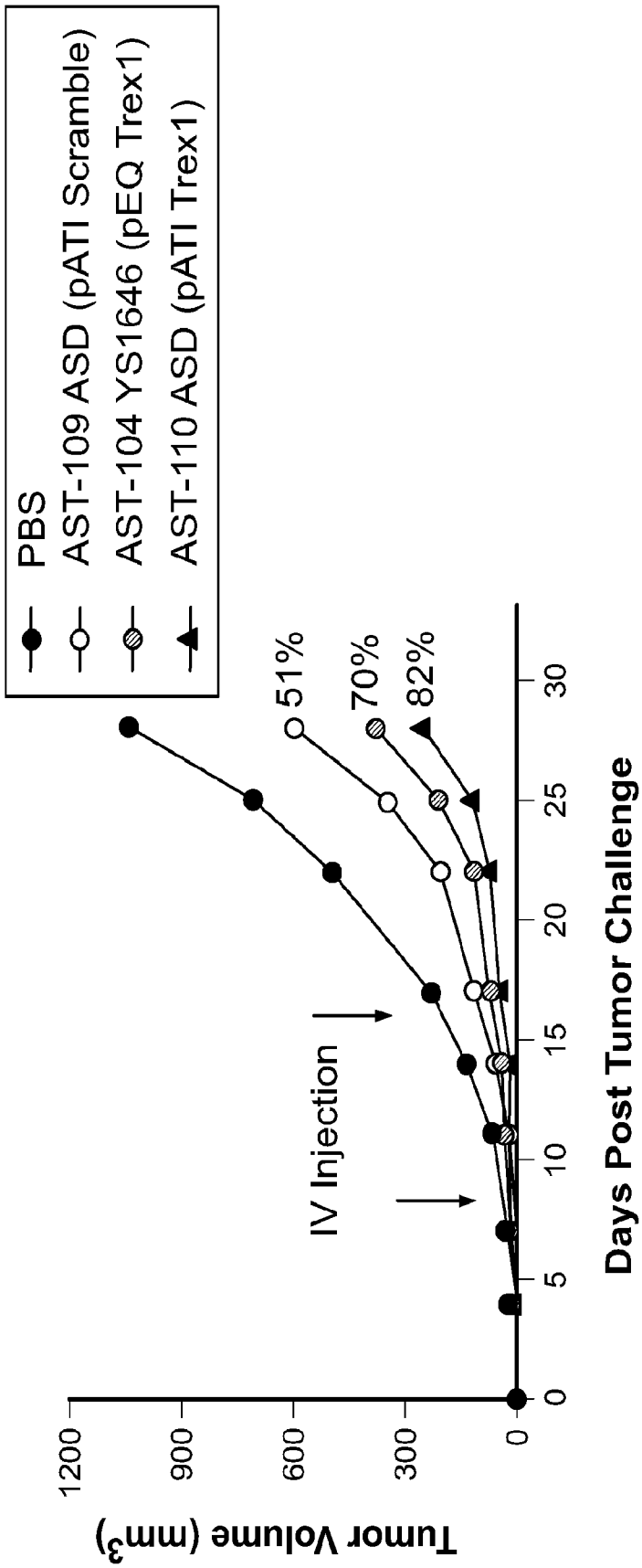

PBS
AST-109 ASD (pATI Scramble)
AST-104 YS1646 (pEQ Trex1)
AST-110 ASD (pATI Trex1)
51%
70%
82%
IV Injection
Tumor Volume (mm3)
1200
900
600
300
0
0
5
10
15
20
25
30
Days Post Tumor Challenge

Figure 46

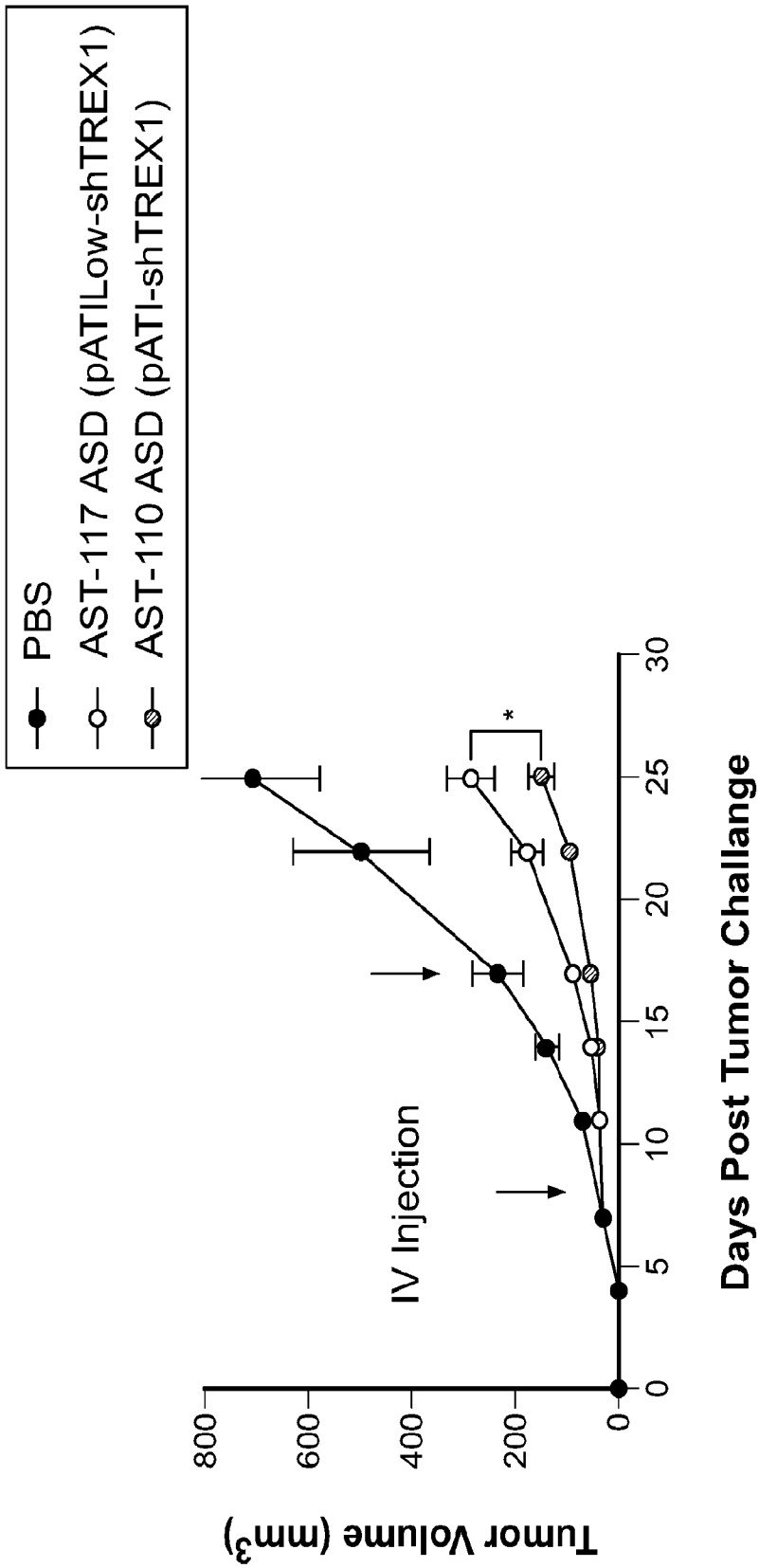

PBS
AST-117 ASD (pATILow-shTREX1)
AST-110 ASD (pATI-shTREX1)
Tumor Volume (mm3)
800
600
400
200
0
IV Injection
*
0
5
10
15
20
25
30
Days Post Tumor Challange

Figure 47

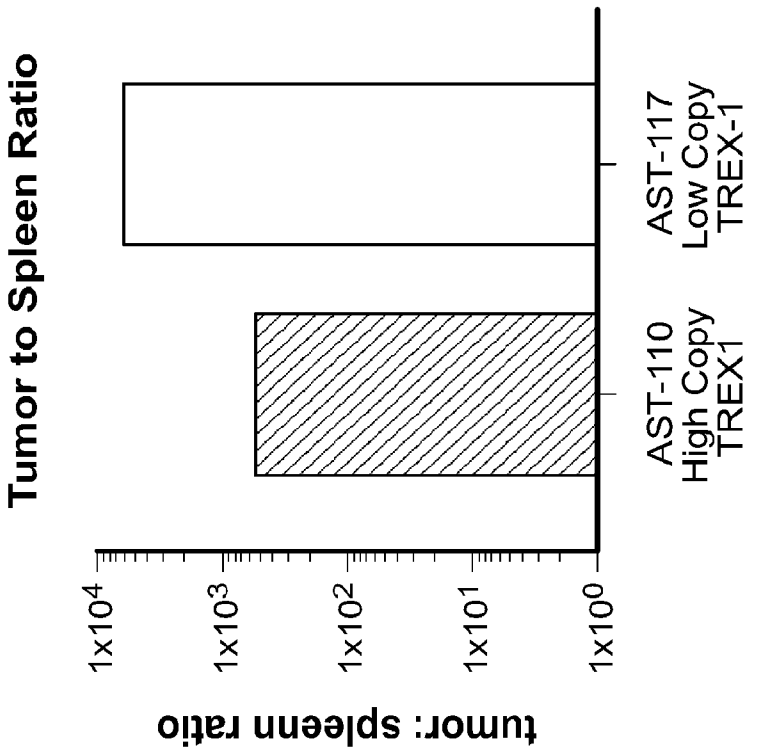



Figure 48B

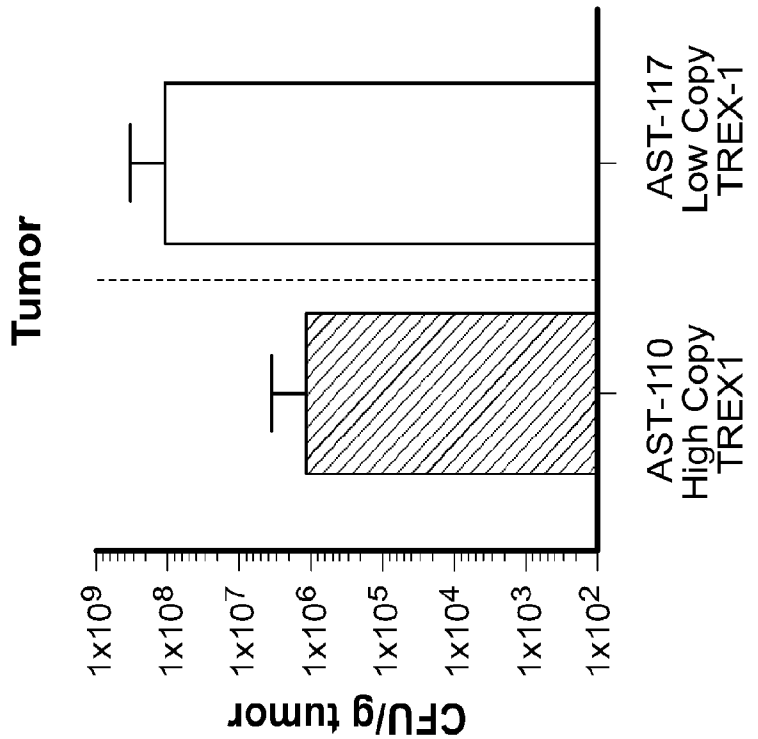



Figure 48A

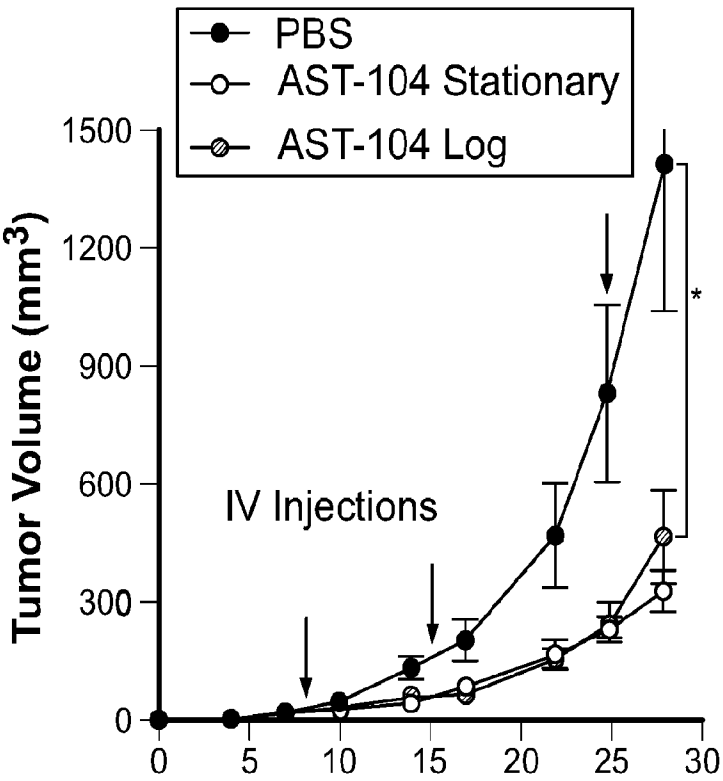



Figure 49A

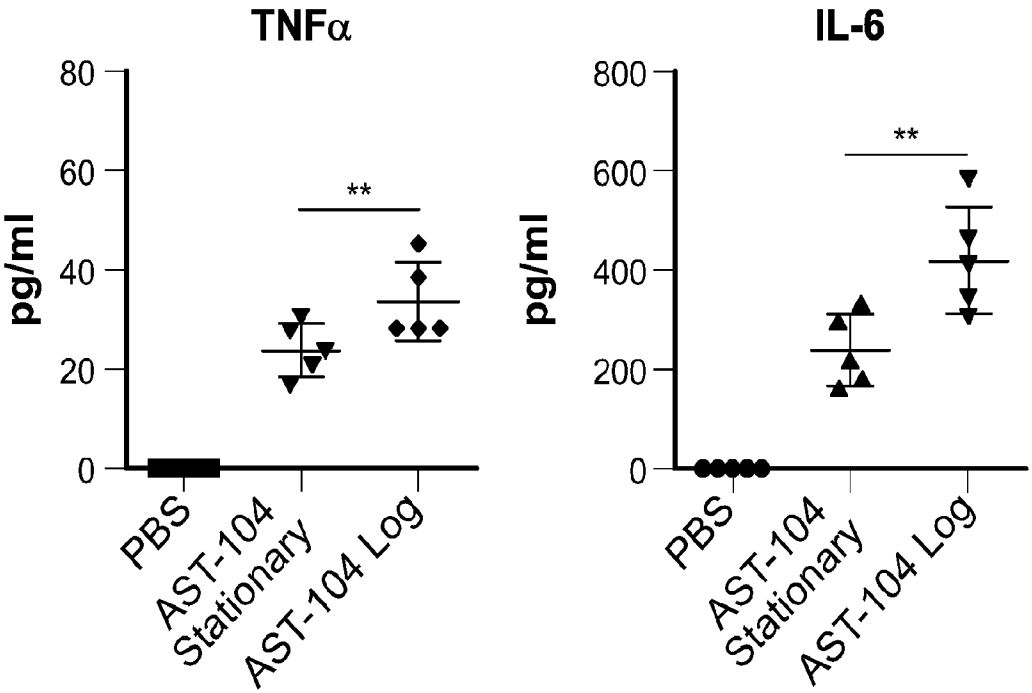



Figure 49B

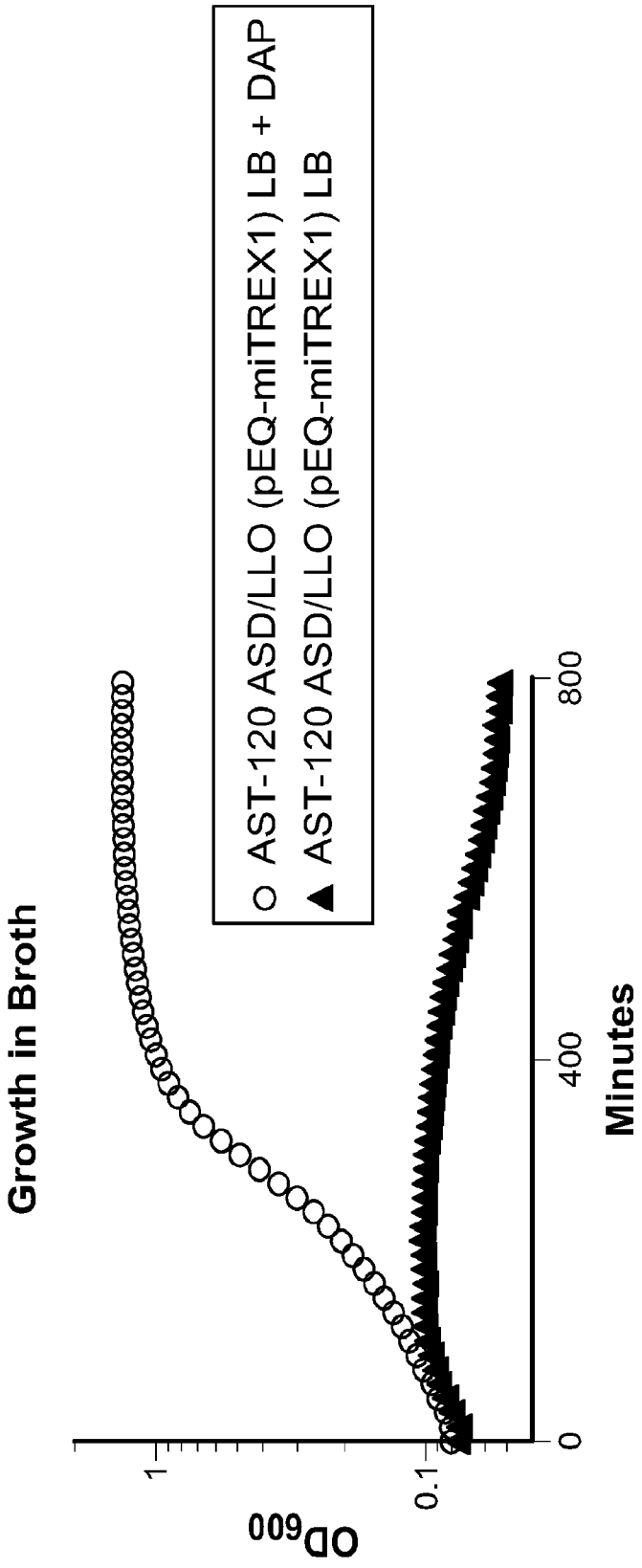

Growth in Broth
OD600
1
0.1
0
400
800
Minutes
AST-120 ASD/LLO (pEQ-miTREX1) LB + DAP
AST-120 ASD/LLO (pEQ-miTREX1) LB

Figure 50

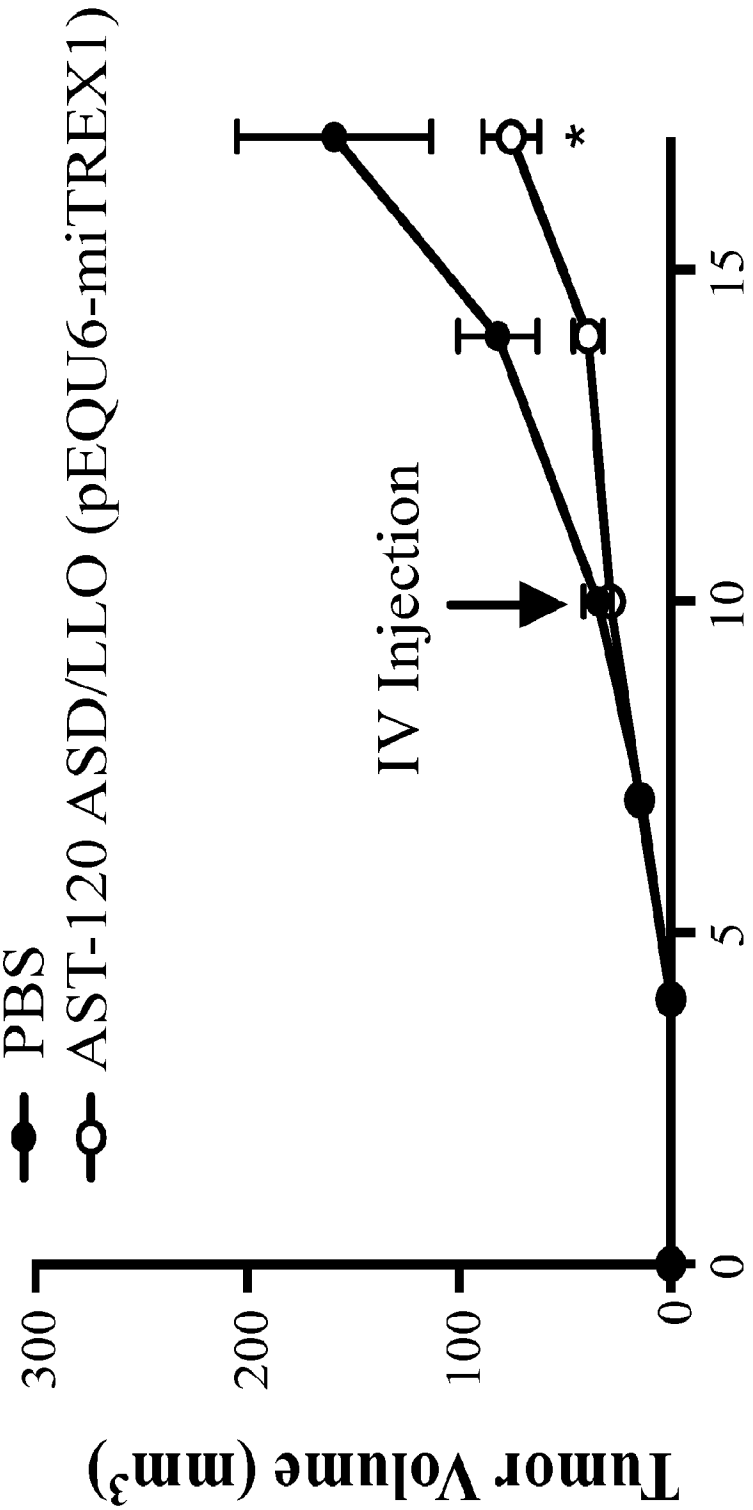

PBS
AST-120 ASD/LLO (pEQU6-miTREX1)
IV Injection
*
Tumor Volume (mm³)
300
200
100
0
0
5
10
15
Days Post Tumor Challenge

Figure 51

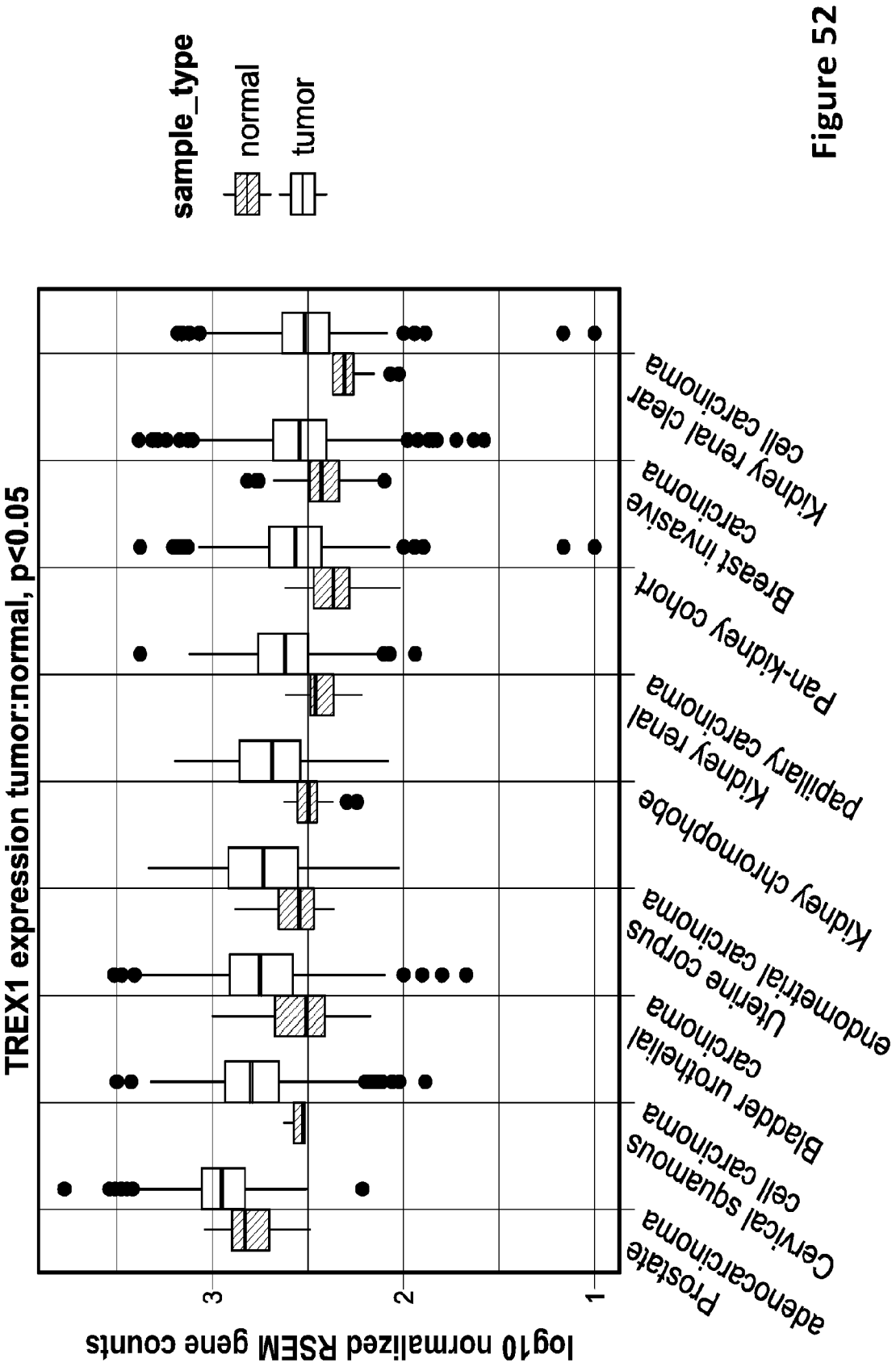

sample_type
normal
tumor
TREX1 expression tumor:normal, p<0.05
log10 normalized RSEM gene counts
3
2
1
Prostate adenocarcinoma
Cervical squamous cell carcinoma
Bladder urothelial carcinoma
Uterine corpus endometrial carcinoma
Kidney chromophobe
Kidney renal papillary carcinoma
Pan-kidney cohort
Breast invasive carcinoma
Kidney renal clear cell carcinoma

Figure 52

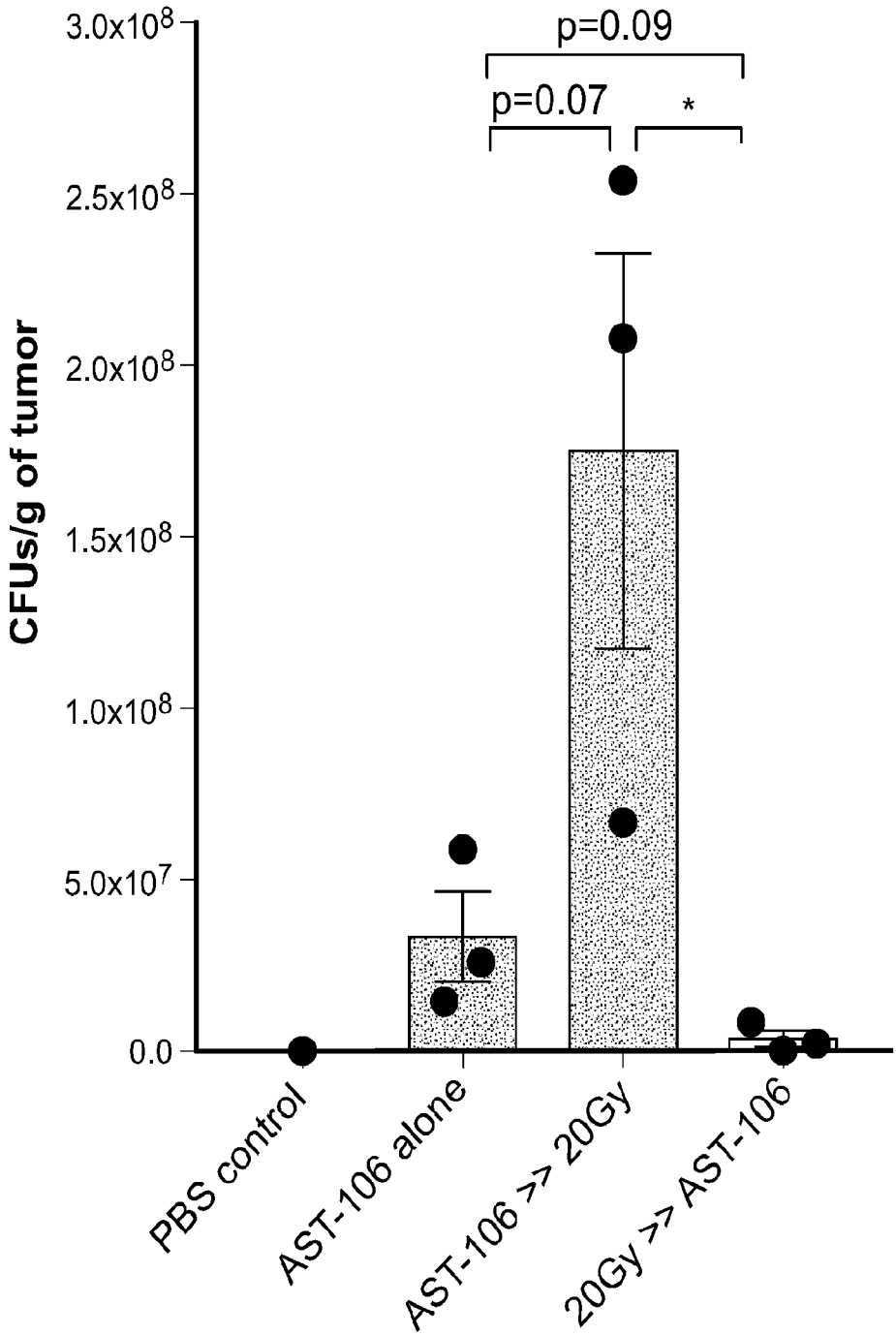

3.0x10^8
2.5x10^8
2.0x10^8
1.5x10^8
1.0x10^8
5.0x10^7
0.0
CFUs/g of tumor
p=0.09
p=0.07
*
PBS control
AST-106 alone
AST-106 >> 20Gy
20Gy >> AST-106

**Figure 53**

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/041713

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [X] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/041713

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

56, 59, 115, 117, 179-184, 186-193, 195, 197-201, 208, 209, 216
217(completely); 1-54, 57, 58, 60-103, 105-114, 119-165, 171-177, 185, 194
196, 202-207, 210-215, 225(partially)

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☒ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/041713

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 C12N1/36 A61K35/74 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 2015/032165 A1 (UNIV HONG KONG [CN])<br>12 March 2015 (2015-03-12)<br><br>page 56 - page 72; claims 31,33,34; table 1<br>-----<br>-/-- | 1,2,<br>5-54,<br>56-103,<br>105-115,<br>117,<br>119-165,<br>171-177,<br>179-217,<br>225 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 19 December 2018 | 03/01/2019 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Bucka, Alexander |

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 2008/091375 A2 (US GOV HEALTH & HUMAN SERV [US]; UNIV JILIN [CN]; UNIV MARYLAND [US];) 31 July 2008 (2008-07-31)<br>claims 1,8; example 1; tables 2,7 | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| A | SHI LEI ET AL: "Combined prokaryotic-eukaryotic delivery and expression of therapeutic factors through a primed autocatalytic positive-feedback loop", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 222, 10 December 2015 (2015-12-10), pages 130-140, XP029382881, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2015.12.005<br>page 136, left-hand column; figures 2,6 | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| A | C. A. BLACHE ET AL: "Systemic Delivery of Salmonella typhimurium Transformed with IDO shRNA Enhances Intratumoral Vector Colonization and Suppresses Tumor Growth", CANCER RESEARCH, vol. 72, no. 24, 22 October 2012 (2012-10-22), pages 6447-6456, XP55305466, US ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-12-0193<br>figures 2-4 | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| Y | CLAIRE VANPOUILLE-BOX ET AL: "DNA exonuclease Trex1 regulates radiotherapy-induced tumour immunogenicity", NATURE COMMUNICATIONS, vol. 8, 9 June 2017 (2017-06-09), page 15618, XP55512079, GB ISSN: 2041-1723, DOI: 10.1038/ncomms15618<br>figures 4,6 | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| Y | US 2014/127284 A1 (CHERESH DAVID [US] ET AL) 8 May 2014 (2014-05-08)<br>claims 6,22 | 41,45, 47,69,70 |

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | LEE ADAM WHEELER ET AL: "TREX1 Knockdown Induces an Interferon Response to HIV that Delays Viral Infection in Humanized Mice", CELL REPORTS, vol. 15, no. 8, 12 May 2016 (2016-05-12), pages 1715-1727, XP55511358, ISSN: 2211-1247, DOI: 10.1016/j.celrep.2016.04.048<br><br>figures 2,4<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| A | TOMICIC MAJA T ET AL: "Human three prime exonuclease TREX1 is induced by genotoxic stress and involved in protection of glioma and melanoma cells to anticancer drugs", BIOCHIMICA ET BIOPHYSICA ACTA. MOLECULAR CELL RESEARCH, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 1833, no. 8, 8 April 2013 (2013-04-08), pages 1832-1843, XP028557924, ISSN: 0167-4889, DOI: 10.1016/J.BBAMCR.2013.03.029<br>figure 8<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| Y | RAEANNA WILSON ET AL: "MicroRNA regulation of endothelial TREX1 reprograms the tumour microenvironment", NATURE COMMUNICATIONS, vol. 7, 25 November 2016 (2016-11-25), page 13597, XP55512101, GB<br>ISSN: 2041-1723, DOI: 10.1038/ncomms13597<br>figures 3-5<br>----- | 41,45, 47,69,70 |
| A | BIN YU ET AL: "Explicit hypoxia targeting with tumor suppression by creating an "obligate" anaerobic Salmonella Typhimurium strain", SCIENTIFIC REPORTS, vol. 2, 1 June 2012 (2012-06-01), XP55176169, DOI: 10.1038/srep00436<br><br>page 8<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |

-/--

3

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | ULRICH METHNER ET AL: "Salmonella Enteritidis with double deletion in phoP fliC - A potential live Salmonella vaccine candidate with novel characteristics for use in chickens", VACCINE, ELSEVIER, AMSTERDAM, NL, vol. 29, no. 17, 24 February 2011 (2011-02-24), pages 3248-3253, XP028380401, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2011.02.030 [retrieved on 2011-02-16] the whole document<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| A | YUE JAI KANG ET AL: "Preventive and therapeutic effects of auxotrophic Edwardsiella tarda mutant harboring CpG 1668 motif-enriched plasmids against scuticociliatosis in olive flounder (Paralichthys olivaceus)", EXPERIMENTAL PARASITOLOGY, vol. 144, 12 June 2014 (2014-06-12), pages 34-38, XP55512164, US ISSN: 0014-4894, DOI: 10.1016/j.exppara.2014.06.007 figures 1,2<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| A | DEWAN MD SAKIB HOSSAIN ET AL: "Leukemia cell-targeted STAT3 silencing and TLR9 triggering generate systemic antitumor immunity", BLOOD, vol. 123, no. 1, 29 October 2013 (2013-10-29), pages 15-25, XP55512071, DOI: 10.1182/blood-2013-07- abstract<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |
| A | PAULINA CHOROBIK ET AL: "Salmonella and cancer: from pathogens to therapeutics", ACTA BIOCHIMICA POLONICA, vol. 60, no. 3, 5 July 2013 (2013-07-05), pages 285-297, XP55511316, tables 2,3<br>----- | 1,2, 5-54, 56-103, 105-115, 117, 119-165, 171-177, 179-217, 225 |

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 2010/057009 A1 (CEQUENT PHARMACEUTICALS INC [US]; FRUEHAUF JOHANNES HEINRICH [US]; VAZ) 20 May 2010 (2010-05-20)<br><br>claims 1,2,20; examples 31,32 | 3-54,57, 58, 60-103, 105-114, 119-165, 171-177, 185,194, 196, 202-207, 210-215, 225 |
| Y | WO 2011/100489 A2 (BRIGHAM & WOMENS HOSPITAL [US]; WALDOR MATTHEW K [US]) 18 August 2011 (2011-08-18)<br><br>paragraph [0040]; claims 1-3 | 3-54,57, 58, 60-103, 105-114, 119-165, 171-177, 185,194, 196, 202-207, 210-215, 225 |
| Y | A. FAULDS-PAIN ET AL: "Flagellin Redundancy in Caulobacter crescentus and Its Implications for Flagellar Filament Assembly", JOURNAL OF BACTERIOLOGY, vol. 193, no. 11, 25 March 2011 (2011-03-25), pages 2695-2707, XP55532473, US ISSN: 0021-9193, DOI: 10.1128/JB.01172-10<br><br>table 1 | 3-54,57, 58, 60-103, 105-114, 119-165, 171-177, 185,194, 196, 202-207, 210-215, 225 |
| A | BROADWAY KATHERINE M ET AL: "Rescuing chemotaxis of the anticancer agent Salmonella enterica serovar Typhimurium VNP20009", JOURNAL OF BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 211, 19 July 2015 (2015-07-19), pages 117-120, XP029273086, ISSN: 0168-1656, DOI: 10.1016/J.JBIOTEC.2015.07.010<br><br>page 118; table 1 | 3-54,57, 58, 60-103, 105-114, 119-165, 171-177, 185,194, 196, 202-207, 210-215, 225 |

-/--

3

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | QIONG LIU ET AL: "Outer membrane vesicles from flagellin-deficient Salmonella enterica serovar Typhimurium induce cross-reactive immunity and provide cross-protection against heterologous Salmonella challenge", SCIENTIFIC REPORTS, vol. 6, no. 1, 4 October 2016 (2016-10-04) , XP55486078, DOI: 10.1038/srep34776<br><br>table 1<br>----- | 3-54,57, 58, 60-103, 105-114, 119-165, 171-177, 185,194, 196, 202-207, 210-215, 225 |
| A | JIN HAI ZHENG ET AL: "Targeted Cancer Therapy Using Engineered Salmonella typhimurium", CHONNAM MEDICAL JOURNAL, vol. 52, no. 3, 23 September 2016 (2016-09-23), page 173, XP55532496, ISSN: 2233-7385, DOI: 10.4068/cmj.2016.52.3.173<br><br>page 179; tables 1,2<br>----- | 3-54,57, 58, 60-103, 105-114, 119-165, 171-177, 185,194, 196, 202-207, 210-215, 225 |

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/041713

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**FURTHER INFORMATION CONTINUED FROM PCT/ISA/** 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 56, 59, 115, 117, 179-184, 186-193, 195, 197-201, 208, 209, 216, 217(completely); 1, 2, 5-54, 57, 58, 60-103, 105-114, 119-165, 171-177, 185, 194, 196, 202-207, 210-215, 225(partially)

An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA, wherein: the RNA inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increase the anti-tumor immune response in a subject;the RNA is encoded on a plasmid in the bacterium; the immunostimulatory bacterium is aspartate-semialdehyde dehydrogenase - (asd-), wherein the RNA inhibits TREX1.

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2-66. claims: 1, 2, 5-54, 57, 58, 60-103, 105-114, 119-165, 171-177, 185, 194, 196, 202-207, 210-215, 225(all partially)

An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA, wherein: the RNA inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increase the anti-tumor immune response in a subject;the RNA is encoded on a plasmid in the bacterium; the immunostimulatory bacterium is aspartate-semialdehyde dehydrogenase - (asd-), wherein in each separate invention the RNA inhibits a target selected from the 65 targets (PD-L1, PD-1, VEGF, etc.) recited in claims 23 and 31, respectively.

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67-71. claims: 3-54, 57, 58, 60-103, 105-114, 119-165, 171-177, 185, 194, 196, 202-207, 210-215, 225(all partially)

An immunostimulatory bacterium, comprising a sequence of nucleotides encoding RNA, wherein: the RNA inhibits, suppresses or disrupts expression of an immune checkpoint;the RNA is encoded on a plasmid in the bacterium; and wherein in each separate invention the plasmid comprises at least one of sequence of nucleic acid encoding (a) listeriolysin O (LLO) protein lacking the signal sequence (cytoLLO), (b) a CpG motif, (c) a DNA nuclear targeting sequence (DTS), (d) a deletion of the gene encoding a flagellin subunit(s), and (e) a retinoic acid-inducible gene-I (RIG-1) binding element.

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72-86. claims: 55(completely); 63-103(partially)

An immunostimulatory bacterium that is a Salmonella typhimurium strain wherein each separate invention is

represented by one of the strains AST-104, AST-105, AST-106, AST-108, AST-109, AST-112, AST-113, AST-115, AST-117, AST-118, AST-119, AST-120, AST-121, AST-122, and AST-123.

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87. claims: 104(completely); 105-114(partially)

An immunostimulatory bacterium that is auxotrophic for adenosine, and comprises: a deletion in its nucleic acid encoding the flagella, and has a deletion in endA; contains a plasmid that encodes CytoLLO, contains a nuclear localization sequence, an asd plasmid complementation system, and encodes RNA that inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increases the anti-tumor immune response in a subject.

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88. claim: 116

A pharmaceutical composition, comprising an immunostimulatory bacterium that is auxotrophic for adenosine but not for adenine in a pharmaceutically acceptable vehicle.

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89. claim: 118

A pharmaceutical composition, comprising an immunostimulatory bacterium that is auxotrophic for adenosine, and is not purr, in a pharmaceutically acceptable vehicle.

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90. claims: 166-170, 178(completely); 171-177(partially)

A method of treating a tumor or metastasis or a hematologic malignancy, comprising: identifying a subject who has a tumor that is cd73+ ; and administering an immunostimulatory bacterium that is auxotrophic for adenosine.

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91-153. claims: 218-224(partially)

Each separate invention is represented by on of the DNA molecules comprising a sequence of nucleic acids as set forth in any of SEQ ID NOs.: 1-30, 36-40 and 75-78 and 195-217.

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154. claim: 226

A method for producing an immunostimulatory bacterium, comprising: culturing an immunostimulatory bacterium, wherein the bacterium comprises a plasmid encoding RNA that

inhibits, suppresses or disrupts expression of an immune checkpoint or other target whose inhibition, suppression or disruption increase the anti-tumor immune response in a subject; and harvesting the bacterium at stationary phase.

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