



(51) International Patent Classification:

A61K 35/74 (2015.01) C12R 1/42 (2006.01)
C07K 14/705 (2006.01)

(21) International Application Number:

PCT/US2020/019112

(22) International Filing Date:

20 February 2020 (20.02.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/809,232 22 February 2019 (22.02.2019) US

(71) Applicant: ARIZONA BOARD OF REGENTS ON BEHALF OF ARIZONA STATE UNIVERSITY [US/US];
1475 N. Scottsdale Road, Suite 200, Scottsdale, AZ 85257 (US).

(72) Inventors: KONG, Wei; 16016 S. 39th Place, Phoenix, AZ 85048 (US). FU, Lingchen; 853 E. Maria Ln., Tempe, AZ 85284 (US). SHI, Yixin; 16016 S. 39th Place, Phoenix, AZ 85048 (US).

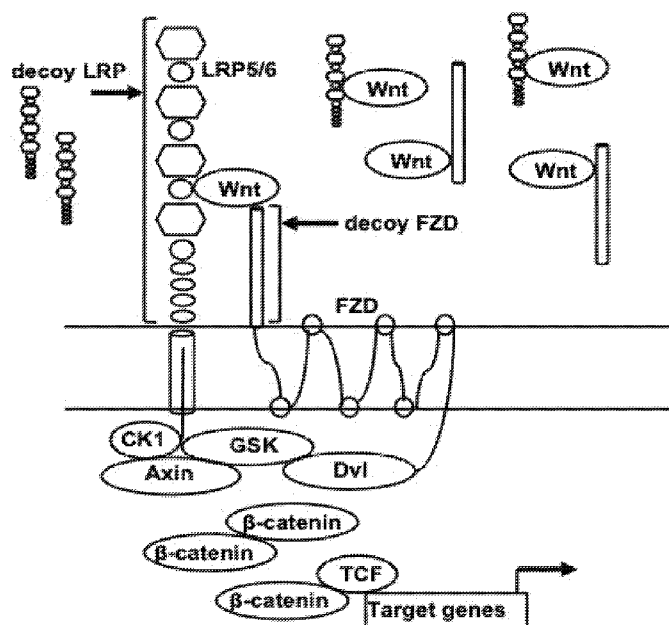
(74) Agent: LEWIS, Jessica, L.; Quarles & Brady LLP, 411 East Wisconsin Avenue, Suite 2350, Milwaukee, WI 53202 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: TUMOR-DELIVERED MULTI-TARGET THERAPEUTICS FOR COLON CANCER

FIG. 1



(57) Abstract: The present disclosure relates to genetically modified strains of *Salmonella*, engineered to be tumor navigating, self-eradicating, and armed with decoy polypeptides that bind endogenous ligand and block activation of signal transduction cascades associated with cancer cell proliferation and tumor growth. Also provided herein are methods of producing and methods of using such genetically modified *Salmonella* strains to treat cancer.

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

TUMOR-DELIVERED MULTI-TARGET THERAPEUTICS FOR COLON CANCER**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of U.S. Provisional Application No. 62/809,232, filed February 22, 2019, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

**STATEMENT REGARDING FEDERALLY FUNDED RESEARCH OR
DEVELOPMENT**

[0002] Not applicable.

SEQUENCE LISTING

[0003] A Sequence Listing accompanies this application and is submitted as an ASCII text file of the sequence listing named “2020-02-17_112624.01168_ST25.txt” which is 79.3KB in size and was created on February 17, 2020. The sequence listing is electronically submitted via EFS-Web with the application and is incorporated herein by reference in its entirety.

BACKGROUND

[0004] Despite many advances in conventional methods such as chemo- and radiation-therapy, cancer treatment is still far from optimal. Current cancer therapies frequently encounter challenges including nonspecific systemic distribution of antitumor agents, inadequate drug concentrations reaching the tumor site, intolerable cytotoxicity and development of multiple drug resistance. Oncolytic bacterial therapy has been extensively studied in recent years to fill the critical unmet needs of cancer patients, where the current treatment options have been exhausted. However, the accumulation of genetic mutations and the potential for acquisition of antibiotic-resistance in the therapeutic bacteria present possible risks for recipients of oncolytic bacterial therapy. Accordingly, there remains a need for improvements to existing oncolytic bacteria-based cancer treatments and, in particular, there is a need to develop new therapeutic methods that achieve precision tumor-navigating and self-eradication of oncolytic *Salmonella* for targeted

delivery of decoy binding partners to target multiple cancer cell-surface receptors and cancer cell-released factors.

SUMMARY OF THE DISCLOSURE

[0005] The present disclosure addresses the aforementioned drawbacks of conventional methods for treating cancer.

[0006] In a first aspect, provided herein is a genetically modified *Salmonella* bacterium comprising, or consisting essentially of, (i) a recombinant gene encoding a human decoy polypeptide; (ii) the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asd::TT\ araC\ P_{BAD}\ c2\ \Delta(araC\ P_{BAD})::P22\ P_R\ araBAD\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA::araC\ P_{BAD}\ lacI\ TT\ \Delta pagP::P_{lpp}\ lpxE\ \Delta endA$; and (iii) one or more of mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg . The decoy polypeptide can disrupt Wnt/ β -catenin signaling. The decoy polypeptide can be selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha. The bacterium can comprise mutation $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$. The bacterium can comprise mutations $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg . The bacterium can be a *Salmonella* strain selected from GMS515(pK-FZD), GMS515(pK-LRP6), GMS515(pK-PD-1), GMS515(pK-SIRP α), GMS515(pK-VEGFR2), GMS515(pK-PDGFR α), and GMS515(pK-FGFR-1).

[0007] In another aspect, provided herein is a genetically modified *Salmonella* bacterium comprising, or consisting essentially of, (i) a recombinant gene encoding a human decoy polypeptide; (ii) the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asdA::TT\ araC\ P_{BAD}\ c2\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA\ \Delta recF\ \Delta sifA\ \Delta endA\ \Delta sseL\ \Delta tlpA\ \Delta P_{hilA}::P_{trc}\ \Delta lacO888\ hilA$; and (iii) one or more of mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg . The decoy polypeptide can disrupt Wnt/ β -catenin signaling. The decoy polypeptide can be selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha. The bacterium can comprise mutation $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$. The bacterium can comprise mutations $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg . The bacterium can be a *Salmonella* strain selected from GMS525(pK-DFZD), GMS525(pK-DLRP6), GMS525(pK-DPD-1), and GMS525(pK-DSIRPa).

[0008] In another aspect, provided herein is a method of treating a tumor in a subject in need thereof comprising administering a genetically modified *Salmonella* bacterium of this

disclosure to the subject, whereby the genetically modified *Salmonella* bacterium treats tumor cells in the subject. Administering can comprise oral administration or intra-tumoral injection of the genetically modified *Salmonella* bacterium.

[0009] In a further aspect, provided herein is a method for stimulating tumoricidal activity in a host comprising, or consisting essentially of, transforming a first recombinant gene into a strain of *Salmonella* forming a strain B, the first recombinant gene encoding a decoy polypeptide; introducing the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asd::TT\ araC\ P_{BAD}\ c2\ \Delta(araC\ P_{BAD})::P22\ P_R\ araBAD\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA::araC\ P_{BAD}\ lacI\ TT\ \Delta pagP::P_{lpp}\ lpxE\ \Delta endA$ into strain B, thereby forming strain C; introducing one or more mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg into strain C, thereby forming strain D; and administering strain D to the host. The decoy polypeptide can disrupt Wnt/ β -catenin signaling. The decoy polypeptide can be selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha. Administering can comprise oral administration or intra-tumoral injection of strain D into the host. Strain D can be a *Salmonella* strain selected from GMS515(pK-FZD), GMS515(pK-LRP6), GMS515(pK-PD-1), GMS515(pK-SIRP α), GMS515(pK-VEGFR2), GMS515(pK-PDGFR α), and GMS515(pK-FGFR-1).

[0010] In another aspect, provided herein is a method for stimulating tumoricidal activity in a host comprising, or consisting essentially of, introducing the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asdA::TT\ araC\ P_{BAD}\ c2\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA\ \Delta recF\ \Delta sifA\ \Delta endA\ \Delta sseL\ \Delta tlpA\ \Delta P_{hilA}::P_{trc}\ \Delta lacO888\ hilA$ into a strain of *Salmonella*, whereby strain E is formed; introducing one or more mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg into strain E, whereby strain F is formed; transforming a recombinant gene into strain F, the recombinant gene encoding a decoy polypeptide; whereby strain G is formed; and administering strain G to the host. The decoy polypeptide can disrupt Wnt/ β -catenin signaling. The decoy polypeptide can be selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha. Administering can comprise oral administration or intra-tumoral injection of strain G into the host. Strain G can be a *Salmonella* strain selected from GMS525(pK-DFZD), GMS525(pK-DLRP6), GMS525(pK-DPD-1), and GMS525(pK-DSIRPa).

[0011] These and other advantages and features of the present disclosure will become more apparent from the following detailed description of the preferred embodiments of the present disclosure when viewed in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 illustrates the disruption of the Wnt/ β -catenin pathway by decoy FZD and decoy LRP5/6.

[0013] FIGS. 2A-2B illustrate (A) interference of the PD-1/PD-L1 interaction by decoy PD-1 receptor and (B) blockage of CD47 by decoy SIRP α .

[0014] FIGS. 3A-3B demonstrate that the reprogramming chemotaxis system of self-eradicating TRAIL-delivering genetically modified *Salmonella* (GMS) strain facilitates their cancer cell-navigating feature. A. Illustration of transwell assay to determine the colony-forming units (CFU) of GMS swimming cross the swimming agar toward cancer cells. B. The CFU of GMS swimming cross the swimming agar toward human colon cancer cell HCT-116 (* $p < 0.05$; $p < 0.0001$).

[0015] FIG. 4 shows immunofluorescence staining to determine the GMS ability to attach and invade to cancer cells. Green, red, and blue fluorescence indicate HCT-116 cell surface attached *Salmonella*, internalized *Salmonella*, and HCT-116 nucleus, respectively.

[0016] FIGS. 5A-5B demonstrate that reprogrammed GMS strains efficiently induced colon cancer cell death *in vitro*. A. Representative patterns of apoptosis assays following HCT-116 cells treated with GMS for 16 hours. B. Percentage of apoptotic cells post-treatment. Annexin V-FITC positive HCT-116 cells were expressed as means.

[0017] FIGS. 6A-6B demonstrate that reprogrammed GMS strains suppressed tumor growth in HCT-116 SQ tumor model *in vivo*. A. A set of representative live imaging of NSGTM mice with SQ injected HCT-116 cells captured before and after IT injections of phosphate-buffered saline (PBS) or GMS strain, as indicated. B. Luciferase activities of tumor cells from NSGTM mice before and after GMS strain IT injections were analyzed. The error bar indicates SEM. ($n = 6$, * $p < 0.05$). Experiments were repeated independently three times.

[0018] FIGS. 7A-7C demonstrate that reprogrammed GMS strains suppressed tumor progression by promoting cancer cell-killing in a transgenic colon tumor mouse model. A. The representative image of colon from t-APC mice orally inoculated with PBS or GMS strains, as

indicated. B. Numbers of polyps from colons of t-APC mice treated with PBS or GMS strains, as indicated (n = 8). C. Survival fraction curves of t-APC mice orally treated with PBS or GMS strains 3 times at 10 days interval (n = 7).

[0019] FIG. 8 demonstrates that reprogrammed GMS strains promoted cancer cell-killing in a transgenic colon tumor mouse model. The representative *Salmonella* staining (left, dark brown) in polypus from colons and rectums of t-APC mice treated with PBS or GMS strains, as indicated. TUNEL staining (right, green).

[0020] FIGS. 9A-9B demonstrate that reprogrammed GMS strains inhibit liver metastasis in an orthotopic human colon cancer mouse model. A. Representative imaging of liver metastasis (top) and liver section H&E staining (bottom) of the mice treated with PBS or GMS strains, as indicated. B. Numbers of liver metastatic tumors in mice treated with PBS or GMS strains, as indicated (n = 12, *** $p < 0.001$).

DETAILED DESCRIPTION

[0021] The present disclosure addresses the aforementioned drawbacks of conventional methods for treating cancer, including current uses of oncolytic bacteria in cancer treatments. In particular, the methods and compositions described herein are based at least in part on the inventor's development of GMS capable of precise navigation to tumors and self-eradication, and armed with decoy peptides that disrupt signal transduction cascades within the tumor microenvironment. The present disclosure provides GMS-based therapeutic strategies for treating cancer based on the ability of tumor-navigating, self-eradicating GMS strains to deliver decoy peptides that disrupt signal transduction cascades within the tumor microenvironment. Without being bound to any particular mode of action or theory, the GMS-based cancer therapeutic strategies of this disclosure are based on the ability of tumor-navigating, self-eradicating GMS strains to deliver decoy receptors to disrupt the Wnt/ β -catenin pathway, to disrupt interaction of PD-L1 and PD-1, to increase macrophage-mediated tumor cell phagocytosis, and to suppress tumor angiogenesis.

[0022] Accordingly, in a first aspect, provided herein is a genetically modified *Salmonella* bacterium, where the bacterium comprises a recombinant gene encoding a decoy polypeptide. Also provided herein is a genetically modified *Salmonella* bacterium, where the bacterium comprises a recombinant gene encoding for increased expression of a methyl-accepting chemotaxis protein

(MCP). Also provided herein is a genetically modified *Salmonella* bacterium, where the bacterium comprises a recombinant gene encoding for reduced toxicity of the bacterium in a plurality of non-tumor cells and for toxicity of the bacterium in tumor cells.

[0023] In some cases, the genetically modified *Salmonella* bacterium comprises a first recombinant gene encoding a decoy peptide, a second recombinant gene encoding for increased expression of a methyl-accepting chemotaxis protein (MCP), and a third recombinant gene encoding for reduced toxicity of the bacterium in a plurality of non-tumor cells and for toxicity of the bacterium in tumor cells. Such genetically modified *Salmonella* bacteria are capable of self-eradication and specific delivery of decoy peptides to tumor cells. Such GMS also exhibit increased chemotaxis to tumor cells and increased tumoricidal activity relative to a *Salmonella* bacterium not comprising the first recombinant gene. Lysis of the genetically modified *Salmonella* of this disclosure releases GMS-expressed decoy peptides, thereby disrupting molecular interactions in targeted signaling cascades within the tumor microenvironment.

[0024] Some embodiments of the instant disclosure comprise a species or subspecies of the *Salmonella* genera. For instance, the recombinant bacterium may be a *Salmonella Enterica* serovar. In an exemplary embodiment, a bacterium of the disclosure may be derived from (i.e., an isolate of) *S. Enterica* serovar Typhimurium, referred to herein as *Salmonella Typhimurium*, and also from *S. Typhi*, *S. Paratyphi*, *S. Enteritidis*, *S. Choleraesuis*, *S. Arizona*, or *S. Dublin*. In an exemplary embodiment, the recombinant bacterium is derived from *S. Typhimurium*. As used herein, “*Salmonella Typhimurium*” refers to an isolate of *Salmonella Typhimurium*. Likewise, the terms “*S. Typhi*,” “*S. Paratyphi*,” “*S. Enteritidis*,” “*S. Choleraesuis*,” “*S. Arizona*,” and “*S. Dublin*” as used herein refer to isolates of *Salmonella Typhi*, *S. Paratyphi*, *S. Enteritidis*, *S. Choleraesuis*, *S. Arizona*, and *S. Dublin*, respectively. As used herein the terms “strain” and “isolate” are used interchangeably.

[0025] As used herein, the term “decoy polypeptide” refers to a polypeptide or fragment thereof that is capable of trapping the ligands of a target molecule (e.g., a cell surface receptor) and thus preventing its activation. Preferably, decoy polypeptides are soluble forms of a target protein. In some cases, a decoy polypeptide is a truncated form of the target protein from which the transmembrane domain has been removed by chemical, proteolytic, or recombinant methods.

[0026] In some cases, the decoy polypeptide is a polypeptide or fragment thereof that disrupts the Wnt/ β -catenin pathway and, consequently, reduces or stops Wnt-regulated gene

expression. Contemplated within the scope of embodiments presented herein are variants of Wnt-binding receptor polypeptides that act as decoys for canonical Wnt ligands and block Wnt/ β -catenin-mediated signaling. In some cases, the decoy polypeptide comprises all or a portion of the extracellular, Wnt-binding domain of a Frizzled (FZD) receptor. For example, as illustrated in FIG. 1, the decoy polypeptide may comprise a human FZD polypeptide, where the polypeptide comprises at least one amino acid modification to increase the affinity of the decoy FZD polypeptide binding to a canonical Wnt ligand as compared to the affinity for Wnts of the corresponding wild-type FZD receptor.

[0027] In some cases, the decoy polypeptide comprises all or a portion of the extracellular Wnt binding domain of LRP6. For example, as illustrated in FIG. 1, the decoy polypeptide may comprise a human LRP6 polypeptide, where the polypeptide comprises at least one amino acid modification to increase the affinity of the decoy LRP6 polypeptide binding to a canonical Wnt ligand as compared to the affinity for Wnts of the corresponding wild-type LRP6 receptor.

[0028] In some cases, the decoy polypeptide is a polypeptide or fragment thereof that blocks molecular interactions between Programmed Cell Death Protein 1 (PD-1) and its ligands, PD-L1 and PD-L2. In some cases, the decoy polypeptide comprises all or a portion of the extracellular ligand-binding domain of a PD-1 receptor. In some cases, the decoy polypeptide is soluble extracellular domain of programmed cell death protein 1 (PD-1) tumor, which serves as a decoy PD-1 receptor to interfere PD-1 and programmed death-ligand 1 (PD-L1) interaction. Without being bound by any particular theory or mode of action, it is believed that expression of a decoy PD-1 receptor reverses T-cell exhaustion and reinvigorating antitumor activity within the tumor microenvironment (see FIGS. 2A).

[0029] In some cases, the decoy polypeptide is a polypeptide or fragment thereof that blocks binding of Cluster of Differentiation 47 (CD47) to its ligand, thereby activating macrophage-mediated tumor cell phagocytosis. CD47 ligands include SIRP-alpha, SIRP-gamma, and thrombospondin-1. Contemplated within the scope of embodiments presented herein are variants of SIRP-alpha polypeptides that act as decoys for CD47 and activate phagocytosis. In some embodiments, a decoy polypeptide comprises a SIRP-alpha polypeptide, wherein the polypeptide comprises at least one amino acid modification to increase affinity of the decoy SIRP-alpha polypeptide binding to CD47 as compared to the affinity for CD47 of the corresponding wild type SIRP-alpha polypeptide. Without being bound by any particular theory or mode of action, it

is believed that expression of a decoy SIRP- α polypeptide interferes with the CD47-SIRP α axis signaling pathway to enhance macrophages-mediated tumor cell phagocytosis within the tumor microenvironment (see FIGS. 2B).

[0030] In some cases, a genetically modified *Salmonella* bacterium of this disclosure is a GMS strain presented in Table 1.

Table 1. GMS-based therapeutics

Strain name; plasmid sequence	Description	Peptide synthesized
GMS515(pK-FZD); SEQ ID NO:1	Wnt pathway-targeting	pYA3681 encoding codon-optimized human Wnt binding domains of FZD
GMS515(pK-LRP6); SEQ ID NO:3	Wnt pathway-targeting	pYA3681 encoding codon-optimized human Wnt binding domains of LRP6
GMS515(pK-PD-1); SEQ ID NO:5	PD-L1-blocking	pYA3681 encoding codon-optimized extracellular domain of human PD-1
GMS515(pK-SIRP α); SEQ ID NO:7	Macrophage phagocytosis enhancing	pYA3681 encoding codon-optimized extracellular domain of human SIRP α
GMS515(pYA3681)	empty vector control	none
GMS515(pK5079)	current death receptor- targeting	pYA3681 encoding human TRAIL (not codon optimized)
GMS525(pK-DFZD); SEQ ID NO:2	Wnt pathway-targeting	pYA4545 encoding human Wnt binding

		domains of FZD
GMS525(pK-DLRP6); SEQ ID NO:4	Wnt pathway-targeting	pYA4545 encoding human Wnt binding domains of LRP6
GMS525(pK-DPD-1); SEQ ID NO:6	PD-L1-blocking	pYA4545 encoding extracellular domain of human PD-1
GMS525(pK-DSIRP α); SEQ ID NO:8	Macrophage phagocytosis enhancing	pYA4545 encoding extracellular domain of human SIRP α
GMS525(pYA4545)	empty vector control	none

[0031] To produce decoy polypeptides, DNA sequences may be codon-optimized for expression in *Salmonella* and synthesized *in vitro*. In some cases, codon-optimized human DNA fragments can be inserted into lysis vector and DNA vaccine vector (e.g., pYA3681 and pYA4545), and then transformed into tumor-navigating strains (e.g., GMS515 and GMS525). In some cases, the resulting strains are those in Table 1.

[0032] As used herein, the terms “genetically modified” and “genetically engineered” are used interchangeably and refer to a prokaryotic cell that includes an exogenous polynucleotide, regardless of the method used for insertion. In some cases, the cell has been modified to comprise a non-naturally occurring nucleic acid molecule that has been created or modified by the hand of man (e.g., using recombinant DNA technology) or is derived from such a molecule (e.g., by transcription, translation, etc.). A cell that contains an exogenous, recombinant, synthetic, and/or otherwise modified polynucleotide is considered to be an engineered cell. The term “altered,” as used herein, refers to any change in the nucleic acid sequence that results in the nucleic acid sequence not being expressed. In an exemplary embodiment, the alteration results in the nucleic acid sequence not being expressed in a host. In one embodiment, the alteration is a deletion. In another embodiment, the alteration places an essential nucleic acid under the control of a regulatable promoter, such that the nucleic acid is not expressed in a host.

[0033] In addition to the introduction of recombinant genes encoding decoy polypeptides as described above, *Salmonella* are also genetically modified to increase navigation of the bacteria

to cancer cells (tumor cells) by modulating the expression of MCP, which are transmembrane chemoreceptors important for taxis (bacterial movement) toward or away from particular substrates. *Salmonella* MCPs include Tar (taxis towards aspartate and maltose, away from nickel and cobalt; aka cheM), Tsr (taxis towards serine, away from leucine, indole and weak acids), Trg (taxis towards sugars, galactose and ribose), Tap (taxis towards dipeptides), McpC (repellent response towards L-cystine), Tip, McpA, and McpB. The coding sequence of Tsr (Methyl-accepting chemotaxis protein) of *Salmonella typhimurium* is accession number A0A0H3NL96. The coding sequence of Tar (Methyl-accepting chemotaxis protein II) of *S. typhimurium* is accession number P02941.

[0034] In some cases, GMS of this disclosure are engineered for increased chemotaxis toward tumor cells by increasing expression of *tar* and/or increasing expression of *tsr*. In some cases, genetic modification further comprises reducing expression of *trg*. For example, a bacterium can be genetically altered to produce modified *Salmonella* having constitutive over-expression of one or more chemoreceptors such as Tar and Tsr. In some cases, a genetically modified *Salmonella* bacterium comprises mutation $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$. In other cases, the genetically modified *Salmonella* bacterium comprises mutations $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$, $\Delta P_{tsr}::P_{trc} \Delta lacO \text{ } tsr$, and Δtrg . In some cases, the genetically modified *Salmonella* bacterium is from strain GMS410(pK5079) or GMS515(pK5079). Such strains include self-eradication vectors, TRAIL, and MCP mutations described herein for increased chemotaxis to tumor cells.

[0035] In some cases, a genetically modified *Salmonella* bacterium comprises: (i) a recombinant gene encoding a human decoy polypeptide; (ii) the following mutations $\Delta P_{murA}::TT \text{ } araC \text{ } P_{BAD} \text{ } murA \text{ } \Delta asd::TT \text{ } araC \text{ } P_{BAD} \text{ } c2 \text{ } \Delta(araC \text{ } P_{BAD})::P22 \text{ } P_R \text{ } araBAD \text{ } \Delta(wza-wcaM) \text{ } \Delta pmi \text{ } \Delta relA::araC \text{ } P_{BAD} \text{ } lacI \text{ } TT \text{ } \Delta pagP::P_{lpp} \text{ } lpxE \text{ } \Delta endA$; and (iii) one or more of mutations selected from $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$, $\Delta P_{tsr}::P_{trc} \Delta lacO \text{ } tsr$, and Δtrg . In some cases, the genetically modified bacterium is a *Salmonella* strain such as GMS515(pK-FZD), GMS515(pK-LRP6), GMS515(pK-PD-1), GMS515(pK-SIRP α), GMS515(pK-VEGFR2), GMS515(pK-PDGFR α), or GMS515(pK-FGFR-1).

[0036] In some cases, a genetically modified *Salmonella* bacterium comprises: (i) a recombinant gene encoding a human decoy polypeptide; (ii) the following mutations $\Delta P_{murA}::TT \text{ } araC \text{ } P_{BAD} \text{ } murA \text{ } \Delta asdA::TT \text{ } araC \text{ } P_{BAD} \text{ } c2 \text{ } \Delta(wza-wcaM) \text{ } \Delta pmi \text{ } \Delta relA \text{ } \Delta recF \text{ } \Delta sifA \text{ } \Delta endA \text{ } \Delta sseL \text{ } \Delta tlpA \text{ } \Delta P_{hilA}::P_{trc} \Delta lacO888 \text{ } hilA$; and (iii) one or more of mutations selected from $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$,

$\Delta P_{tsr}::P_{trc} \Delta lacO \text{ } tsr$, and Δtrg . In some cases, the genetically modified *Salmonella* bacterium is a *Salmonella* strain such as GMS525(pK-DFZD), GMS525(pK-DLRP6), GMS525(pK-DPD-l), or GMS525(pK-DSIRPa).

[0037] The “ Δ ” as used herein, refers to gene deletion; The “ $::$ ” as used herein, refers to gene insertion; The “*asdA*” as used herein, refers to a gene encoding aspartate-semialdehyde dehydrogenase. The *asdA* mutants of Gram-negative bacteria have an obligate requirement for diaminopimelic acid (DAP), which is an essential constituent of the peptidoglycan layer of the cell wall of these organisms. The “*murA*” refers to a gene required for the synthesis of the peptidoglycan layer of the bacterial cell wall. Like *asdA* mutants, *murA* mutants (“ $\Delta murA$ ”) are deficient in bacterial cell wall synthesis.

[0038] In another aspect, provided herein are methods for producing genetically modified *Salmonella* bacteria having increased tumoricidal activity. In exemplary embodiments, the method comprises: introducing one or more mutations into a first strain of *Salmonella*, whereby strain B is formed and exhibits (i) reduced toxicity in a non-tumor cell relative to a *Salmonella* control strain that does not comprise the one or more mutations; and (ii) increased toxicity in a tumor cell relative to a *Salmonella* control strain that does not comprise the one or more mutations. The mutations can be introduced as a first recombinant nucleic acid.

[0039] In some cases, a second recombinant gene is introduced into strain B, thus forming strain C, where the second recombinant gene encodes a protein that promotes chemotaxis of the strain C toward a plurality of tumor cells. In some cases, the second recombinant gene encodes for the synthesis of Tar or synthesis of Tsr.

[0040] In some cases, a third recombinant gene is introduced into strain C, thus forming strain D, where the third recombinant gene encodes a decoy polypeptide. Preferably, the third recombinant gene encodes a decoy polypeptide. In some cases, the third recombinant gene is introduced in a lysis vector. In other cases, the third recombinant gene is introduced in a DNA vaccine vector. In some cases, strain D comprises mutation $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$, or comprises mutations $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$, $\Delta P_{tsr}::P_{trc} \Delta lacO \text{ } tsr$, and Δtrg . In some cases, strain D is a strain in Table 1.

[0041] In certain embodiments, a genetically modified bacterium of the disclosure may also be attenuated. As used herein, the term “attenuated” refers to the state of the bacterium wherein the bacterium has been weakened from its wild-type fitness by some form of recombinant

or physical manipulation such that the bacterium's virulence is reduced relative to a control (a non-recombinant/non-manipulated bacterium). This includes altering the genotype of the bacterium to reduce its ability to cause disease. However, the bacterium's ability to colonize the tumor is, preferably, not substantially compromised. For instance, in one embodiment, regulated attenuation allows the recombinant bacterium to express one or more nucleic acids encoding products important for the bacterium to withstand stresses encountered in the host after immunization. This allows efficient invasion and colonization of tumor tissues before the genetically modified bacterium is regulated to display the attenuated phenotype. As used herein in this context, the term "reduce/reduced" means a reduction of at least 10%, preferably 25%, even more preferably 50%, still more preferably 60%, even more preferably 70%, still more preferably 80%, even more preferably 90% and most preferably of 100% as compared to the appropriate control.

[0042] The genetically modified *Salmonella* described herein can be used in a variety of applications. For example, the genetically modified *Salmonella* can be used in therapeutic methods to treat cancer or a cancer-associated condition. In some cases, a method of treating cancer in a subject in need thereof will comprise administering an effective amount of a modified *Salmonella* bacterium having the genetic modifications described herein and, thus, being tumor navigating, self-eradicating, and armed with one or more decoy polypeptides, to the subject, whereby the genetically modified *Salmonella* bacterium treats cancer in the subject.

[0043] As used herein, the term "effective amount" means, in the context of a composition, an amount of an immunogenic composition capable of inducing an immune response that reduces the incidence of or lessens the severity of infection or incident of disease in an animal. Alternatively, in the context of a therapy, the term "effective amount" refers to the amount of a therapy which is sufficient to reduce or ameliorate the severity or duration of a disease or disorder (e.g., cancer), or one or more symptoms thereof, prevent the advancement of a disease or disorder, cause the regression of a disease or disorder, prevent the recurrence, development, onset, or progression of one or more symptoms associated with a disease or disorder, or enhance or improve the prophylaxis or treatment of another therapy or therapeutic agent. The effective amount to be administered will depend upon the host receiving the modified bacteria as well as factors such as the size, weight, and age of the host.

[0044] As used herein, "subject" refers to an animal or a patient for whom the described treatment is intended. In exemplary embodiments, subjects treated according to the methods

provided herein are human. In other cases, subjects treated according to the methods provided herein are non-human mammals, including by way of example and not limitation, members of rodentia (e.g., mouse, rat, guinea pig), lagomorpha (e.g., rabbits, hares), perissodactyla (e.g., horses, donkeys, etc.), artodactyla (e.g., pigs, cows, sheep), carnivora (e.g., cats, canines), and primates (e.g., apes, monkeys, baboons, and humans).

[0045] As used herein, the terms "treat" and "treating" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to treat, rescue, ameliorate, or otherwise lessen an undesired symptom or condition associated with cancer or any condition associated with aberrant cell proliferation. In some cases, the term "treated" refers to any beneficial effect on the progression of a disease or condition. Beneficial effects can include reversing, alleviating, inhibiting the progress of, preventing, or reducing the likelihood of the disease or condition to which the term applies or one or more symptoms or manifestations of such a disease or condition. Where the disease or condition is cancer or a cancer-associated condition, treating can refer to the management and care of a patient for the purpose of combating cancer, and can include reversing, alleviating, inhibiting the progress of, preventing, or reducing the likelihood of, or lessening the severity of any aspect of the cancer or cancer-associated condition (e.g., metastasis, tumor growth). As used herein, the terms "preventing" and "prevent" refer not only to a complete prevention of a certain disease or condition, but also to partially or substantially attenuating, reducing the risk of, or delaying the development or recurrence of the disease or condition to which the term applies.

[0046] In some cases, the methods provided herein are directed to treating or preventing a cancer in a subject by administering a composition provided herein. In other cases, the present disclosure provides a method of inhibiting, retarding, or preventing the growth of a tumor or tumor cells in a subject. In exemplary embodiments, colon cancer (colorectal cancer) is treated using the methods provided herein. Examples of other cancers appropriate for methods of treating or preventing as provided herein include, without limitation, lung cancer, pancreatic cancer, prostate cancer, skin cancer, bladder cancer, kidney cancer, ovarian cancer, colorectal cancer, breast cancer, cervical cancer, brain cancer, esophageal cancer, and stomach cancer. Other diseases or conditions appropriate for methods of treating or preventing as provided herein include, without limitation, lymphoma and chronic and acute leukemia.

[0047] Any appropriate route or mode of administration to the subject can be employed according to a method provided herein. In some cases, administering comprises oral administration of the genetically modified *Salmonella* bacterium. In other cases, administering comprises intratumoral injection of the genetically modified *Salmonella* bacterium. The mode of administration can be determined based on the physical location, type, or the number of tumors in the subject's body.

[0048] Clinicians, physicians, and other health care professionals can administer genetically modified *Salmonella* bacteria to a subject in need thereof according to a method provided herein. In some cases, a single administration of the composition may be sufficient. In other cases, more than one administration of the composition is performed at various intervals (*e.g.*, once per week, twice per week, daily, monthly) or according to any other appropriate treatment regimen. The duration of treatment can be a single dose or periodic multiple doses for as long as the administration of a composition provided herein is tolerated by the subject.

[0049] Any appropriate method can be practiced to determine, detect, or monitor a subject's response to treatment according to a method provided herein. As used herein, "determining a subject's response to treatment" refers to the assessment of the results of a therapy in a subject in response to administration of a composition provided herein or to treatment according to a method provided herein. For example, a subject's condition can be monitored continuously or evaluated at appropriate time intervals (*e.g.*, at regular or irregular time points) to detect and/or monitor any changes in disease progression (*e.g.*, change in tumor size) as an indicator of the subject's response to a composition comprising genetically modified *Salmonella* bacteria as described herein. In some cases, tumors can be measured to detect or monitor any change in, for example, tumor size or tumor growth rate (*e.g.*, tumor expansion or shrinkage, inhibited or accelerated tumor growth rate). For example, detection methods such as computed tomography (CT), magnetic resonance imaging (MRI) scanning, and x-ray (*e.g.*, chest x-ray) can be used. In some cases, ultrasound examinations can be used to detect and measure tumor regression or to detect the progression of lesions. In other cases, evaluation of a tumor can involve cytology or histology of, for example, biopsy samples. For solid tumors, evaluation of a subject's response to treatment as provided herein can include assessing RECIST ("Response Evaluation Criteria in Solid Tumors"). RECIST criteria can be used to evaluate a subject's response to the

therapy used to treat their disease or condition. See, for review, Therasse *et al.*, *J. Natl. Cancer Inst.* 92:205-16, 2000.

[0050] The term “promoter”, as used herein, may mean a synthetic or naturally-derived molecule which is capable of conferring, activating or enhancing expression of a nucleic acid in a cell. A promoter may comprise one or more specific transcriptional regulatory sequences to further enhance expression and/or to alter the spatial expression and/or temporal expression of the same.

[0051] The terms “nucleic acid” and “nucleic acid molecule,” as used herein, refer to a compound comprising a nucleobase and an acidic moiety, e.g., a nucleoside, a nucleotide, or a polymer of nucleotides. Nucleic acids generally refer to polymers comprising nucleotides or nucleotide analogs joined together through backbone linkages such as but not limited to phosphodiester bonds. Nucleic acids include deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) such as messenger RNA (mRNA), transfer RNA (tRNA), etc. Typically, polymeric nucleic acids, e.g., nucleic acid molecules comprising three or more nucleotides are linear molecules, in which adjacent nucleotides are linked to each other via a phosphodiester linkage. In some embodiments, “nucleic acid” refers to individual nucleic acid residues (e.g. nucleotides and/or nucleosides). In some embodiments, “nucleic acid” refers to an oligonucleotide chain comprising three or more individual nucleotide residues. As used herein, the terms “oligonucleotide” and “polynucleotide” can be used interchangeably to refer to a polymer of nucleotides (e.g., a string of at least three nucleotides). In some embodiments, “nucleic acid” encompasses RNA as well as single and/or double-stranded DNA. Nucleic acids may be naturally occurring, for example, in the context of a genome, a transcript, an mRNA, tRNA, rRNA, small interfering RNA (siRNA), small nuclear RNA (snRNA), a plasmid, cosmid, chromosome, chromatid, or other naturally occurring nucleic acid molecules. On the other hand, a nucleic acid molecule may be a non-naturally occurring molecule, e.g., a recombinant DNA or RNA, an artificial chromosome, an engineered genome, or fragment thereof, or a synthetic DNA, RNA, DNA/RNA hybrid, or include non-naturally occurring nucleotides or nucleosides. Furthermore, the terms “nucleic acid,” “DNA,” “RNA,” and/or similar terms include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, etc. Where appropriate, e.g., in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, and backbone

modifications. A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, a nucleic acid is or comprises natural nucleosides (e.g. adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

[0052] Nucleic acids and/or other constructs of the disclosure may be isolated. As used herein, "isolated" means to separate from at least some of the components with which it is usually associated whether it is derived from a naturally occurring source or made synthetically, in whole or in part.

[0053] The terms "protein," "peptide," and "polypeptide" are used interchangeably herein and refer to a polymer of amino acid residues linked together by peptide (amide) bonds. The terms refer to a protein, peptide, or polypeptide of any size, structure, or function. Typically, a protein, peptide, or polypeptide will be at least three amino acids long. A protein, peptide, or polypeptide may refer to an individual protein or a collection of proteins. One or more of the amino acids in a protein, peptide, or polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a hydroxyl group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. A protein, peptide, or polypeptide may also be a single molecule or may be a multi-molecular complex. A protein, peptide, or polypeptide may be just a fragment of a naturally occurring protein or peptide. A protein, peptide, or polypeptide may be naturally occurring, recombinant, or synthetic, or any combination thereof. A protein may comprise different domains, for example, a nucleic acid binding domain and a nucleic acid cleavage domain. In some embodiments, a protein comprises a proteinaceous part, e.g., an amino acid sequence constituting a nucleic acid binding domain.

[0054] Nucleic acids, proteins, and/or other moieties of the disclosure may be purified. As used herein, purified means separate from the majority of other compounds or entities. A compound or moiety may be partially purified or substantially purified. Purity may be denoted by weight measure and may be determined using a variety of analytical techniques such as but not limited to mass spectrometry, HPLC, etc.

[0055] In interpreting this disclosure, all terms should be interpreted in the broadest possible manner consistent with the context. It is understood that certain adaptations of the disclosure described in this disclosure are a matter of routine optimization for those skilled in the art, and can be implemented without departing from the spirit of the disclosure, or the scope of the appended claims.

[0056] So that the compositions and methods provided herein may more readily be understood, certain terms are defined:

[0057] As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. Any reference to “or” herein is intended to encompass “and/or” unless otherwise stated.

[0058] The terms “comprising,” “comprises” and “comprised of” as used herein are synonymous with “including,” “includes” or “containing,” “contains”, and are inclusive or open-ended and do not exclude additional, non-recited members, elements, or method steps. The phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of “including,” “comprising,” “having,” “containing,” “involving,” and variations thereof, is meant to encompass the items listed thereafter and additional items. Embodiments referenced as “comprising” certain elements are also contemplated as “consisting essentially of” and “consisting of” those elements. Use of ordinal terms such as “first,” “second,” “third,” etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed. Ordinal terms are used merely as labels to distinguish one claim element having a certain name from another element having the same name (but for the use of the ordinal term), to distinguish the claim elements.

[0059] The terms “about” and “approximately” shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typical, exemplary degrees of error are within 10%, and preferably within 5% of a given value or range of

values. Alternatively, and particularly in biological systems, the terms “about” and “approximately” may mean values that are within an order of magnitude, preferably within 5-fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term “about” or “approximately” can be inferred when not expressly stated.

[0060] Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. As used herein and in the claims, the singular forms “a,” “an,” and “the” include the singular and the plural reference unless the context clearly indicates otherwise. Thus, for example, a reference to “an agent” includes a single agent and a plurality of such agents. Any reference to “or” herein is intended to encompass “and/or” unless otherwise stated.

[0061] Various exemplary embodiments of compositions and methods according to this disclosure in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and the following examples and fall within the scope of the appended claims. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the disclosure described herein. Such equivalents are intended to be encompassed by the following claims.

EXAMPLES

[0062] The following examples will enable one of skill in the art to more readily understand the principles thereof. The following examples are presented by way of illustration and are not meant to be limiting in any way.

[0063] The inventor previously developed a self-destructing *Salmonella* lysis system in which the bacteria are attenuated, yet capable of synthesizing a selected protein or harboring a DNA vaccine, to serve as vaccine delivery platforms against various infectious diseases. The *Salmonella* lysis system contained two components: a lysis *Salmonella* strain and a lysis vector. The *Salmonella* lysis strains harbor a deletion of *asdA* and the arabinose-regulated expression of *murA*, two genes required for the synthesis of the peptidoglycan layer of the bacterial cell wall. They also contain additional mutations intended to enhance bacterial cell lysis and antigen or DNA vaccine delivery. The lysis vector cooperatively works with its host *Salmonella* lysis strain to facilitate the arabinose-dependent bacterial cell wall synthesis needed for bacterial reproduction.

Upon invasion of host tissues, which is an arabinose-free environment, synthesis of the bacterial cell wall eventually ceases. This leads to bacterial cell lysis to release cell contents after bacteria accumulate in host tissues and accomplish *Salmonella* self-eradicating. Experiments were undertaken to genetically engineer the lysis strains into a versatile set of tumor navigating anti-cancer material delivering vehicles.

[0064] Five to six percent of individuals will develop colorectal cancer (CRC) over their lifetime in the United States. The heavy burden that CRC imposes on our society emphasizes the need to develop effective strategies to prevent and treat this disease. It has been reported that mutations of the adenomatous polyposis coli (APC) gene predispose individuals to familial adenomatous polyposis (FAP), characterized by multiple tumors in the large intestine. Mice carrying a *CDX2P-NLS-Cre* recombinase transgene and a *loxP*-targeted *Apc* allele develop mainly colorectal tumors after tamoxifen induction. A transgenic *Apc^{flox/flox}/CDX2-CRE* colon tumor mouse models greatly mimic human FAP-associated colorectal cancer and sporadic colorectal cancer. Moreover, direct orthotopic cell microinjection, between the mucosa and the muscularis externa layers of the cecal wall of immunocompromised NOD. *Cg-Prkdcscid Il2rgtm1Wjl/SzJ* (NSGTM) mice, induces tumor foci in the most relevant metastatic sites observed in humans. The application of this procedure to the human colorectal cancer cell lines HCT-116 yielded high tumor takes and dissemination rates, replicating the metastatic spread to lymph nodes, liver, lung, and peritoneum observed in advanced human colorectal cancer. To faithfully recapitulate human CRC, in addition to allograft and xenograft subcutaneous tumor models, the transgenic and orthotopic colon tumor mouse models were used to evaluate our re-engineered GMS therapeutic strains on inhibition of tumor growth and cancer metastasis.

[0065] The *Salmonella* chemotaxis system was engineered to develop tumor navigating, self-eradicating, and TRAIL-armed genetically engineered *Salmonella*. These GMS hold tumor-navigating features and are able to release TRAIL into tumor bed via *Salmonella* cell lysis leading to the induction of tumor cell apoptosis. These GMS were comprehensively evaluated to assure the safety and demonstrate their efficacy on the suppression of cancer growth and metastasis in subcutaneous, orthotopic, and transgenic colon cancer mouse models. These GMS dramatically induced a variety of types of cancer cell death *in vitro*. Intra-tumor (IT) injected GMS significantly reduced tumor growth in both allograft and xenograft subcutaneous colon cancer mouse models. Moreover, oral administrated (OR), a convenient and less toxic route than parenteral

administration, GMS reduced significant tumor growth in the transgenic CRC mouse model and inhibited metastasis in the xenograft orthotopic colon cancer mouse model.

[0066] RESULTS

[0067] ***Reprogramming Salmonella chemotaxis system for tumor-navigating***

[0068] We have improved our self-eradicating *Salmonella* strains to better serve the delivery purpose. Lysis strain GMS409 was engineered to not only harbor the genetic attributes for self-eradicating feature, but also to display genetic characteristics for regulated delayed attenuation, delayed antigen synthesis, and reduced endotoxic activity. However, such GMS strain could not target either cancer cells or tumors. To transform a vaccine delivery strain GMS409 into a universal tumor-navigating delivery vehicle for cancer therapy, our approach was to reprogram the *Salmonella* chemotaxis system to enhance its chemotaxis toward particular tumor secreting amino acids. Such a strategy will allow maximized GMS tumor-eradicating and release of an anti-cancer agent inside of the tumor during the self-eradicating process to trigger bacteria-based oncolysis.

[0069] In order to achieve this goal, we first replaced the promoters of the genes encoding chemoreceptors Tar (*tar*) and Tsr (*tsr*), respectively, with the P_{trc} promoter for constitutive chemoreceptor synthesis. *Salmonella* strains GMS371 carrying single deletion-insertion mutation $\Delta P_{tar}::P_{trc} \Delta lacO \text{ } tar$ and GMS372 harboring single deletion-insertion mutation $\Delta P_{tsr}::P_{trc} \Delta lacO \text{ } tsr$ were created using *Salmonella* wild-type strain $\chi 3761$. The constitutive overexpression of chemoreceptors Tar and Tsr in GMS371 and GMS372, respectively, was confirmed by SDS electrophoresis and western blot assay. In addition, strains GMS371 and GMS372 showed similar growth and swimming speed comparing to their wild-type *Salmonella* parent strain $\chi 3761$. Chemotaxis assay was performed to demonstrate the ideal enhancement of chemotaxis caused by each deletion-insertion mutation. We found that GMS371 and GMS372 are significantly more attracted to aspartate and serine, respectively, than the wild-type strain $\chi 3761$. To further enhance the *Salmonella* accumulation in the layer of tumor quiescent cells, other than the necrotic core, the ribose/galactose receptor *trg* gene was deleted. The strain with Δtrg deletion is much less attracted to galactose than wild-type strain as desired. To finally create tumor-navigating, self-eradicating GMS strains, which hopefully will be able to efficiently navigate tumor and release cancer-killing material in the tumor bed, the single mutation

$\Delta P_{tar::P_{trc\Delta lacO}} tar$ or triple mutations $\Delta P_{tar::P_{trc\Delta lacO}} tar$, $\Delta P_{tsr::P_{trc\Delta lacO}} tsr$, and Δtrg were introduced into GMS409 to achieve strains GMS410 and GMS515 (Table 1).

Table 2. Primers for Construction of Suicide Vectors

Name	Sequence
pK-FZD	
FZD NcoI Primer 1	CGCCATGGACCACGGCTTCTGC (SEQ ID NO: 9)
FZD XmaI Primer 2	GCCCCGGGCTATTACGGCGCGC (SEQ ID NO: 10)
pK-LRP6	
LRP6P NcoI Primer 1	CGCCATGGGCATCGTACCCGAA (SEQ ID NO: 11)
LRP6P XmaI Primer 2	CCCCGGGCTATTAGCATGACAA (SEQ ID NO: 12)
pK-PD-I	
PD1 NcoI Primer 1	CGCCATGGGCTTAGACTCCCCA (SEQ ID NO: 13)
PD1 XmaI Primer 2	GCCCCGGGCTATTAGGGTGAGG (SEQ ID NO: 14)
pK-SIRPα	
SPRI α NcoI Primer 1	CGCCATGGGCGTTGCGGGCGAA (SEQ ID NO: 15)
SPRI α XmaI Primer 2	GCCCCGGGCTATTAACGTTCGT (SEQ ID NO: 16)
pK-DFZD	
FZD KpnI Primer 1	GGGGTACCACCACCATGGACCACGGCTTCTGCCAGCC (SEQ ID NO: 17)
FZD XhoI Primer 2	GCCGCTCGAGCTATTAGGGAGCTCCGTCCTCGGAGT (SEQ ID NO: 18)
pK-DLRP6	
LRP6P KpnI Primer 1	GGGGTACCACCATGGGCATTGTCCCAGAGGCTTTCCT (SEQ ID NO: 19)
LRP6P XhoI Primer 2	GCCGCTCGAGTTCAAGATGAGCTATCATGTTAATAG (SEQ ID NO: 20)
pK-DPD-I	
PD1 KpnI Primer 1	GGGGTACCACCATGGGCTTAGACTCCCCAGACAGGCC (SEQ ID NO: 21)

PD1 XhoI Primer 2	GCCGCTCGAGCTATTAGGGTGAGGGGCTGGGGTGG (SEQ ID NO: 22)
pK-DSIRPα	
SPRI α KpnI Primer 1	GGGGTACCACCATGGGAGTGGCGGGTGAGGAGGAG (SEQ ID NO: 23)
SPRI α XhoI Primer 2	GCCGCTCGAGCTATTACCGTTCATTAGATCCAGTGT (SEQ ID NO: 24)

[0070] ***Building up tumor-targeting self-eradicating TRAIL delivery vehicles***

[0071] A human TRAIL-expressing lysis vector pK5079 was constructed by inserting the TRAIL coding sequence into lysis vector pYA3681 to assemble a self-eradicating *Salmonella* lysis system for cancer therapy. The repressor LacI, expressed from the built-in chromosomal *lacI* gene under arabinose-regulated *araC* P_{BAD} promoter, will turn off TRAIL synthesis *in vitro* to avoid the reduced growth rates and a compromised ability to colonization caused by high-level production of foreign protein. Then the tumor-navigating strains GMS410 and GMS515 were armed with TRAIL by carrying plasmid pK5079 for enhanced cancer cell-killing. The strain GMS409(pK5079) was built, without tumor-navigating feature, to serve as a negative control. The expression of TRAIL by pK5079 in GMS strains was confirmed through western blotting analysis.

[0072] ***Reprogrammed chemotaxis system endues GMS strains superior ability of cancer cell seeking, attaching and invading***

[0073] To validate whether the GMS with chemoreceptor modifications could obtain cancer cell-navigating feature, a transwell culture system was used. A swimming agar layer was used as a barrier between GMS strains and colon cancer cells. GMS strains were cultured in the upper compartment of the transwell culture system, while human colon cancer HCT-116 cells were grown in the lower compartment. The swimming agar layer and micropores in the insert membrane allow GMS strains to cross freely (FIG. 3A). It was observed that significantly higher numbers of GMS410(pK5079) and GMS515(pK5079) swam across the swimming agar layer toward HCT-116 cells, whereas very little numbers of GMS409(pK5079) did, indicating that reprogrammed chemotaxis system in GMS410(pK5079) and GMS515(pK5079) endue them the cancer cell-navigating ability to seek cancer cells (FIG. 3B). The capability of GMS strains

attaching to and invading cancer cells was also examined. The GMS strains were incubated with HCT-116 cells. We found that more GMS410(pK5079) and GMS515(pK5079) attached to and invaded into CT-26 cells or HCT-116 cells (FIG. 4) comparing to the control strain GMS409(pK5079). These data suggest that chemotaxis system reprogramming in GMS410(pK5079) and GMS515(pK5079) strains to enable them to be better attracted to cancer cells leading to efficient attachment and invasion compared with their parent strain GMS409(pK5079) without genetically engineered chemotaxis system. Overall, the GMS strains with reprogrammed chemotaxis systems possess superior ability to navigate, attach, and invade colon cancer cells.

[0074] ***Reprogrammed GMS strains efficiently induced colon cancer cell death in vitro***

[0075] To examine whether the reprogrammed GMS strains have the potential for cancer treatment, the multiple cytotoxic features against cancer cells built into the reprogrammed GMS strains were evaluated *in vitro*. We first validated the level of activated caspase-3, which is the key “executioner” caspase in the apoptotic cascade, after incubating CT-26 and HCT-116 cells with GMS strains, respectively. It was observed that both cancer cells co-incubated with GMS410(pK5079) or GMS515(pK5079) had higher levels of active caspase-3 protein and lower levels of pro-caspase-3, comparing to the cells co-incubated with GMS409(pK5079). Our results indicate that the self-eradicating TRAIL delivering GMS strains, with reprogrammed chemotaxis system, are able to promote the apoptotic cascade through caspase-3 activation. To further validate the cancer cell-killing features in reprogrammed GMS strains, the apoptosis assays were performed using the same colon cancer cell lines. Significantly higher percentage of cell death was observed in the HCT-116 samples co-incubated with GMS515(pK5079) and GMS410(pK5079) than the control strain GMS409(pK5079) (FIGS. 5A-5B). Collectively, the data suggest that the self-eradicating TRAIL-delivering GMS strains with reprogrammed chemotaxis systems hold remarkable cancer cell-killing ability.

[0076] ***Reprogrammed GMS suppress tumor growth in vivo***

[0077] The engineered TRAIL-delivering GMS strains with a reprogrammed chemotaxis system, displaying multiple cancer-killing features, have the potential to function as cancer therapeutics. Therefore, the impact of reprogrammed GMS-based therapy on tumor growth, following intra-tumor injection, was evaluated in an allograft colon cancer mouse model. The CT-26 cells were subcutaneously (SQ) injected into the flank area of BALB/c mice. First, the

colonization of GMS strains in tumor versus spleen was determined nine days post-intratumoral injection (IT) of 10^8 CFU bacteria. We found that the reprogrammed GMS410(pK5079) and GMS515(pK5079) strains preferably accumulated in the tumors, after injection of bacteria into the tumors on the mice, growing the bacterial density up to 5,000 -13,000 times higher comparing to the density of bacteria found in the spleen. In contrast, strain GMS409(pK5079) without chemoreceptor modification selectively accumulated in *Salmonella* preferred colonization organ, spleen. These data suggested that the reprogrammed chemotaxis system in GMS410(pK5079) and GMS515(pK5079) increased their capacity of tumor specific accumulation that is a key safety feature required for efficient *Salmonella*-based cancer therapy. We then tested whether the GMS strains specifically accumulated in tumor would suppress tumor growth. Phosphate-buffered saline (PBS), GMS409(pK5079), GMS410(pK5079), and GMS515(pK5079) were administrated by IT injection. The tumor sizes were measured every three days post-IT injection of bacteria. The tumor size of mice treated with GMS410(pK5079) or GMS515(pK5079) was significantly smaller than that treated with PBS or control strain GMS409(pK5079) after three days following IT injection. Moreover, both GMS410(pK5079) and GMS515(pK5079) treatments prolonged the lifespan of tumor-bearing mice. The lifespan of tumor-bearing mice was significantly prolonged, which was correlated with suppression of tumor growth, was ascribed to tumor-navigating GMS-mediated oncolysis. To test the hypothesis, immunochemistry staining of Ki67 (an indicator of cancer cell proliferation) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling to detect DNA fragmentation as a hallmark of apoptosis) assays were carried out. Many more Ki67 positive cancer cells were observed in the PBS and GMS409(pK5079)-treated tumor samples comparing to the tumor samples from the groups treated with GMS410(pK5079) or GMS515(pK5079). Meanwhile, more apoptotic cells were observed in the tumor sections treated with GMS410(pK5079) or GMS515(pK5079) than in the PBS- or control strain-treated tumors.

[0078] We further evaluated the efficacy of strains GMS410(pK5079) and GMS515(pK5079) on cancer therapy *in vivo* using a human colon cancer HCT-116 cell xenograft mouse model. HCT-116 cells, which stably express luciferases, were subcutaneously injected into the flank area of immunocompromised NOD. Cg-*Prkdcscid* *Il2rgtm1Wjl/SzJ* (NSGTM) mice. PBS, GMS410(pK5079), and GMS515(pK5079) were IT injected into the NSGTM mice carrying tumors. The tumor growth was monitored through measuring cancer cell luciferase activity using

a live imaging system following IT injection. As shown in Figs. 6A-6B, the tumor luciferase activity of mice treated with GMS410(pK5079) or GMS515(pK5079) is significantly lower than that in the control tumors (PBS-treated), suggesting that GMS410(pK5079) and GMS515(pK5079) inhibited HCT-116 cancer cell growth *in vivo*. In addition, Ki67 staining demonstrated that the proliferated cancer cells are much less in tumors treated with GMS410(pK5079) or GMS515(pK5079) than that in the tumors treated with PBS, which confirmed that GMS410(pK5079) and GMS515(pK5079) were also able to inhibit human colon cancer cell growth *in vivo*. Furthermore, TUNEL assays showed more apoptotic cells in the tumor sections treated with GMS410(pK5079) or GMS515(pK5079) than that in PBS-treated tumor sections. Taken together, these observations suggested that reprogramming of the chemotaxis system was an essential component of the GMS anti-cancer effect and the self-eradicating GMS could effectively deliver TRAIL into the tumor microenvironment, and trigger *Salmonella*- and TRAIL-mediated tumor cell death.

[0079] ***Evaluation of reprogrammed GMS strains using a transgenic colon tumor mouse model***

[0080] In addition to allograft and xenograft subcutaneous tumor models, we evaluated our GMS strains in a transgenic *Apc*^{flax/flax}/*CDX2-CRE* colon tumor mouse model, which mimic human FAP associated colorectal cancer and sporadic colorectal cancer. Ten days after tamoxifen induction, mice were orally inoculated with PBS, GMS409(pK5079), GMS410(pK5079), and GMS515(pK5079). Tumors in the colons and rectums were counted 10 days post-GMS treatment. As shown in Figs. 7A-7B, the number of polyps is significantly less in the mice treated with either GMS410(pK5079) or GMS515(pK5079), compared to the number of polyps in the mice treated with the PBS or GMS409(pK5079). Moreover, the survival time of the tamoxifen induced *Apc*^{flax/flax}/*CDX2-CRE* mice treated with GMS410(pK5079) and GMS515(pK5079) was dramatically increased when compared with the control group (Fig. 7C). In addition, more positive anti-*Salmonella* immunostaining was observed in the intestinal polyps treated with GMS410(pK5079) and GMS515(pK5079) strains than that in the samples treated with control strain GMS409(pK5079) (Fig. 8). These results suggest that the reprogramming of the chemotaxis system enables GMS410(pK5079) and GMS515(pK5079) to navigate and colonize in tumor tissue following oral inoculation. Furthermore, a TUNEL assay was performed to detect apoptotic cells in the colon polypus. More apoptotic cells were discovered in polypus

from the mice treated with GMS410(pK5079) and GMS515(pK5079) than that in the polypus from the mice treated with control GMS409(pK5079) (Fig. 8). Overall, these data further demonstrate that GMS410(pK5079) and GMS515(pK5079) are able to navigate to the tumor and efficiently induce tumor cell apoptosis *in vivo*.

[0081] ***Reprogrammed GMS strains-based therapy for metastatic cancer in an orthotopic xenograft mouse model***

[0082] Colorectal cancer is one of the leading causes of cancer mortality because of its metastasis. Liver is the most common organ for colon cancer metastasis. To investigate whether GMS410(pK5079) and GMS515(pK5079) are able to inhibit liver metastasis from orthotopically implanted colon cancer, the HCT-116 cells expressing luciferase were injected into the cecum wall of NSGTM mice. At day 7 post-surgery, mice were orally inoculated with PBS, GMS409(pK5079), GMS410(pK5079), or GMS515(pK5079) once per week for 5 weeks. Tumor growth and metastasis were monitored using a live imaging system. Metastatic tumor number and size were analyzed at week 5 post-inoculation. As shown in Figs. 9A-9B, colon cancer cells grew from cecum and metastasized to adjacent tissue and distant organ liver in the groups treated with PBS and GMS409(pK5079). However, much less local and distance metastasis was observed in the mice treated with GMS410(pK5079) or GMS515(pK5079). These data indicate that both GMS410(pK5079) and GMS515(pK5079) are capable of reducing tumor metastasis.

[0083] **DISCUSSION**

[0084] Despite many advances in conventional methods such as chemo- and radiation-therapy, cancer treatment is still far from optimal. Current cancer therapies frequently encounter challenges including nonspecific systemic distribution of antitumor agents, inadequate drug concentrations reaching the tumor site, intolerable cytotoxicity and development of multiple drug resistance. As with any cancer therapy, the key issue is to achieve the desired concentration of the therapeutic agent specifically in tumor sites, thereby destroying cancerous cells while minimizing damage to normal cells. Bacterial cancer therapies offer unique features that can overcome these obstacles. However, intrinsic bacterial toxicity and tumor-targeting efficiency are two major concerns for the bacterial approach in cancer therapy. We report here that we have now addressed the concerns by constructing GMS strains with enhanced chemotaxis systems that are attracted by tumor-released small molecules to confer tumor-navigating features. Moreover, the regulated delayed attenuation and programmed self-eradicating features designed into these

S. Typhimurium strains to enable them to efficiently colonize in tumors and allow the release of a target agent (e.g., a tumoricidal protein such as TRAIL, another protein) after cell lysis. As proof of concept, we have demonstrated that the genetically engineered tumor navigating and self-eradicating GMS410(pK5079) and GMS515(pK5079) strains not only improve the safety of cancer treatment, but also efficiently target tumor tissue and release a target agent into the tumor tissues to significantly affect tumor growth and extend the survival rate in both allograft and xenograft colon cancer mouse models. We also validate the efficacy of anti-cancer metastasis using *Salmonella* based-cancer therapies in the orthotopic human colon cancer xenograft mouse model created through cecum wall surgical microinjection, which drives tumor foci to the most relevant metastatic sites observed in humans. Most importantly, orally administrated GMS410(pK5079) and GMS515(pK5079) successfully achieved metastasis blockage in such mouse models. In addition, we are the first to evaluate *Salmonella*-based cancer therapeutics in an inducible APC gene mutation mouse model, which can better mimic human familial adenomatous polyposis disease. The results proved that GMS410(pK5079) and GMS515(pK5079) strains effectively suppressed tumor progression. As such, these GMS strains show tremendous potential, either alone or in combination with other treatments, to make an important contribution in cancer therapy.

[0085] The present disclosure has described one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the disclosure.

CLAIMS

We claim:

1. A genetically modified *Salmonella* bacterium comprising:
 - (i) a recombinant gene encoding a human decoy polypeptide;
 - (ii) the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asd::TT\ araC\ P_{BAD}\ c2\ \Delta(araC\ P_{BAD})::P22\ P_R\ araBAD\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA::araC\ P_{BAD}\ lacI\ TT\ \Delta pagP::P_{lpp}\ lpxE\ \Delta endA$; and
 - (iii) one or more of mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg .
2. The genetically modified *Salmonella* bacterium of claim 1, wherein the decoy polypeptide disrupts Wnt/ β -catenin signaling.
3. The genetically modified *Salmonella* bacterium of claim 1, wherein the decoy polypeptide is selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha.
4. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacterium comprises mutation $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$.
5. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacterium comprises mutations $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg .
6. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacterium is a *Salmonella* strain selected from GMS515(pK-FZD), GMS515(pK-LRP6), GMS515(pK-PD-1), GMS515(pK-SIRP α), GMS515(pK-VEGFR2), GMS515(pK-PDGFR α), and GMS515(pK-FGFR-1).
7. A genetically modified *Salmonella* bacterium comprising:
 - (i) a recombinant gene encoding a human decoy polypeptide;

- (ii) the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asdA::TT\ araC\ P_{BAD}\ c2\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA\ \Delta recF\ \Delta sifA\ \Delta endA\ \Delta sseL\ \Delta tlpA\ \Delta P_{hilA}::P_{trc}\ \Delta lacO888\ hilA$; and
- (iii) one or more of mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg .

8. The genetically modified *Salmonella* bacterium of claim 7, wherein the decoy polypeptide disrupts Wnt/ β -catenin signaling.
9. The genetically modified *Salmonella* bacterium of claim 7, wherein the decoy polypeptide is selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha.
10. The genetically modified *Salmonella* bacterium of claim 7, wherein the bacterium comprises mutation $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$.
11. The genetically modified *Salmonella* bacterium of claim 7, wherein the bacterium comprises mutations $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg .
12. The genetically modified *Salmonella* bacterium of claim 7, wherein the bacterium is a *Salmonella* strain selected from GMS525(pK-DFZD), GMS525(pK-DLRP6), GMS525(pK-DPD-I), and GMS525(pK-DSIRPa).
13. A method of treating a tumor in a subject in need thereof comprising administering a genetically modified *Salmonella* bacterium of any one of claims 1-12 to the subject, whereby the genetically modified *Salmonella* bacterium treats tumor cells in the subject.
14. The method of claim 13, wherein administering comprises oral administration or intra-tumoral injection of the genetically modified *Salmonella* bacterium.
15. A method for stimulating tumoricidal activity in a host comprising:

transforming a first recombinant gene into a strain of *Salmonella* forming a strain B, the first recombinant gene encoding a decoy polypeptide;

introducing the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asd::TT\ araC\ P_{BAD}\ c2\ \Delta(araC\ P_{BAD})::P22\ P_R\ araBAD\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA::araC\ P_{BAD}\ lacI\ TT\ \Delta pagP::P_{lpp}\ lpxE\ \Delta endA$ into strain B, thereby forming strain C;

introducing one or more mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg into strain C, thereby forming strain D; and

administering strain D to the host.

16. The method of claim 15, wherein the decoy polypeptide disrupts Wnt/ β -catenin signaling.

17. The method of claim 15, wherein the decoy polypeptide is selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha.

18. The method of claim 15, wherein administering comprises oral administration or intra-tumoral injection of strain D into the host.

19. The method of claim 15, wherein strain D is a *Salmonella* strain selected from GMS515(pK-FZD), GMS515(pK-LRP6), GMS515(pK-PD-1), GMS515(pK-SIRP α), GMS515(pK-VEGFR2), GMS515(pK-PDGFR α), and GMS515(pK-FGFR-1).

20. A method for stimulating tumoricidal activity in a host comprising:

introducing the following mutations $\Delta P_{murA}::TT\ araC\ P_{BAD}\ murA\ \Delta asdA::TT\ araC\ P_{BAD}\ c2\ \Delta(wza-wcaM)\ \Delta pmi\ \Delta relA\ \Delta recF\ \Delta sifA\ \Delta endA\ \Delta sseL\ \Delta tlpA\ \Delta P_{hilA}::P_{trc}\ \Delta lacO888\ hilA$ into a strain of *Salmonella*, whereby strain E is formed;

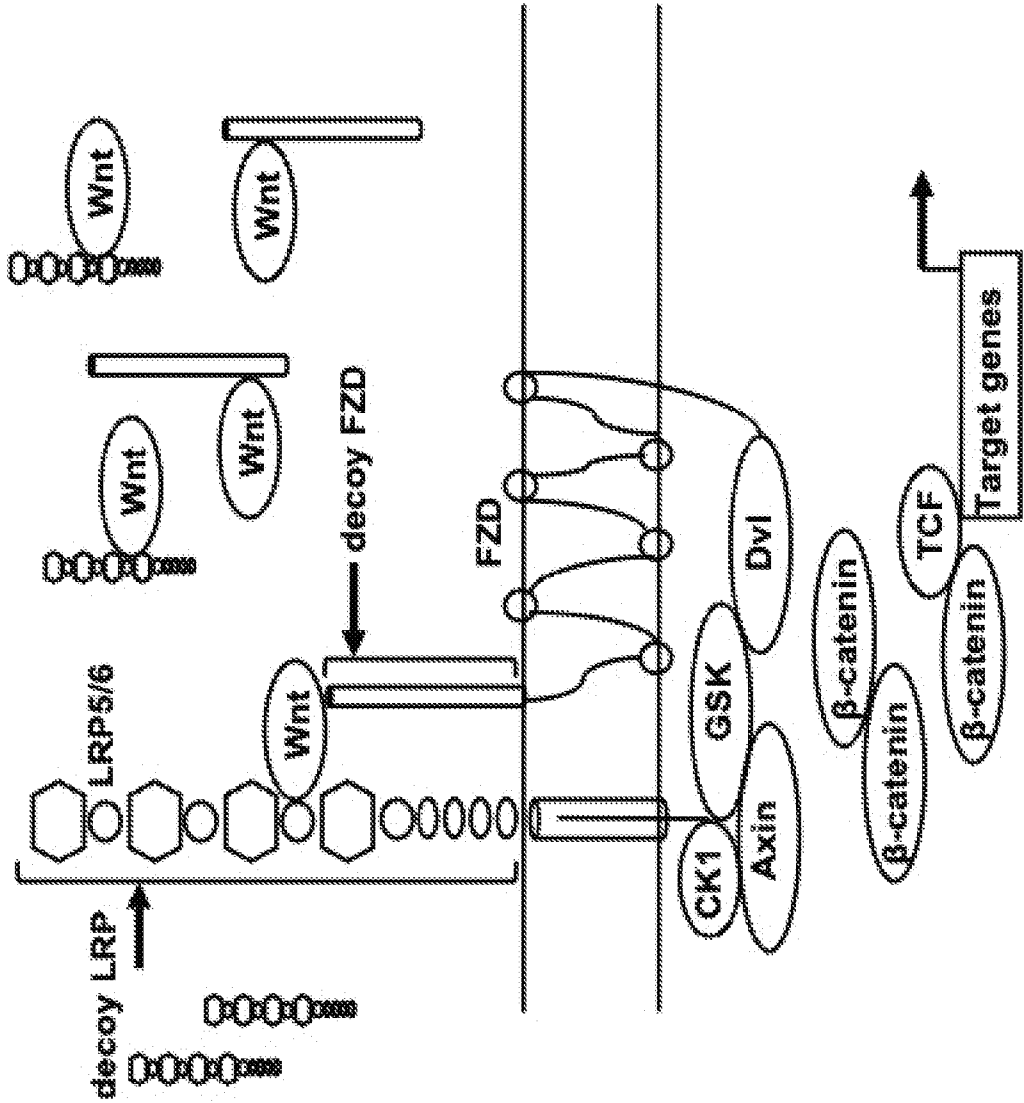
introducing one or more mutations selected from $\Delta P_{tar}::P_{trc}\ \Delta lacO\ tar$, $\Delta P_{tsr}::P_{trc}\ \Delta lacO\ tsr$, and Δtrg into strain E, whereby strain F is formed;

transforming a recombinant gene into strain F, the recombinant gene encoding a decoy polypeptide; whereby strain G is formed; and

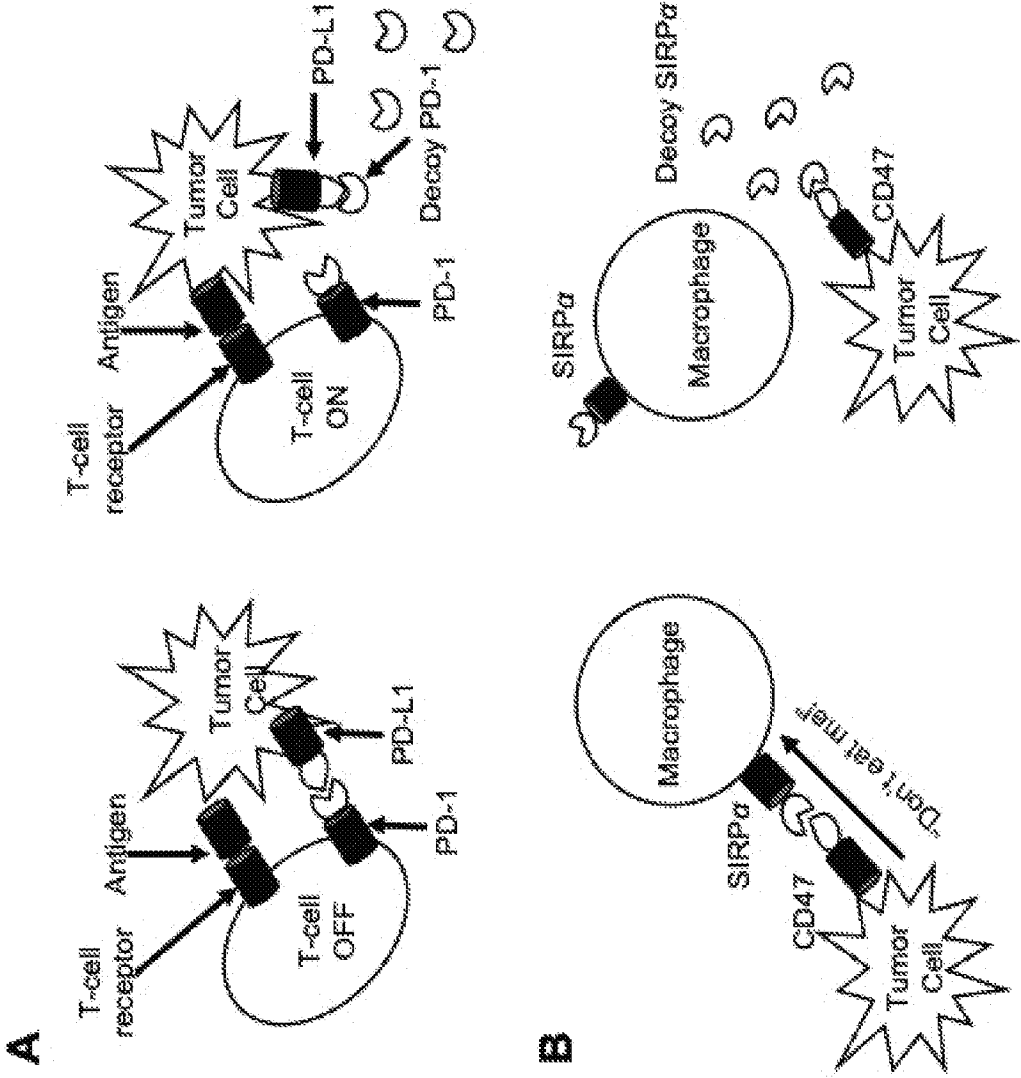
administering strain G to the host.

21. The method of claim 20, wherein the decoy polypeptide disrupts Wnt/ β -catenin signaling.
22. The method of claim 20, wherein the decoy polypeptide is selected from a soluble form of human frizzled (FZD) receptor, a soluble form of human LRP6, a soluble form of human PD-1, and a soluble form of human SIRP-alpha.
23. The method of claim 20, wherein administering comprises oral administration or intra-tumoral injection of strain G into the host.
24. The method of claim 20, wherein strain G is a *Salmonella* strain selected from GMS525(pK-DFZD), GMS525(pK-DLRP6), GMS525(pK-DPD-1), and GMS525(pK-DSIRPa).

FIG. 1



FIGS. 2A-2B



FIGS. 3A-3B

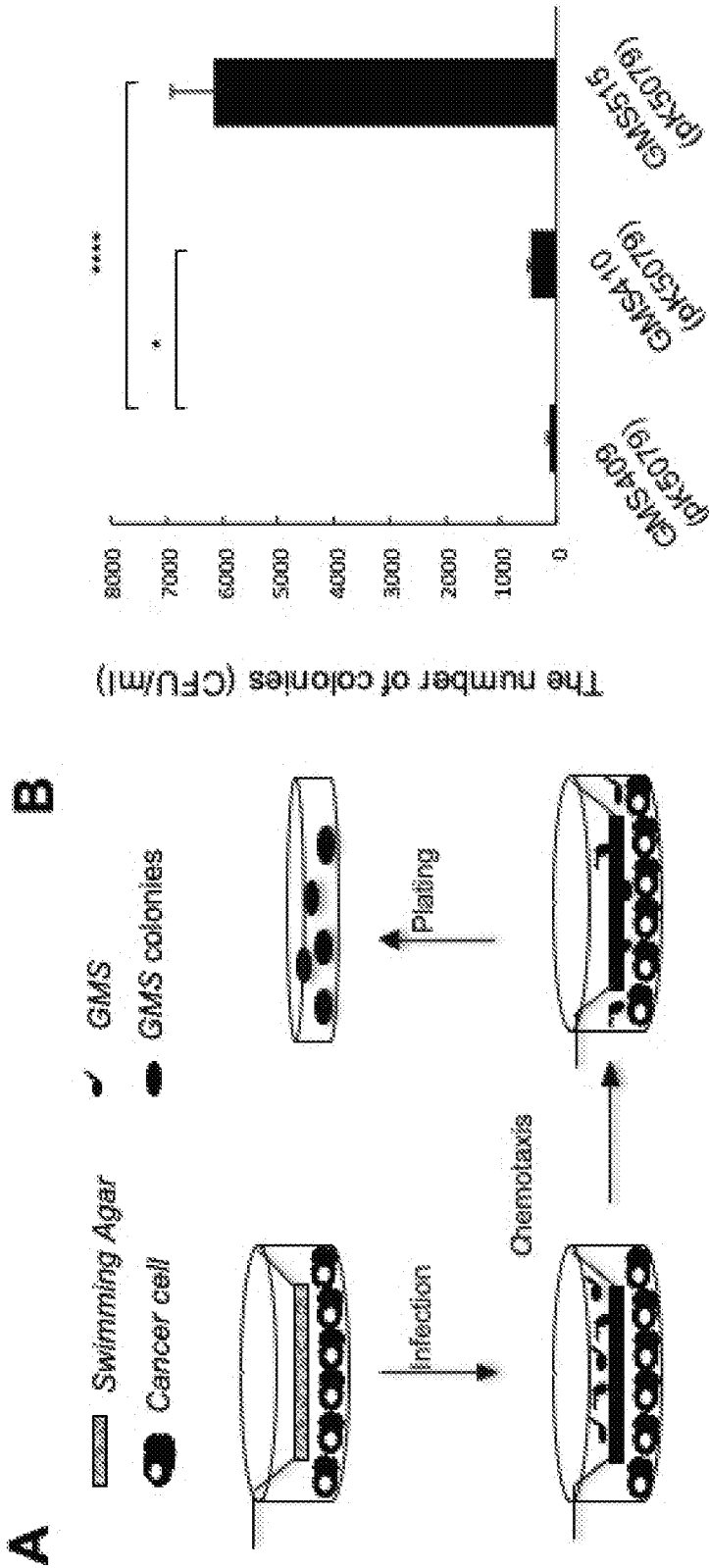
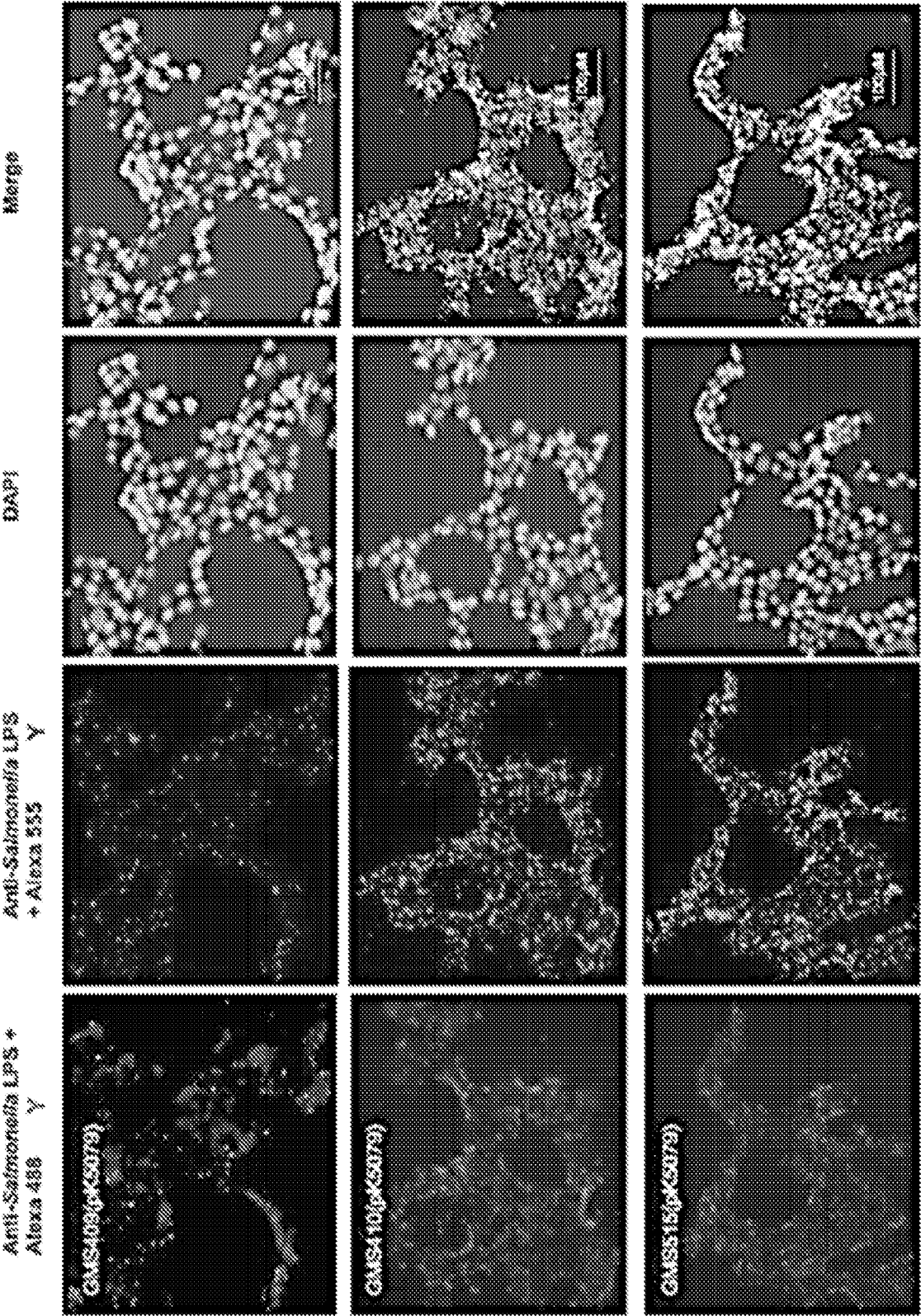


FIG. 4



FIGS. 5A-5B

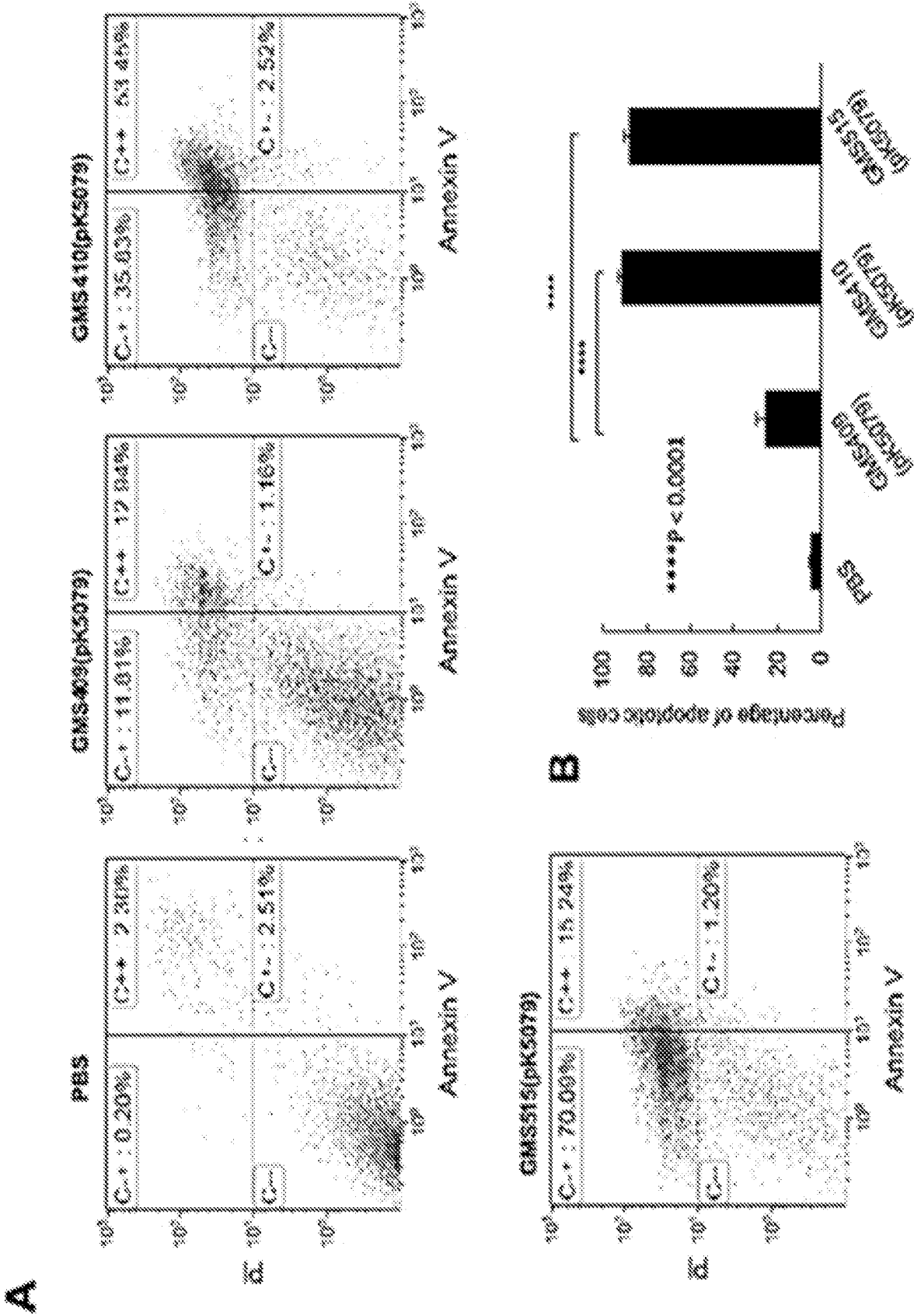


FIG. 6A

A

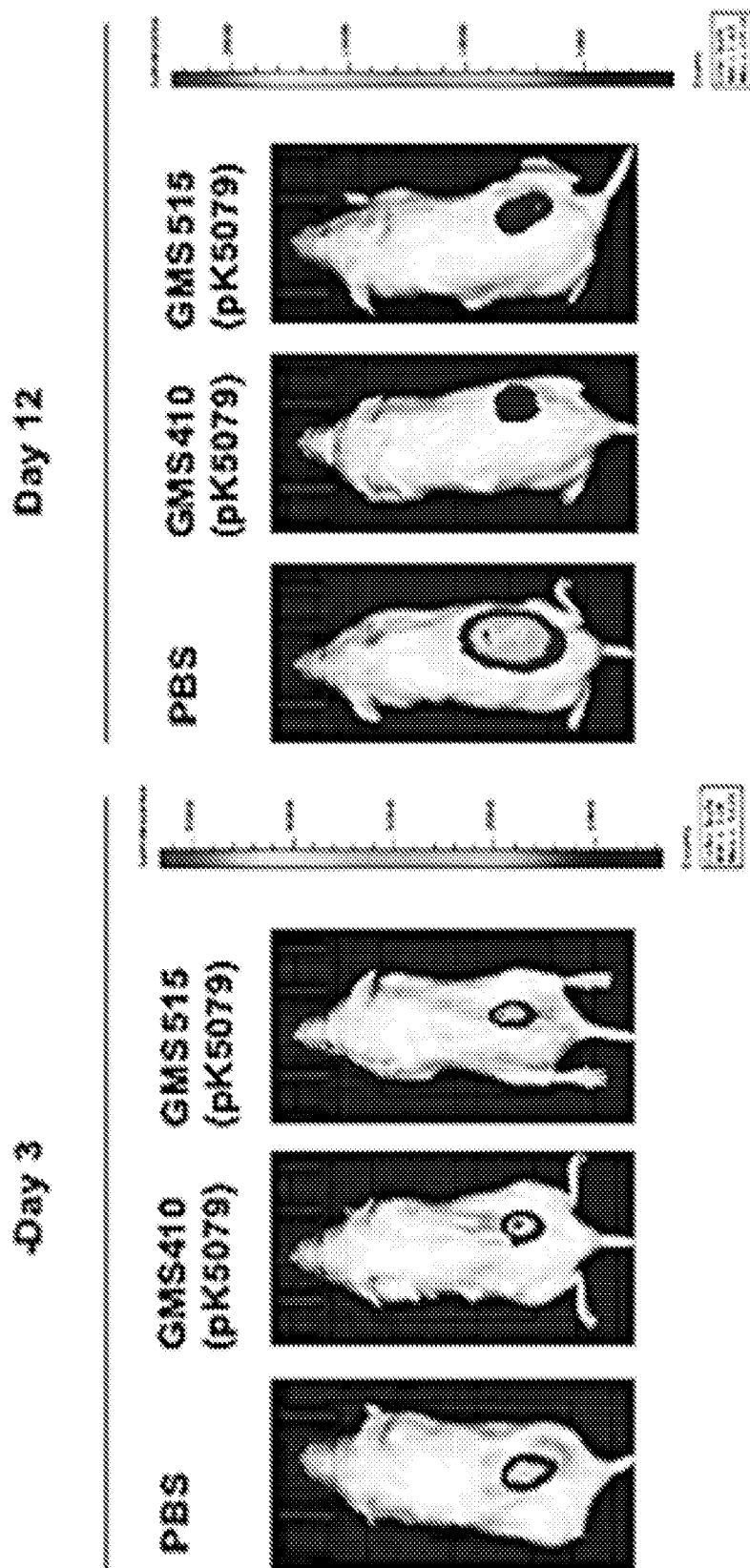
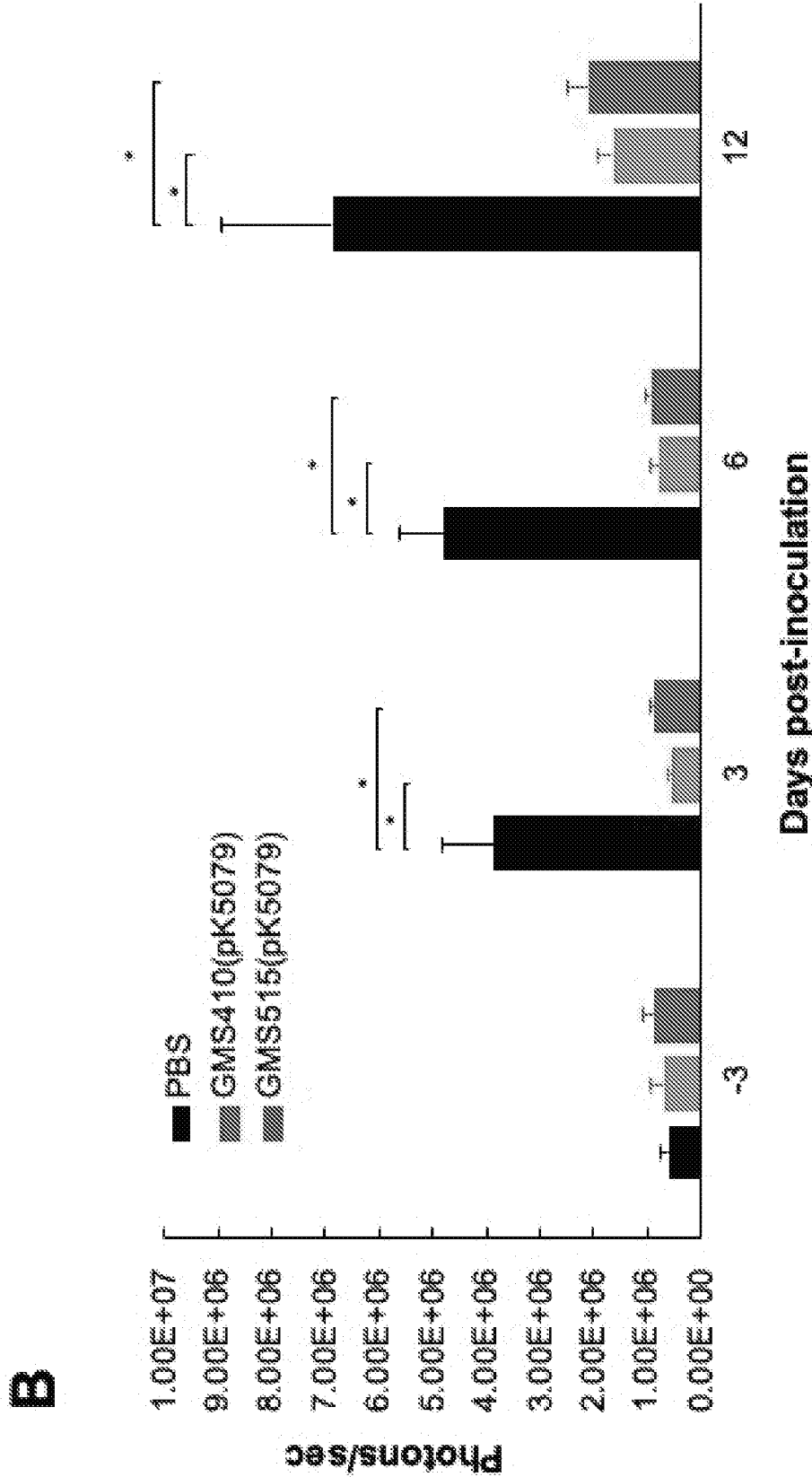


FIG. 6B



FIGS. 7A-7C

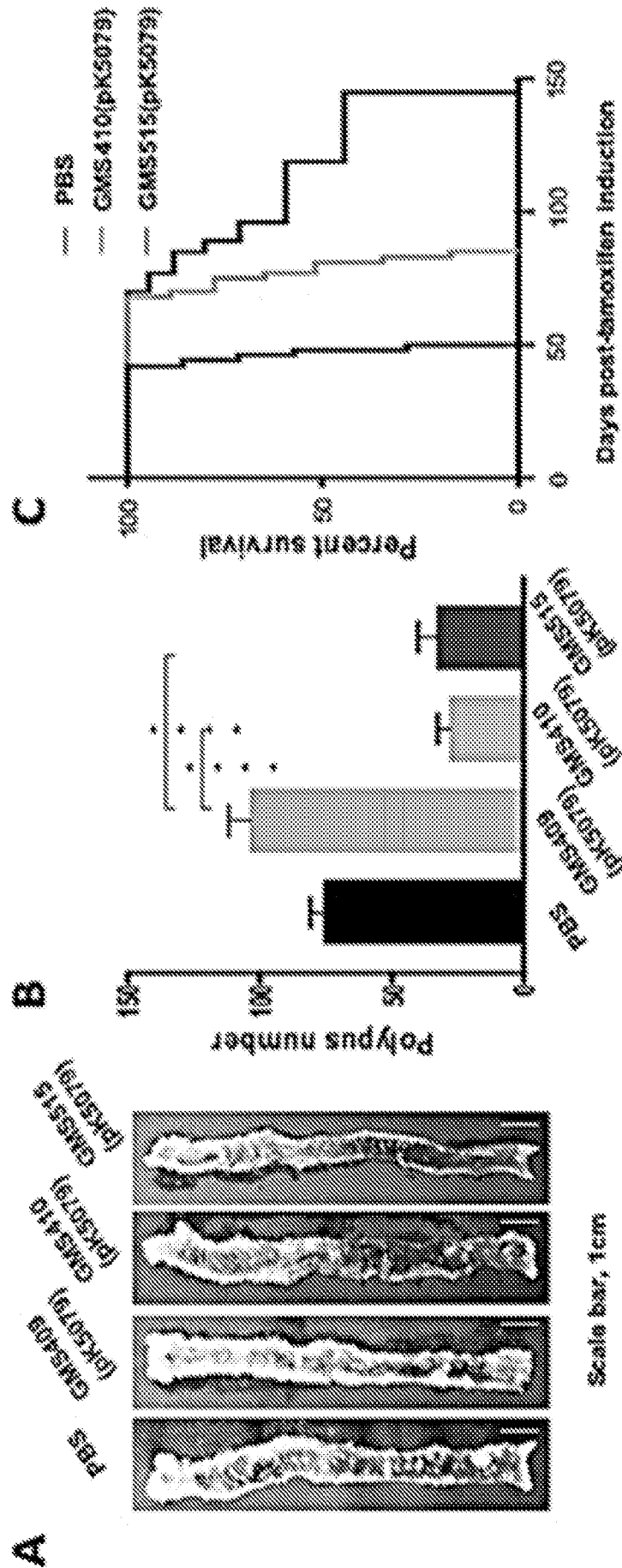


FIG. 8

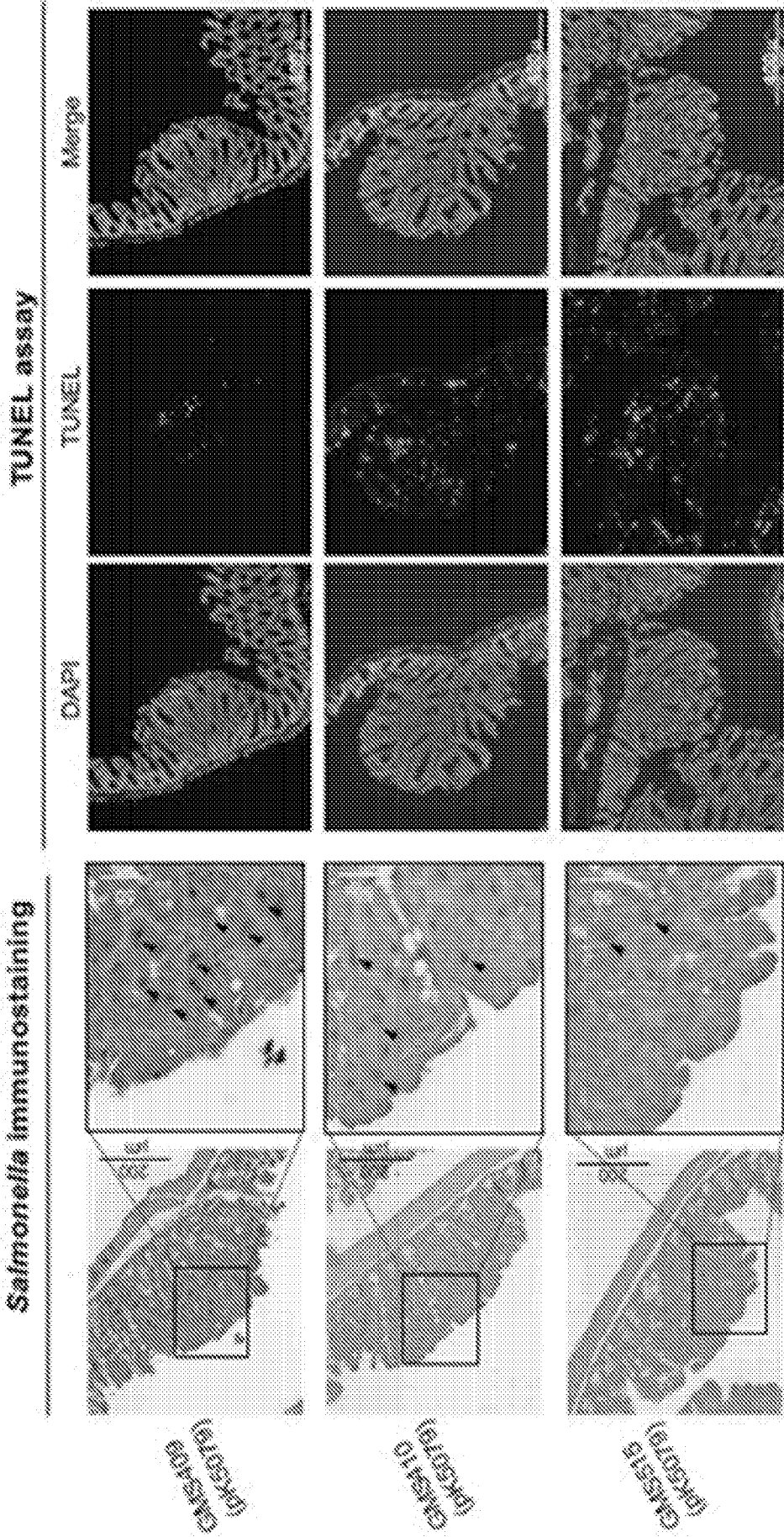
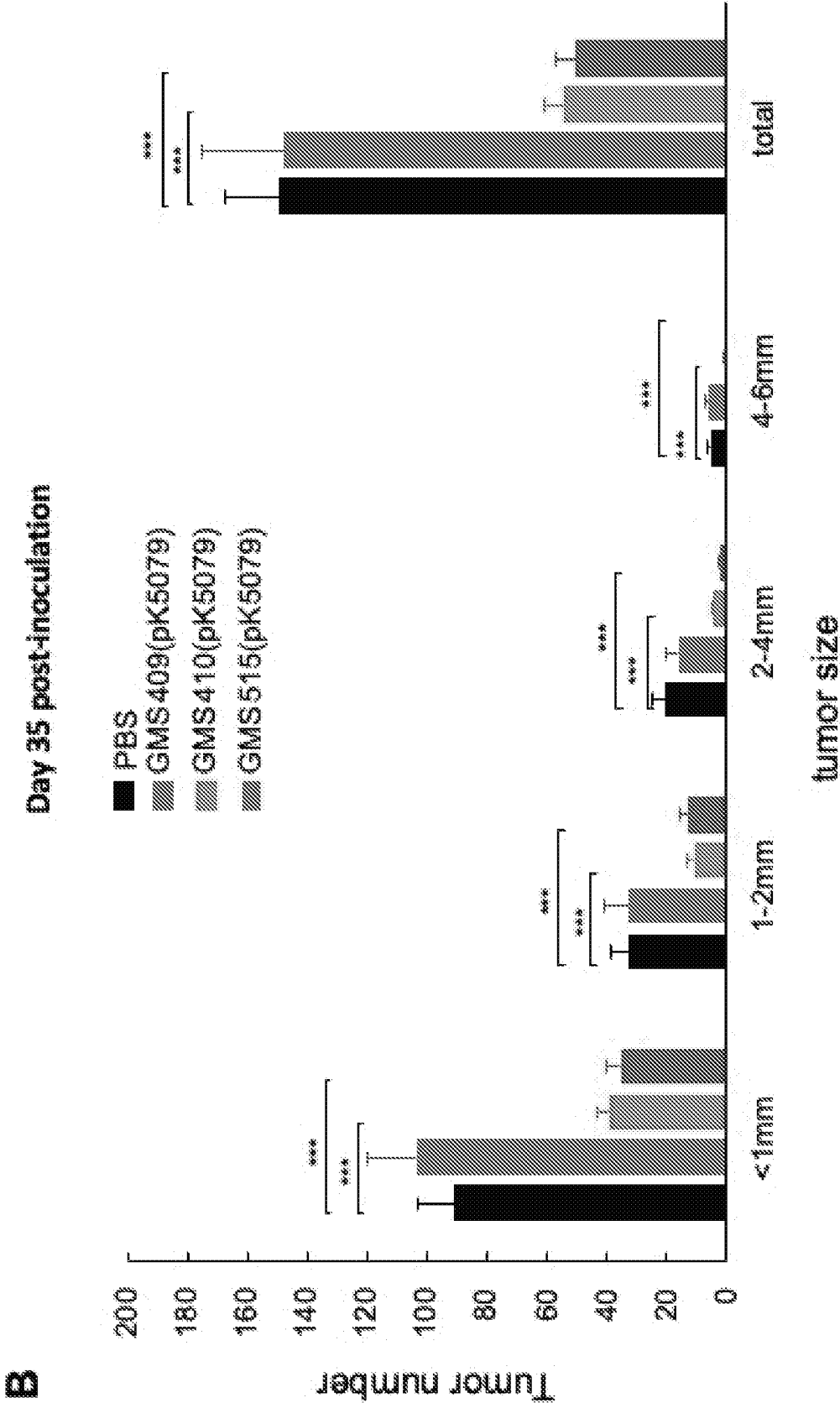


FIG. 9B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/019112

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. ☒ forming part of the international application as filed:

☒ 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*. I(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*. I(a)).

☐ on paper or in the form of an image file (Rule 13*ter*. I(b) and Administrative Instructions, Section 713).

2. ☐ 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:

SEQ ID NO: 1 was searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/019112

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/74; C07K 14/705; C12R 1/42 (2020.01)

CPC - A61K 35/74; C07K 14/70503; C12N 15/74; C12R 1/42 (2020.05)

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)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/93.2; 424/258.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2017/0306338 A1 (THE ARIZONA BOARD OF REGENTS FOR AND ON BEHALF OF ARIZONA STATE UNIVERSITY) 26 October 2017 (26.10.2017) entire document	1-5, 7-11, 13-18, 20-23
Y	US 2017/0275340 A1 (GENEMEDICINE CO., LTD.) 28 September 2017 (28.09.2017) entire document	1-5, 7-11, 13-18, 20-23
Y	US 2017/0224806 A1 (THE ARIZONA BOARD OF REGENTS FOR AND ON BEHALF OF ARIZONA STATE UNIVERSITY) 10 August 2017 (10.08.2017) entire document	7-11, 20-23
Y	US 2010/0317084 A1 (CURTISS, III) 16 December 2010 (16.12.2010) entire document	7-11, 20-23
A	WO 2018/197621 A1 (HELMHOLTZ-ZENTRUM FÜR INFektionsFORSCHUNG GMBH) 01 November 2018 (01.11.2018) entire document	1-24
A	US 2003/0165500 A1 (RHEE et al) 04 September 2003 (04.09.2003) entire document	1-24

☐ Further documents are listed in the continuation of Box C.☐ 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

19 May 2020

Date of mailing of the international search report

11 JUN 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774