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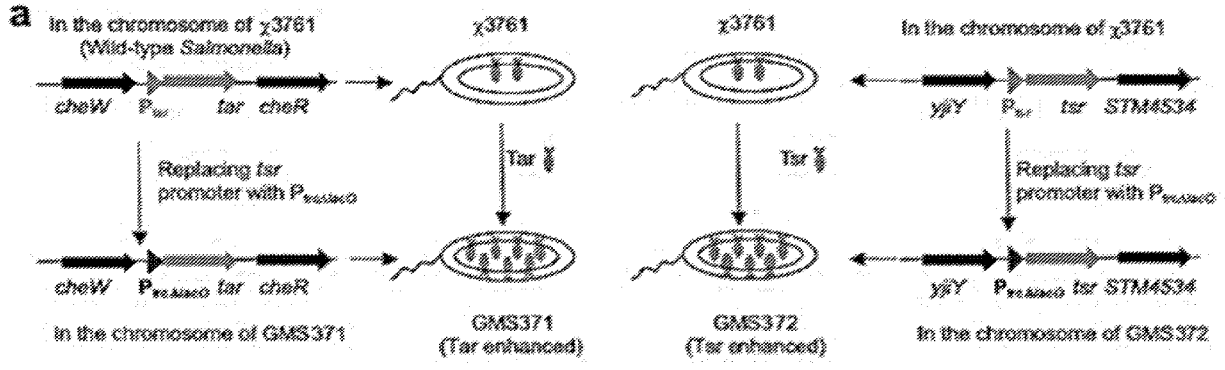
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(54) Title: TUMOR-NAVIGATING, SELF-ERADICATING, TRAIL-ARMED *SALMONELLA* FOR PRECISION CANCER THERAPY

FIG. 1A



(57) Abstract: The present disclosure relates to genetically modified strains of *Salmonella*, engineered to be tumor navigating, self-eradicating, and armed with TRAIL to trigger tumor cell apoptosis. Also provided herein are methods of producing and methods of using such genetically modified *Salmonella* strains to treat cancer.

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TUMOR-NAVIGATING, SELF-ERADICATING, TRAIL-ARMED *SALMONELLA* FOR PRECISION CANCER THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 62/809,222, filed February 22, 2019, which is incorporated by reference herein as if set forth in its entirety.

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under R21 CA152456 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] 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. The self-propelling feature of facultative anaerobe bacteria enables them to establish and maintain a high density in the tumor tissue. *Salmonella* Typhimurium showed a pronounced potential for cancer treatment since they preferably accumulate in large established tumors leading to active suppression of tumor growth. In addition, *S.* Typhimurium exhibits the properties to invade and affect metastases. However, the mechanism of oncolytic *Salmonella*-based therapy *in vivo*, especially the toxicity caused by *Salmonella* infection and tumor lysis, is not yet clearly understood. Although the current therapeutic *Salmonella* strains were most often modified to reduce virulence factors and endotoxins, the accomplishment of tumor-navigating and self-eradicating of *Salmonella* remains the critical challenge for *Salmonella*-based cancer treatment.

[0004] Currently, there is 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*.

SUMMARY OF THE DISCLOSURE

[0005] The present disclosure addresses the aforementioned drawbacks of conventional methods for treating cancer. In a first aspect, provided herein is a genetically modified *Salmonella* bacterium, where the bacterium comprises a recombinant gene encoding human TRAIL. 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. The bacterium can comprise a recombinant gene encoding human TNF-related Apoptosis-inducing Ligand (TRAIL); the following mutations ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asd*::TT *araC* P_{BAD} *c2* Δ(*araC* P_{BAD})::P22 P_R *araBAD* Δ(*wza-wcaM*) Δ*pmi* Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*pagP*::P_{lpp} *lpxE* Δ*endA;* and one or more of the following mutations: ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*, ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*, and Δ*trg*. In some cases, the genetically modified *Salmonella* bacterium further 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, whereby the modified bacterium is capable of self-eradication in non-tumor cells. The genetically modified *Salmonella* (GMS) bacterium can comprise mutations ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar,* ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*, and Δ*trg*. The bacterium can comprise pK5079 (SEQ ID NO:7). The genetically modified *Salmonella* bacterium can be strain GMS410(pK5079). The genetically modified *Salmonella* bacterium can be bacterium is strain GMS515(pK5079). The bacterium can be *S. Typhimurium.*

[0006] In another aspect provided herein is a method of treating cancer in a subject in need thereof. The method can comprise or consist essentially of administering a genetically modified *Salmonella* bacterium of this disclosure to the subject, whereby the genetically modified *Salmonella* bacterium treats cancer in the subject. Administering can comprise oral administration or intra-tumoral injection of the genetically modified *Salmonella* bacterium.

[0007] In a further aspect, provided herein is a method for stimulating tumoricidal activity in a subject. The method can comprise or consist essentially of administering a genetically

modified *Salmonella* bacterium of this disclosure to the subject, whereby the genetically modified *Salmonella* bacterium induces tumoricidal activity in the subject. Administering can comprise oral administration or intra-tumoral injection of the genetically modified *Salmonella* bacterium. The subject can have cancer.

[0008] 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

[0009] FIGS. 1a-1e demonstrate that genetic manipulation of *Salmonella* chemoreceptor synthesis enhanced beneficial chemotaxis for tumor-navigation. **a**. Schematic illustration of experimental design to confer high level constitutive chemoreceptor synthesis. **b**. Growth curves of *Salmonella* chemoreceptor-enhanced strains GMS371, GMS372, and their wild-type parent χ3761. Data are presented as the means, and the error bar represents the standard deviation (SD) from three independent experiments. **c**. Swimming rate of strains GMS371, GMS372, and χ3761. Shown are the means of distance *Salmonella* traveled on swimming agar plates and SD from three independent experiments. **d**. Chemotaxis assay of strains GMS371 and χ3761 with aspartate as a chemoattractant. The representative images (left) of χ3761 (top) and GMS371 (bottom) were captured after 12 h of incubation. Histogram (right) of swim ring size represents three independent experiments, each in triplicate. Error bars represent SD ($n = 3$, $*\rho = 0.03$). **e**. Chemotaxis assay of strains GMS372 and χ3761 with serine as a chemoattractant. The picture (left) presented is a representative image of χ3761 (top) and GMS372 (bottom). Histogram data (right) are presented as the means of swimming distance, and the SD is from three independent experiments ($n = 4$, $**\rho = 0.002$).

[0010] FIGS. 2a-2c demonstrate improved tumor-navigating, attaching, and invading ability in self-eradicating TRAIL-delivering GMS strain. **a.** Transwell assay (illustrated in the left) to determine the colony-forming units (CFU) of *Salmonella* swimming across the swimming agar toward mouse colon cancer cell CT-26 (middle, $**\rho < 0.01$) and human colon cancer cell HCT-116 (right, $*\rho < 0.05$, $****\rho < 0.0001$). The data presented are representatives of three independent experiments. **b**. *Salmonella* attachment and invasion assay through anti-*Salmonella* antibody

immunofluorescence staining. Green, red, and blue fluorescence indicate *Salmonella* attached to CT-26 cell surface, *Salmonella* internalized into CT-26 cell, and nucleus of CT-26 cell, respectively. **c**. Immunofluorescence staining. Green, red, and blue fluorescence indicate *Salmonella* attached to HCT-116 cell surface, *Salmonella* internalized into HCT-116 cell, nucleus of HCT-116 cells, respectively.

[0011] FIGS. 3a-3f demonstrate that reprogrammed GMS strains efficiently triggered caspase-3 activation and colon cancer cell death *in vitro*. **a.** Schematic model of apoptosis assays to detect cancer cell killing. **b**. Representative image of western blotting (left) and quantification (right) to determine the active caspase 3 levels of CT-26 and HCT-116 treated with different GMS strains. Actin was used as a loading control. Normalized active caspase-3 levels were expressed as means, and the error bar represents SD from three different replicates (*$\rho < 0.05$). **c**. Representative patterns of Annexin V/PI apoptosis assays following CT-26 cells treated with GMS for 16 hours. **d.** Percentage of apoptotic cells after treatment. Annexin V-FITC positive CT-26 cells were expressed as means, and the error bar represents SD from three different replicates (***$\rho <0.001$). **e.** Representative patterns of apoptosis assays following HCT-116 cells treated with GMS for 16 hours. **f.** Percentage of apoptotic cells after treatment. Annexin V-FITC positive HCT-116 cells were expressed as means, and the error bar represents SD from three different replicates (****$\rho < 0.0001$).

[0012] FIGS. 4a-4h demonstrate that reprogrammed GMS strains favored tumor accumulation in CT-26 SQ tumor model and suppressed tumor growth *in vivo*. **a.** Schematic illustration of the events of tumor regression of reprogrammed GMS in CT-26 SQ tumor model. **b.** Schematic route and timeline of GMS-based therapy. **c**. Reprogrammed GMS strains favored tumor accumulation versus spleen ($n = 3$, *$\rho < 0.05$, ***$\rho < 0.001$). **d.** The growth curve of SQ CT-26 tumor in BALB/c mice following IT injection of PBS or GMS strains, as described in **4b**. The error bar indicates ± SEM. ($n = 11$, $\rho < 0.05$). **e.** Survival fraction curves for the mice described in **4b.** Death was recorded post-treatments. In addition, the mice were euthanized when tumor size had reached a volume of more than 2 cm^3 followed the guideline, and then considered as death ($n = 11$, $\rho < 0.01$). **f.** The representative immunostaining of Ki67 (dark brown in nucleus) in CT-26 tumor section of BALB/c mice described in **4b**. **g.** TUNEL staining (green) in CT-26 tumor section of BALB/c mice described in **4b**. Nucleus staining is blue color. **h**. Frequencies of isolated intra-tumoral $CD107a^+CD8^+$ T cells (upper, left panel, $p = 0.018$), $IFN\gamma^+CD8^+$ T cells (upper, right

panel, $p = 0.033$), Ki67 positive cell in NK cells (lower, left panel, $p = 0.045$), and IFNγ^+ positive cell in NK cells (lower, right panel, $p = 0.038$) from mice bearing tumors treated with PBS (n = 4 tumors) and GMS (n = 4 tumors).

[0013] FIGS. 5a-5e demonstrate reprogrammed GMS strains suppressed tumor growth in HCT-116 SQ tumor model *in vivo*. **a.** Schematic route and timeline of GMS-based therapy. **b.** A set of representative live imaging of NSG™ mice with SQ injected HCT-116 cells captured before and after GMS strain IT injections, as described in **5a**. **c.** Luciferase activities of tumor cells from NSG™ mice before and after GMS strain IT injections were analyzed. The error bar indicates SEM. (n = 6, *$\rho < 0.05$). Experiments were repeated independently for three times. **d.** The representative immunostaining of Ki67 staining of the tumor sections of NSG™ mice described in **5a**. **e.** TUNEL staining of the tumor sections of NSG™ mice described in **5a.**

[0014] FIGS. 6a-6g demonstrate reprogrammed GMS strains suppressed tumor progression by promoting cancer cell-killing in a transgenic colon tumor mouse model. **a.** Schematic route and timeline of GMS-based therapy. **b.** The representative image of colon from *Apc*$^{flox/flox}$/*CDX2-CRE* mice orally inoculated with PBS or GMS strains, as described in **6a**. **c.** Numbers of polyps from colons of *Apc*$^{flox/flox}$/*CDX2-CRE* mice described in **6a**. The error bar indicates SEM. (n = 8, ****$\rho < 0.0001$). **d.** Survival fraction curves of *Apc*$^{flox/flox}$/*CDX2-CRE* mice orally treated with PBS or GMS strains 3 times at 10 days interval (n = 7, ****$\rho < 0.0001$). **e.** The representative *Salmonella* staining (dark brown) in polyps from colons and rectums of *Apc*$^{flox/flox}$/*CDX2-CRE* mice described in **6a**. **f.** TUNEL staining (green) in polyps from colons and rectums of *Apc*$^{flox/flox}$/*CDX2-CRE* mice described in **6a**. **g**. Polyps were isolated from *Apc*$^{flox/flox}$/*CDX2-CRE* mice on day ten post-treatment, and then weighed, minced, and digested. Tumor-infiltrating lymphocytes were isolated for flow cytometry analysis: NK cells (left panel) and CD8$^+$ T cells (right panel). The error bar indicates SEM. (n = 5, *** $p < 0.001$).

[0015] FIGS. 7a-7d demonstrate that reprogrammed GMS strains inhibit liver metastasis in a mouse model of orthotopic human colon cancer. **a.** Schematic route and timeline of GMS-based therapy. **b.** Representative live imaging of NSG™ mice, with cecally injected HCT-116 cells, orally treated with PBS or GMS strains**. c.** Representative imaging of liver metastasis and liver section H&E staining of the mice described in **7a**. **d.** Numbers of liver metastatic tumors in mice (described in Fig. **7a**) treated with PBS or GMS strains. The error bar indicates ± SEM. (n = 12,*** $\rho < 0.001$).

[0016] FIGS. 8a-8d illustrate genetic engineering and characterization of GMS strains to enhance beneficial chemotaxis. **a**. Schematic illustration of experimental design to tag chromosomal chemoreceptor genes and quantify chemoreceptor synthesis. **b.** Western blot assay (using antibodies against Flag-tag and c-Myc-tag) on the constitutive over-expression of Tar or Tsr chemoreceptor in strains GMS371 and GMS372, compared to their wild-type parent strain χ3761. **c.** Schematic illustration of experimental design to delete ribose/galactose chemoreceptor *trg* gene. **d**. Chemotaxis assay of strains GMS524 and χ3761 with galactose as a chemoattractant. The representative images (left) of χ3761 (top) and GMS524 (bottom) were captured after 12 hours of incubation. Histogram of swim ring size (right) represents three independent experiments, each in triplicate. Error bars represent SD ($n = 3$, $\rho = 0.0008$).

[0017] FIGS. 9a-9c are schematic illustrations of regulatory interactions on TRAIL expression and delivery. **a**. A schematic diagram of regulatory interactions for delayed TRAIL synthesis in the pK5079 harboring GMS strains. LacI, expressed from a chromosomal arabinose-regulated repressor gene, regulates the TRAIL expression from P_{trc} promoter in pK5079. In the presence of arabinose (top), LacI is produced, which block TRAIL synthesis. *In vivo,* an arabinose-poor environment (bottom), the concentration of LacI decreases with each bacterial cell division, allowing increased TRAIL synthesis. **b**. Western blotting assay showing TRAIL expressions in strains GMS409(pK5079), GMS410(pK5079), GMS515(pK5079), and their precursor strains using an antibody against human TRAIL. Strain χ11021 (pYA3681) is an empty plasmid control. **c**. A schematic diagram of regulatory interactions for TRAIL delivery. In the presence of arabinose, *asdA*, *murA,* and *c2* are transcribed, allowing for bacterial growth and repression of the P22 P_R promoter by C2. In the absence of arabinose, the P_{BAD} promoters cease to be active, with no further synthesis of Asd and MurA or C2. The concentrations of Asd, MurA, and C2 decrease due to cell division, leading to reduced synthesis of DAP, muramic acid, and imbalanced synthesis of the rigid layer of the cell wall. As the C2 concentration drops, P22 P_R is de-repressed, resulting in P_R-directed synthesis of antisense mRNA, which blocks translation of residual *asdA* and *murA* mRNA. These concerted activities lead to cell lysis and release of TRAIL.

[0018] FIG. 10 is an illustration of a plasmid map for pK5079.

DETAILED DESCRIPTION

[0019] 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 genetically modified *Salmonella* (GMS) capable of precise navigation to tumors and self-eradication. The GMS described herein armed with TRAIL to trigger cancer cell apoptosis. As demonstrated in the Examples, genetically modified *Salmonella* of this disclosure navigate to cancer cells, self-lyse, release TRAIL, and induce cancer cell apoptosis *in vitro*. Moreover, intratumorally injected GMS effectively suppress subcutaneous tumor growth, and orally administrated GMS reduce the number of polyps and prolong lifespan in an *in vivo* model of colon cancer.

[0020] Accordingly, in a first aspect, provided herein is a genetically modified *Salmonella* bacterium, where the bacterium comprises a recombinant gene encoding human TRAIL. 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.

[0021] In some cases, the genetically modified *Salmonella* bacterium comprises a first recombinant gene encoding human TRAIL, 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. Such genetically modified *Salmonella* bacteria are capable of self-eradication *in vivo*. Such GMS also exhibit increased chemotaxis to tumor cells and increased tumoricidal activity relative to a *Salmonella* bacterium not comprising the first recombinant gene. As demonstrated in the Examples, lysis of the genetically modified *Salmonella* of this disclosure releases TRAIL, thus inducing apoptosis of cancer cells targeted by the modified bacteria. In some cases, other members of the tumor necrosis factor family, such as Tumor Necrosis Factor α (TNFα) and Fas Ligand (FasL), may be used in place of TRAIL. In such cases, TNFα or FasL is released by lysis of the genetically modified *Salmonella* to induce apoptosis of cancer cells targeted by the modified bacteria.

[0022] 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 exogenous nucleic acid under the control of a regulatable promoter, such that the nucleic acid is not expressed in a host.

[0023] 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. Choleraesius*, *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. Choleraesius*," "*S. Arizona,"* and "*S. Dublin*" as used herein refer to isolates of *Salmonella Typhi, S. Paratyphi, S. Enteritidis, S. Choleraesius, S. Arizona*, and *S. Dublin*, respectively. As used herein the terms "strain" and "isolate" are used interchangeably.

[0024] As described and demonstrated herein, *Salmonella* are 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.

[0025] In some cases, a recombinant bacterium of this disclosure is engineered for increased chemotaxis toward tumor cells by increasing expression of Tar and/or increasing expression or Tsr. In some cases, the genetic modification further comprise 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 $\Delta P_{tar}::P_{trc\ \Delta lacO}$ *tar*. In other cases, the genetically modified *Salmonella* bacterium comprises mutations $\Delta P_{tar}::P_{trc\ \Delta lacO}$ *tar*, $\Delta P_{tsr}::P_{trc\ \Delta lacO}$*tsr*, and Δ*trg*. In some cases, the genetically modified *Salmonella* bacterium is from strain GMS410(pK5079) or GMS515(pK5079). A plasmid map for pK5079 is provided in FIG. 10. Such strains include self-eradication vectors, TRAIL, and MCP mutations described herein for increased chemotaxis to tumor cells.

[0026] In certain embodiments, a recombinant 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 recombinant 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.

[0027] For genetically modified *Salmonella*, non-limiting examples of nucleic acid sequences which may be used for attenuation include: a *pab* nucleic acid sequence, a *pur* nucleic acid sequence, an *aro* nucleic acid sequence, an *asdA* nucleic acid sequence, *murA, nadA, pncB, galE, pmi, fur, rpsL, ompR, htrA, hemA, cdt, cya, crp, dam, phoP, phoQ, rfc, poxA, galU, mviA,*

sodC, recA, ssrA, sirA, inv, hilA, rpoE, flgM, tonB, slyA, and any combination thereof. Generally, the nucleic acids provided above as non-limiting examples encode "attenuation proteins," meaning any protein the absence of which attenuates a bacterium. The "Δ" as used herein, refers to gene deletion. The "::" as used herein, refers to gene insertion. The "asd" refers to a gene encoding aspartate-semialdehyde dehydrogenase. The *asd* mutants ("Δ*asd*") 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 ("Δ*murA*") are deficient in bacterial cell wall synthesis.

[0028] In some cases, the genetically modified bacterium is further modified such that the recombinant bacterium is capable of regulated attenuation. Generally speaking, the bacterium comprises a chromosomally integrated regulatable promoter. The promoter replaces the native promoter of, and is operably linked to, at least one nucleic acid sequence encoding an attenuation protein, such that the absence of the function of the protein renders the bacterium attenuated. In some embodiments, the promoter is modified to optimize the regulated attenuation.

[0029] In some cases, the genetically modified bacterium is further modified such that the recombinant bacterium exhibits a reduced expression of immunosuppressive membrane proteins, which are typically overexpressed during *Salmonella* infection. Generally speaking, the bacterium comprises chromosomal gene mutations, largely originates from the *Salmonella* pathogenicity island 2 (SPI2), which encodes proteins associated with induction of programmed death ligand 1 (PD-L1) expression. PD-L1 is an immunosuppressive membrane protein that binds to T cells via the PD-1 receptor and thereby halts their activation. PD-L1 expression plays an essential role in the immunological tolerance of self-antigens but is also exploited for immune evasion by pathogen-infected cells and cancer cells. It has been demonstrated that *Salmonella* infection of intestinal epithelial cells combined with gamma interferon (IFNγ) causes the synergistic induction of PD-L1. The increased expression of PD-L1 through *Salmonella* infection was seen in both human and rat intestinal epithelial cell lines. It was determined that cellular invasion by the bacteria is necessary for PD-L1 induction, potentially indicating that *Salmonella* strains are delivering mediators from inside the host cell that triggers the increased PD-L1 expression. In addition, *Salmonella* plus IFNγ induction of PD-L1 decreased the cytokine production of activated T cells. Knockout mutants, including but not limited Δ*sseL,* Δ*spvD* or Δ*ssrAB*, cause the absence of the

function of specific proteins to prevent the induction of PD-L1 expression. In some embodiments, the mutations are combined to maximize the effect of selected mutations. In some cases, a genetically modified *Salmonella* bacterium comprises one or more mutations selected from Δ*sseL*, Δ*spvD*, and/or Δ*ssrAB*. The term "*sseL*" refers to a gene encoding sulfatase/phosphatase/protease, which acts as a deubiquitinase in infected host cells. The term "*spvD*" refers to a gene encoding cysteine hydrolase, which negatively regulates the NF-κB signaling pathway and promotes virulence of *S.* Typhimurium in mice. The term "*ssrAB*" refers to chromosomal loci located within SPI-2, which encodes two-component regulatory system SsrAB, which regulates the expression of several operons in SPI-2 and, in addition, a large number of genes for non-SPI2-encoded effector proteins.

[0030] In another aspect, provided herein are methods for producing genetically modified *Salmonella* bacteria having increased tumoricidal activity. In exemplary embodiments, the method comprises: transforming a first recombinant gene into a regulated attenuation strain of *Salmonella* forming a strain B, the first recombinant gene encoding for chemotaxis of the strain B toward a plurality of tumor cells; transforming a second recombinant gene into the strain B forming a strain C, the second recombinant gene encoding for (i) reduced toxicity of strain C in a plurality of non-tumor cells; and (ii) toxicity of strain C in the plurality of tumor cells; and transforming a third recombinant gene into the strain C forming a strain D, the third recombinant gene encoding for activation of tumoricidal activity. In some cases, the first recombinant gene encodes for synthesis of Tar or synthesis of Tsr. Preferably, the third recombinant gene can encode human TRAIL. In some cases, strain D comprises mutation $\Delta P_{tar}::P_{trc\ \Delta lacO}$ *tar*, or comprises mutations $\Delta P_{tar}::P_{trc\ \Delta lacO}$ *tar*, $\Delta P_{tsr}::P_{trc\ \Delta lacO}$*tsr*, and Δ*trg*. In some cases, strain D is GMS410(pK5079). In other cases, strain D is GMS515(pK5079).

[0031] The genetically modified *Salmonella* described herein can be used in any of 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 TRAIL to trigger tumor cell apoptosis, to the subject, whereby the genetically modified *Salmonella* bacterium treats cancer in the subject. 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.

[0032] 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).

[0033] 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 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 a cancer or 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.

[0034] 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 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.

[0035] 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 intra-tumoral injection of the genetically modified *Salmonella* bacterium. The mode of administration can be determined based on the physical location, type, or number of tumors in the subject's body.

[0036] 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 administration of a composition provided herein is tolerated by the subject.

[0037] 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 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.

[0038] 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 same.

[0039] 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 molecule. 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-aminoadeno sine, 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).

[0040] 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.

[0041] 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.

[0042] 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.

[0043] 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.

[0044] So that the compositions and methods provided herein may more readily be understood, certain terms are defined:

[0045] 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.

[0046] 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 a same name (but for use of the ordinal term), to distinguish the claim elements.

[0047] 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.

[0048] 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.

[0049] 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

[0050] 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.

[0051] 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.

[0052] 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 *lox*P-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-*Prkdc*scid *Il2rg*tm1Wjl/SzJ (NSG™) 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.

[0053] 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.

[0054] MATERIALS AND METHODS

[0055] *Attachment and invasion assay of GMS strain in cancer cells*

[0056] This assay was performed following the protocol previously described by S. Stender et al., with minor modifications. Briefly, $0.1x10^6$ HCT-116 or CT-26 cells were seeded into a 4-well Nunc® Lab-Tek® Chamber Slide (154526, Thermo Fisher Scientific) for 24 hours in a tissue culture medium containing 10% FBS. GMS strains were grown overnight in LA broth and then diluted 1:20 into a fresh LA medium, grown until OD_{600} nm reached 0.9. To start the assay, bacteria were spun down and re-suspended with 500 µl of tissue culture medium without FBS. HCT-116 or CT-26 cells were incubated with the bacteria suspension at a multiplicity of infection (MOI; the number of bacteria per cell) of 200:1 at 37°C, 5 % CO_2 for 90 minutes. Then the cells were washed three times with phosphate-buffered saline (PBS) and fixed in PBS containing 3.7% paraformaldehyde. Extracellular bacteria were stained with a mouse monoclonal anti-*Salmonella* antibody (NB110-16952, Novus Biological, 1:200) and a secondary anti-mouse-Alexa Fluor 488 conjugate (A11001, Thermo Fisher Scientific, 1:200). After permeabilization of the cell membrane (3 minutes in PBS, 0.1% Triton X-100), intracellular bacteria were stained with the anti-*Salmonella* antibody (NB110-16952, Novus Biological, 1:200) and a secondary anti-mouse-Alexa Fluor 555 conjugate (A21422, Thermo Fisher Scientific, 1:200). The nuclei of cancer cells were stained with 4,6-diamidino-2-phenylindole (DAPI). Samples were mounted and analyzed using an EVOSTM FL Auto Imaging System (Thermo Fisher Scientific).

[0057] *Apoptosis cell detection by flow cytometry analysis*

[0058] Cancer cells were harvested after being treated with the control vehicle or desired GMS stains at a MOI of 20:1 for 16 hours. Cell death was detected using the Annexin V-FITC kit

(4830-01-K, Trevigen) or FITC Annexin V Apoptosis Detection Kit (640914, Biolegend) following the manufactory protocols. Samples were then analyzed by flow cytometry (Beckman counter). The percentage of dead cells from each sample was analyzed using Kaluza Analysis Software (Beckman counter).

[0059] *Transwell chemotaxis assays*

[0060] $0.5x10^6$/well of HCT-116 or CT-26 cells were cultured in a 6-well plate with a medium containing 10% FBS for 24 hours before the assay, and then the cells were cultured in the 1.5 mL tissue medium without FBS during the assay. GMS strains were grown overnight in LA broth, and then diluted 1:20 into fresh LA medium grown until OD600 nm reached 0.9. Next, 200 µl bacteria solutions from each GMS strain were span down and re-suspended into 200 µl tissue culture media, then added to the top of the insert wells (3 µm pore) (353092, BD Falcon) and covered with an 800 µl soft swimming agar layer (0.25% agar, 1% tryptone). The insert wells with GMS strains were put into the 6-well plates with HCT-116 or CT-26 cells to incubate at 37°C for 6 hours. 100 µl culture media from the bottom section were 182 sub-cultured on a LA agar plate at 37°C for 16 hours. Colonies from each plate were counted.

[0061] *Animal models*

[0062] All animal experiments conform to our animal protocols approved by the Institutional Animal Care and Use Committee. We aimed for at least three animals per group (range 3-12 mice) to allow basic statistical inference while using a justifiable number of mutant mice. Mice of similar ages were randomly allocated into different groups.

[0063] Subcutaneous CT-26 tumors in BALB/c mice: BALB/c mice were purchased from Charles River Laboratories (Worcester, MA). $1x10^5$ CT-26 cells were injected into the flanks of the BALB/c mice at 6-8 weeks old. For the colonization assay, 1×10^8 CFU of each GMS strain in 20 µl of PBS was intra-tumor injected when the tumor size reached 0.3 mm^3 (3 mice/group). Mice were euthanized at day 9 post-inoculation, and their spleens and tumors were collected aseptically. Tissues were homogenized and plated on LB agar with 0.2% arabinose to evaluate colonization and persistence, and onto LB agar plates without arabinose to confirm arabinose dependency. For the safety and anti-cancer efficacy assays, mice were treated with 1×10^8 CFU GMS strain in 20 µl of PBS by intra-tumor injection when the tumor size reached 0.3 mm^3.

[0064] Subcutaneous HCT-116 tumors in NSG™ mice: NSG™ mice were obtained from Jackson Laboratory. About 1×10^6 HCT-116 cells were injected into the flanks of NSG™ mice at

6-8 weeks old. These mice were treated with GMS strains (20 µl, 1 x 10^9 CFU in PBS) by intra-tumor injection when the tumor size reached 0.3 mm^3.

[0065] Transgenic *APC$^{flox/flox}$* and *CDX2-CRE* tumor models: *APC$^{flox/flox}$* and *CDX2-CRE* mice (Jackson Laboratory) were crossed to generate C57BL/6J (*Apc$^{flox/flox}$/CDX2-CRE*) mice. At 8 weeks old, *Apc$^{flox/flox}$/CDX2-CRE* mice were injected with Tamoxifen (T5648-19, Sigma-Aldrich) (IP, 25 mg/kg body weight) in sunflower seed oil (S1929, Spectrum) to induce polypus formation in the large intestine. These mice were orally treated with GMS strains (30 µl, 1x10^9 CFU) 10 days post Tamoxifen induction. Mice were euthanized 10 days after inoculation, and the colorectal polypi were analyzed. For survival assay, mice were treated orally with GMS every 10 days for three treatments, beginning 10 days post-Tamoxifen injection.

[0066] Liver metastasis in NSG™ orthotopic mouse models: about 5 x 10^4 HCT-116 cells were injected into the cecal wall of NSG™ mice at 8 weeks old. Mice were orally treated with GMS strains (30 µl, 1x10^9 CFU) 7 days after surgery. Mice were euthanized 35 days after inoculation, and the tumor numbers in livers were recorded.

[0067] *Immunohistochemical staining*

[0068] Paraffin-embedded tissue sections (5 µm thick) were stained with an anti-Ki67 rabbit antibody (12202, 1:400, cell signaling technology) or an anti-*Salmonella* rabbit antibody (NB600-1087, 1:200, Novus Biologicals) overnight at 4°C. The immunohistochemical staining was completed by using a VECTASTAIN Elite ABC HRP Kit (PK-6100, Vector laboratory) and a DAB Peroxidase (HRP) Substrate Kit (SK-4100, Vector laboratory) following the manufacturer's protocols. Samples were mounted and analyzed using an EVOSTM 227 FL Auto Imaging System (Thermo Fisher Scientific).

[0069] *Isolation of immunocytes from tumor and flow cytometry analysis*

[0070] CT-26 cells (approximately 1×10^5) were implanted in the hind flanks of BALB/c mice. When tumors reached 150 mm^3 in volume (day 0), mice were treated with PBS or GMS515(pK5097). Tumor tissues were carefully separated on day seven post-treatment. For transgenic *Apc$^{flox/flox}$/CDX2-CRE* mice, polyps were isolated under the dicing microscope were weighted and minced. Then tumor tissues were minced and digested using digestion buffer (RPMI medium containing 5% FBS, 200 units/ml of collagenase, and 25 U/ml DNase I) (Gibco) for 1 hour at 37°C under slow rotation. Discontinuous (44% and 67%) percoll (GE) separation method was used to enrich immunocytes. Immunocyte cells isolated from tumor were incubated for 30

min on ice with the appropriate combination of the following antibodies (Biolegend) in staining buffer (PBS with 0.1% BSA) at the following dilution: CD45-PB (1:200), or CD45-FITC (1:200), CD3-PB (1:100), CD8a-PE-Cy7 (1:100), CD107a-FITC (1:50), IFN-γ-FITC (1:50), CD335-PE (1:100). For analysis of IFNγ positive T cells and NK cells, isolated colonic immune cells (approximately 5 x 10^6 cells/well) were incubated with CT-26 tumor cells (approximately 1 x 10^6 cells/well) in a 6-well plate with brefeldin A, 5 μg/ml (Biolegend) in RPMI supplemented medium with 10% FBS. Cells were incubated at 37°C for 4 hours, then were washed and stained with cell surface markers. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD biosciences) followed by intracellular cytokine detection with anti-IFNγ-FITC (1:50) in a permeabilization buffer at 4°C for 30 minutes. After the cells were washed twice with 1 ml of the labeling buffer, they were analyzed on a Gallios flow cytometer (Beckman Coulter).

[0071] *Bacterial strains and plasmids*

[0072] Bacterial strains, plasmids, and primers used in this study are provided herein. *S.* Typhimurium strains with *asdA* gene deletions were grown at 37°C in LB broth or on LB agar supplemented with 50 μg/ml DAP. Transformants containing *araC* P_{BAD} *asdA murA* plasmids were selected on LB agar plates containing 0.2% arabinose (LA). LB agar, containing 5% sucrose and no sodium chloride, was used for *sacB* gene-based counter-selection in allelic exchange experiments. When required, 25 μg/ml chloramphenicol was added to the culture media. For mouse inoculation, *Salmonella* strains were grown with aeration in LA broth to an optical density at 600 nm (OD_{600}) of 0.9 from a non-aerated static overnight culture. *Salmonella* cells were resuspended in PBS at appropriate CFU. The exact CFU of each inoculum were determined by titer tests after inoculation.

[0073] *General DNA Procedures*

[0074] DNA manipulations were carried out as described by Sambrook et al. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT). *Escherichia coli* and *Salmonella* were transformed by electroporation. Suicide vector technology was used to generate precise deletion/deletion-insertion mutations. Conjugational transfer of suicide vectors was performed using the suicide vector donor strain χ7213. PCR amplification was used to obtain DNA fragments for cloning and verification of chromosomal deletion mutations. All plasmid constructs and chromosomal deletion mutations were further verified by nucleotide sequencing.

[0075] *Construction of tumor-navigating strains GMS410 and GMS515*

[0076] Suicide vectors pK4946 (ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*) and pK4947 (ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*) were constructed using primers listed in Table 1. Briefly, DNA cassettes (the upstream DNA flanking sequence promoter $P_{trc\ \Delta lacO}$ - downstream flanking sequence) were inserted into the suicide vector pRE112 (Table 1). Suicide vector pK4948 (for mutation Δ*trg*) was constructed by inserting the flanking sequence of gene *trg* into suicide vector pRE112. To test the tumor-navigating feature of mutations ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*, ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*, and Δ*trg*, each single mutation was created in *Salmonella* by conjugating wild-type strain χ3761 with *E. coli* strain χ7213 carrying suicide vector pK4946, pK4947, or pK4948. The resulting strains were named GMS371, GMS372, and GMS525 (Table 1). Then, single mutation ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar* or triple mutations ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*, ΔP_{tsr}::$P_{trc\ \Delta lacO}$*tsr*, and Δ*trg*, were introduced into strain GMS409 by conjugation to achieve the tumor-navigating strains GMS410 and GMS515, respectively.

[0077] The promoter sequence for $P_{trc\ \Delta lacO}$ DNA cassette is set forth in the accompanying sequence listing as SEQ ID NO:1:

5'-ATTCTGAAATGAGCTG**TTGACA**ATTAATCATCCGGCTCG**TATAAT**GTGT**AGATGCG TAGGCACC**

-35 -10 random sequence

TGTTACGACGAACCACAC**AGGA**AACAGACC-3' (SEQ ID NO:1)

SD

[0078] Nucleic acid sequences for plasmids pK4946, pK4947, and pK4948 are provided as SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:6, respectively.

[0079] *Construction of TRAIL expressing lysis vector pK5079*

[0080] The cDNA fragment of human TRAIL gene (GenBank: ABM84955.1) was derived from plasmid pDNR-Dual_with_human_insert (Clone ID: HsCD00001122, DNASU). The amplified TRAIL sequence was inserted into lysis vector pYA3681 to achieve TRAIL expressing lysis vector pK5079 (see FIG. 10). Nucleic acid sequence for plasmid pK5079 is provided as SEQ ID NO:7. Then, pK5079 was transformed into strains GMS409, GMS410, and GMS515. The final tumor-navigating GMS strains evaluated in this study were GMS410(pK5079) and GMS515(pK5079), with GMS409(pK5079) serving as a negative control of chemoreceptor modification. The expression of the TRAIL protein in the isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced GMS cells was verified by SDS/PAGE and western blot analysis.

[0081] *Construction of suicide vectors to create GMS strains preventing Salmonella-induced PD-L1 overexpression.*

[0082] Suicide vectors pK4951 (Δ*sseL*), pK4952 (Δ*spvD*), and pK4953 (Δ*ssrAB*) were constructed using primers listed in Table 1. Briefly, DNA cassettes (the upstream DNA flanking sequence- downstream flanking sequence) were inserted into the suicide vector pRE112 (Table 1). Suicide vectors pK4951 (for mutation SseL), pK4952 (for mutation SpvD), and pK4953 (for mutation SsrAB) were constructed by inserting the flanking sequence of genes *sseL, spvD,* and *ssrAB* into suicide vector pREl12, respectively. To test the effect of these mutations on *Salmonella*-induced PD-L1 overexpression, Δ*sseL*, Δ*spvD*, and Δ*ssrAB*, each single mutation will be created in *Salmonella* by conjugating wild-type strain χ3761 with *E. coli* strain χ7213 carrying suicide vector pK4951, pK4952, or pK4953. Then, single mutation Δ*sseL*, Δ*spvD*, and Δ*ssrAB* will be introduced into strain GMS515 by conjugation, respectively.

[0083] Nucleic acid sequences for plasmids pK4951, pK4952, and pK4953 are provided as SEQ ID NO:41, SEQ ID NO:42, and SEQ ID NO:43, respectively.

[0084] *Strain characterization*

[0085] The GMS strains were compared with vector controls for the stability of plasmid maintenance over 50 generations, arabinose-dependent growth, and protein synthesis. Molecular genetic attributes were confirmed by PCR and sequencing with appropriate primers. Lipopolysaccharide profiles of GMS strains were examined. For detection of constitutive over-expression of chemoreceptors Tar and Tsr in GMS strains, 12 μl and 2 μl of bacterial cultures at an OD_{600} of 0.9 were subjected to an SDS/PAGE and immunoblot analysis, respectively.

[0086] *Growth curve of GMS strains*

[0087] Cells were grown in LB-broth at 37°C until reaching the early stationary phase. A 1:100 dilution of a saturated culture in LB was incubated at 185 rpm. The OD_{600} was measured at different time points over 8 hours. The data are presented as the means and SD from three independent experiments.

Table 1. Bacterial strains, plasmids, and primers used in this study

Strain or Plasmid	**Description**	**Source**
Escherichia coli		
χ6212	Δ*asd*- DH5α derivative	Kang HY, Srinivasan J, Curtiss R, 3rd. Immune responses to recombinant pneumococcal PspA antigen

		delivered by live attenuated *Salmonella enterica* serovar typhimurium vaccine. *Infect Immun 2002;70:1739-49.*
χ7213	F−*supE42* λ− T3r*thi-1 thr-1 leuB6 supE44 tonA21 fhuA21 lacY1 recA1 RP4* 2-Tc::Mu (λ*pir*) Δ*asdA4* Δ(*zhf-2*::Tn*10*)	Roland K, Curtiss R, 3rd, Sizemore D. Construction and evaluation of a delta cya delta crp *Salmonella* typhimurium strain expressing avian pathogenic *Escherichia coli* O78 LPS as a vaccine to prevent airsacculitis in chickens. *Avian Dis 1999;43:429-41.*
***Salmonella enterica* serovar Typhimurium**		
χ3761	Wild-type	Kong W, Wanda SY, Zhang X, et al. Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment. *Proc Natl Acad Sci U S A 2008;105:9361-6.*
GMS371	ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*	This study (previously referred to as χ11371)
GMS372	ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*	This study (previously referred to as χ11372)
GMS525	Δ*trg*	This study (previously referred to as χ11525)
GMS526	*tar*-Flag-tag in χ3761	This study (previously referred to as χ3761 tar-tag)
GMS527	*tsr*-c-Myc-tag in χ3761	This study (previously referred to as χ3761 tsr-tag)
GMS528	ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar* -Flag-tag in GMS371	This study (previously referred to as χ11371 tar-tag)
GMS529	ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr* c-Myc-tag in GMS372	This study (previously referred to as χ11372 tsr-tag)
χ11021	ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asdA*::TT *araC* P_{BAD} *c2* Δ*araBAD* Δ(*gmd-fcl*) Δ*pmi* Δ*relA*::TT *araC* P_{BAD} *lacI*	Juarez-Rodriguez MD, Yang J, Kader R, et al. Live attenuated *Salmonella* vaccines displaying regulated

		delayed lysis and delayed antigen synthesis to confer protection against *Mycobacterium tuberculosis*. *Infect Immun 2012;80:815-31.*
GMS409	ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asd*::TT *araC* P_{BAD} *c2* Δ(*araC* P_{BAD})::P22 P_R *araBAD* Δ(*wza-wcaM*) Δ*pmi* Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*pagP*::P_{lpp} *lpxE* Δ*endA*	This study (previously referred to as χ11409)
GMS410	ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asd*::TT *araC* P_{BAD} *c2* Δ(*araC* P_{BAD})::P22 P_R *araBAD* Δ(*wza-wcaM*) Δ*pmi* Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*pagP*::P_{lpp} *lpxE* Δ*endA* ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*	This study (previously referred to as χ11410)
GMS515	ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asd*::TT *araC* P_{BAD} *c2* Δ(*araC* P_{BAD})::P22 P_R *araBAD* Δ(*wza-wcaM*) Δ*pmi* Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*pagP*::P_{lpp} *lpxE* Δ*endA* ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar* ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr* Δ*trg*	This study (previously referred to as χ11515)
***S.* Typhimurium GMS strains**		
GMS409(pK5079)	GMS409 carrying TRAIL-expressing plasmid pK5079	This study
GMS410(pK5079)	GMS410 carrying TRAIL-expressing plasmid pK5079	This study
GMS515(pK5079)	GMS515 carrying TRAIL-expressing plasmid pK5079	This study
Plasmids		
Asd^+/$MurA^+$ expression lysis vectors		
pYA3681	Asd^+/$MurA^+$ expression lysis vector containing pBR *ori araC* P_{BAD} SD-GTG *asdA* SD-GTG *murA* P22P_R anti-sense mRNA prokaryotic expression cassette	Kong W, Wanda SY, Zhang X, et al. Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment. *Proc Natl Acad Sci U S A 2008;105:9361-6.*
pK5079	Asd^+/$MurA^+$ expression lysis vector containing pBR *ori araC* P_{BAD} SD-GTG	This study (previously referred to as pYA5079)

	asdA SD-GTG *murA* P22P_R anti-sense mRNA prokaryotic expression cassette of human TRAIL	
Suicide vectors		
pK4946	To create genome mutation $\Delta P_{tar}::P_{trc\ \Delta lacO}$ *tar*	This study (previously referred to as pYA4946)
pK4947	To create genome mutation $\Delta P_{tsr}::P_{trc\ \Delta lacO}$ *tsr*	This study (previously referred to as pYA4947)
pK4948	To create genome mutation Δ*trg*	This study (previously referred to as pYADtrg or pYA5077)
pK4949	To create genome Tar-Flag-tag	This study
pK4950	To create genome Tsr-c-Myc-tag	This study
pK4951	To create genome mutation Δ*sseL*	This study
pK4952	To create genome mutation Δ*spvD*	This study
pK4953	To create genome mutation Δ*ssrAB*	This study

Primers	
Name	Sequence
A. Construction of plasmid pK5079	
C-Nco I TRAIL 3681 5'	5'GACGTCCCATGGCTATGATGGAGGTCCAGGGG (SEQ ID NO:8)
C-Xma I TRAIL 3681 3'	5'CTGCAGCCCGGGCTAGCCAACTAAAAAGGCCCC (SEQ ID NO:9)
B. Construction of suicide vectors	
pK4946	
tar/cheM Primer 1	5'CATCGCCAATACACCGGCCTTTATAAA (SEQ ID NO:10)
tar/cheM Primer 2	5'CTACACATTATACGAGCCGGATGATTAATTGTCAACAG CTCATTTCAGAATCGCGGGCGATGAAGAGGCACTCTC (SEQ ID NO:11)
tar/cheM Primer 3	5'GGCTCGTATAATGTGTAGATGCGTAGGCACCTGTTACG ACGAACCACACAGGAAACAGACCATGTTTAACCGTATCC GCGTTGTCAC (SEQ ID NO:12)
tar/cheM Primer 4	5'GAAGTAGGCATCCATATTGCCATTGTC (SEQ ID NO:13)
pK4947	
tsr Primer 1	5'GTCGTTATTGATAACCGCCGGCGTCGC (SEQ ID NO:14)
tsr Primer 2	5'CTACACATTATACGAGCCGGATGATTAATTGTCAACAG CTCATTTCAGAATATCACATAAAATAGCCCACGCCCTCC (SEQ ID NO:15)
tsr Primer 3	5'GGCTCGTATAATGTGTAGATGCGTAGGCACCTGTTACG ACGAACCACACAGGA AACAGACCATGTTAAAGCGAATTAAAATTGTTACC (SEQ ID NO:16)

tsr Primer 4	5'CTCGTTAATCTTACCCGCGCCAAGAAG (SEQ ID NO:17)
pK4948	
D-Trg Up 5'	5'GACGTTATTGTTGAGCTTACTGATGGC (SEQ ID NO:18)
D-Trg Up 3'	5'GCTGGCGCGACGCTTACACATTTTCACAACATTCATTC (SEQ ID NO:19)
D-Trg Down 5'	5'TAAGCGTCGCGCCAGCGGGCTATTTTA (SEQ ID NO:20)
D-Trg Down 3'	5'TTAGTGGTACGCCCCGGTCATCCCCTG (SEQ ID NO:21)
pK4949	
Tar plus Flag Primer 1	5'CGGGAGAGCAGGGGCGCGGTTT (SEQ ID NO:22)
Tar plus Flag Primer 2	5'TCGTCATCGTCTTTATAATCGAAGGTTTCCCAGTTCGCATCA (SEQ ID NO:23)
Tar plus Flag Primer 3	5'GATTATAAAGACGATGACGATAAGTGATCGACGTGCGCTGTCGGTTA (SEQ ID NO:24)
Tar plus Flag Primer 4	5'-CCGTGGCGGCGACGCGCATGTTC (SEQ ID NO:44)
pK4950	
Tsr plus c-Myc Primer 1	5'-CGCGCGCAGGCGAGCAGGGACGC (SEQ ID NO:25)
Tsr plus c-Myc Primer 2	5'-CTTCGGAGATCAGTTTCTGTTCAAATGTTTCCCAATTATCGC (SEQ ID NO:26)
Tsr plus c-Myc Primer 3	5'GAACAGAAACTGATCTCCGAAGAGGATCTGTAAGGGCGTAGTGGTGAACAGT (SEQ ID NO:27)
Tsr plus c-Myc Primer 4	5'-GATTTTGCAGAGCACGCCCTGCG (SEQ ID NO:28)
pK4951	
sseL Primer 1	5'-CCCAAGCTTGCTGCGGCATTGCCGGTACGTATGGATTTA (SEQ ID NO:29)
sseL Primer 2	5'-GGGATAGGCTCTAAGTACTCACCACTCTTCTGTATATAAGCTGTGAAAT (SEQ ID NO:30)
sseL Primer 3	5'-TTTCACAGCTTATATACAGAAGAGTGGTGAGTACTTAGAGCCTATCCC (SEQ ID NO:31)
sseL Primer 4	5'-CGGGAATTCGGGGAGAATCGACAACTATATGGC (SEQ ID NO:32)

pK4952	
spvD Primer 1	5'-CCCAAGCTTCTCAGGGCAAATTTGCCGGTGACA (SEQ ID NO:33)
spvD Primer 2	5'-TAAAATGAATATTTAAAAAAGTTAAGTTACACTACCTCAATAAAATGC (SEQ ID NO:34)
spvD Primer 3	5'-GCATTTTATTGAGGTAGTGTAACTTAACTTTTTTAAATATTCATTTTA (SEQ ID NO:35)
spvD Primer 4	5'-CCCAAGCTTGCTGTACACAAAACGGACTGCACC (SEQ ID NO:36)
pK4953	
ssrAB Primer 1	5'-CGGGAATTCGCTACTACTTGTGGTATAATAACC (SEQ ID NO:37)
ssrAB Primer 2	5'-CTTAATACCATCGGACGCCCCTGGAATGCTTCCCTCCAGTTGCCTGTT (SEQ ID NO:38)
ssrAB Primer 3	5'-AACAGGCAACTGGAGGGAAGCATTCCAGGGGCGTCCGATGGTATTAAG (SEQ ID NO:39)
ssrAB Primer 4	5'-CGGGAATTCTGATCCGAGAGATTCCATCCGCTA (SEQ ID NO:40)

[0088] RESULTS

[0089] ***Reprogramming Salmonella chemotaxis system for tumor-navigating***

[0090] 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 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.

[0091] 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 $\Delta P_{tar}::P_{trc\ \Delta lacO}$ *tar* and GMS372 harboring single deletion-insertion mutation $\Delta P_{tsr}::P_{trc\ \Delta lacO}$ *tsr* were created using *Salmonella* wild-type strain χ3761 (Table 1). 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 χ3761. 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 χ3761. 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 (Fig. 8C). The strain with Δ*trg* deletion is much less attracted to galactose than wild-type strain as desired (Fig. 8D). 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 Δ*trg* were introduced into GMS409 to achieve strains GMS410 and GMS515 (Table 1).

[0092] ***Building up tumor-targeting self-eradicating TRAIL delivery vehicles***

[0093] 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. The diagram of the model illustrating the regulatory of TRAIL synthesis in the GMS strain is shown in Fig. 9a. 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.

[0094] ***Reprogrammed chemotaxis system endues GMS strains superior ability of cancer cell seeking, attaching and invading***

[0095] 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 mouse colon cancer CT-26 or 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. 2A, left panel). It was observed that significantly higher numbers of GMS410(pK5079) and GMS515(pK5079) swam across the swimming agar layer toward CT-26 or 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. 2A, right panel). The capability of GMS strains attaching to and invading cancer cells were also examined. The GMS strains were incubated with CT-26 and HCT-116 cells, respectively. We found that more GMS410(pK5079) and GMS515(pK5079) attached to and invaded into CT-26 cells (Fig. 2B) or HCT-116 cells (Fig. 2C) comparing to the control strain GMS409(pK5079). These data suggest that chemotaxis system reprogramming in GMS410(pK5079) and GMS515(pK5079) strains 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 system possess superior ability to navigate, attach, and invade colon cancer cells.

[0096] ***Reprogrammed GMS strains efficiently induced colon cancer cell death in vitro***

[0097] 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 (Fig. 3B). To further validate the cancer cell-killing features in reprogrammed GMS strains, the apoptosis assays were performed using same colon cancer cell lines. After co-incubating CT-26 cells with GMS515(pK5079) and GMS410(pK5079), it was found that over 99% and 35% of cells were undergoing apoptosis, respectively, which is significantly higher than the sample co-incubated with the control strain GMS409(pK5079) (Figs. 3C-3D). Furthermore, a 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. 3E-3F). Collectively, the data suggest that the self-eradicating TRAIL-delivering GMS strains with reprogrammed chemotaxis systems hold remarkable cancer cell-killing ability.

[0098] ***Reprogrammed GMS suppress tumor growth in vivo***

[0099] The engineered TRAIL-delivering GMS strains with reprogrammed chemotaxis system, displaying multiple cancer-killing features, have the potential to function as cancer therapeutics (Fig. 4A). Therefore, the impact of reprogrammed GMS-based therapy on tumor growth, following intra-tumor injection, was evaluated in an allograft colon cancer mouse model (Fig. 4B). 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 (Fig. 4C). In contrast, strain GMS409(pK5079) without chemoreceptor modification selectively accumulated in *Salmonella* preferred colonization organ, spleen. These data suggested that 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. As shown in Fig. 4D, 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 (Fig. 4E). 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. It was found that much more Ki67 positive cancer cells were present in the PBS and GMS409(pK5079)-treated tumor samples comparing to the tumor samples from the groups treated with GMS410(pK5079) or GMS515(pK5079) (Fig.4F). 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 (Fig.4G). Recent studies have highlighted the importance of *Salmonella*-induced systemic anti-tumor immunity. Systemic administration of *Salmonella* triggers immune cell infiltration and induction of proinflammatory cytokine expressions. The enhanced expressions of IFN-inducible chemokines may play a crucial role in the recruitment of activated $CD8^+$ T cells and an increase in intra-tumoral activated NK cells. Therefore, flow cytometry analysis was performed for lymphocytes isolated from treated tumors of mice injected with GMS515(pK5079), compared to phosphate buffered saline (PBS) control. It showed significantly increased frequencies of activated $CD107a^+CD8^+$ T cells (FIG. 4H, upper-left panel) as well as a trend towards an increased rate of $IFN\gamma^+CD8^+$ T cells (FIG. 4H, upper-right panel). Additionally, we observed a significantly higher percentage of proliferating Ki67 positive NK cells (FIG. 4H, lower-left panel) and $IFN\gamma^+$ positive NK cells (FIG. 4H, lower-right panel). Overall, these results indicate that GMS515(pK5079) could safely promote local tumor regression while also induced systemic tumor-specific antitumor immunity. The tumor-specific T cells will likely mediate the prevention of metastasis (FIG. 4H).

[00100] 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 (Fig. 5A). HCT-116 cells, which stably express luciferases, were subcutaneously injected into the flank area of immunocompromised NOD. Cg-*Prkdc*scid *Il2rg*tm1Wjl/SzJ (NSG™) mice. PBS, GMS410(pK5079), and GMS515(pK5079) were IT injected into the NSG™ 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 Fig. 5B and 5C, 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* (Fig. 5D). 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 (Fig. 5E). Taken together, these observations suggested that the reprogramming of chemotaxis system was an essential component of 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.

[00101] ***Evaluation of reprogrammed GMS strains using a transgenic colon tumor mouse model***

[00102] In addition to allograft and xenograft subcutaneous tumor models, GMS strains were evaluated in a transgenic *Apc*$^{flox/flox}$/*CDX2-CRE* colon tumor mouse model, which mimic human FAP associated colorectal cancer and sporadic colorectal cancer (Fig. 6A). 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. 6B and 6C, 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*$^{flox/flox}$/*CDX2-CRE* mice treated with GMS410(pK5079) and GMS515(pK5079) was dramatically increased when compared with the control group (Fig. 6D). 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. 6E). These results suggest that the reprogramming 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. 6F). Overall, these data further demonstrate that GMS410(pK5079) 409 and GMS515(pK5079) are able to navigate to the tumor and efficiently induce tumor cell apoptosis *in vivo*. Moreover, it was observed that GMS515(pK5079) significantly recruited natural killer cells (NKs) and $CD8^+$ T-cells to the polyps, which may contribute to the observed suppression of tumor growth (Fig. 6G). Overall, these data further demonstrate that engineered *Salmonella* of strain GMS515(pK5079) navigate to the tumor, efficiently induce tumor cell apoptosis, and induce systemic tumor-specific antitumor immunity *in vivo*.

[00103] ***Reprogrammed GMS strains-based therapy for metastatic cancer in an orthotopic xenograft mouse model***

[00104] 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 NSG™ 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 (Fig. 7A). Tumor growth and metastasis were monitored using a live imaging system (Fig. 7B). Metastatic tumor number and size were analyzed at week 5 post-inoculation. As shown in Figs. 7C and 7D, 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.

[00105] DISCUSSION

[00106] 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 feature. 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 the tumoricidal contents, TRAIL, after cell lysis. 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, release TRAIL into the tumor tissues, induce significant tumor regression 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. In addition, GMS515(pK5079) successfully recruited and activated significant anti-cancer immune cells to the tumor microenvironment. As such, these GMS strains show tremendous potential, either alone or in combination with other treatments, to make an important contribution in cancer therapy.

[00107] 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, wherein the bacterium comprises

a recombinant gene encoding human TNF-related Apoptosis-inducing Ligand (TRAIL);

the following mutations ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asd*::TT *araC* P_{BAD} *c2* Δ(*araC* P_{BAD})::P22 P_R *araBAD* Δ(*wza-wcaM*) Δ*pmi* Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*pagP*::P_{lpp} *lpxE* Δ*endA;* and

one or more of the following mutations: ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*, ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*, and Δ*trg*.

2. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacterium further 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, whereby the modified bacterium is capable of self-eradication in non-tumor cells.

3. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacterium comprises mutations ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar,* ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*, and Δ*trg*.

4. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacteria comprise SEQ ID NO:7.

5. The genetically modified *Salmonella* bacterium of claim 1, wherein the bacteria are *S. Typhimurium.*

6. A genetically modified *Salmonella* bacterium, wherein the bacterium comprises a recombinant gene encoding human TNF-related Apoptosis-inducing Ligand (TRAIL); the following mutations ΔP_{murA}::TT *araC* P_{BAD} *murA* Δ*asd*::TT *araC* P_{BAD} *c2* Δ(*araC* P_{BAD})::P22 P_R *araBAD* Δ(*wza-wcaM*) Δ*pmi* Δ*relA*::*araC* P_{BAD} *lacI* TT Δ*pagP*::P_{lpp} *lpxE* Δ*endA*; and one or more of the following mutations: ΔP_{tar}::$P_{trc\ \Delta lacO}$ *tar*, ΔP_{tsr}::$P_{trc\ \Delta lacO}$ *tsr*, and Δtrg; and one or more of the following mutations: Δ*sseL*, Δ*spvD*, and Δ*ssrAB*.

7. The genetically modified *Salmonella* bacterium of claim 6, wherein the bacterium further 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, whereby the modified bacterium is capable of self-eradication in non-tumor cells.

8. The genetically modified *Salmonella* bacterium of claim 6, wherein the bacterium comprises mutations Δ*sseL*, Δ*spvD*, and Δ*ssrAB*.

9. The genetically modified *Salmonella* bacterium of claim 6, wherein the bacteria comprise SEQ ID NO:7.

10. The genetically modified *Salmonella* bacterium of claim 6, wherein the bacteria are *S. Typhimurium*.

11. A method of treating cancer in a subject in need thereof comprising administering an effective amount of the genetically modified *Salmonella* bacterium of any one of claims 1-10 to the subject, whereby the genetically modified *Salmonella* bacterium treats cancer in the subject.

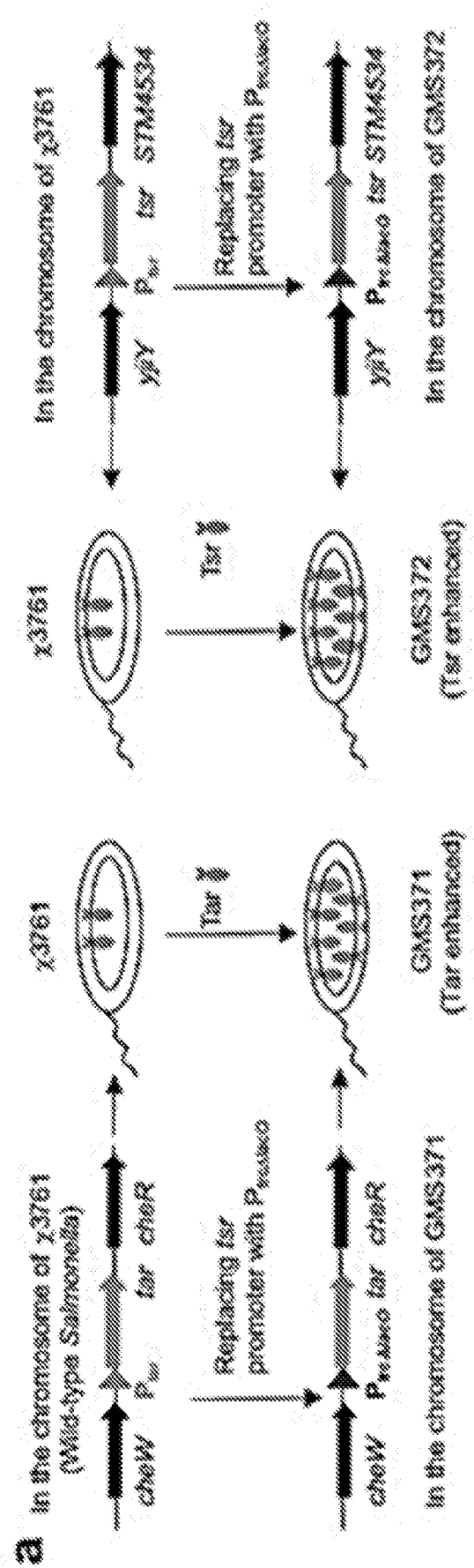
12. The method of claim 11, wherein administering comprises oral administration or intra-tumoral injection of the genetically modified *Salmonella* bacterium.

13. A method for stimulating tumoricidal activity in a subject comprising administering an effective amount of the genetically modified *Salmonella* bacterium of any one of claims 1-10 to the subject, whereby the genetically modified *Salmonella* bacterium induces tumoricidal activity in the subject.

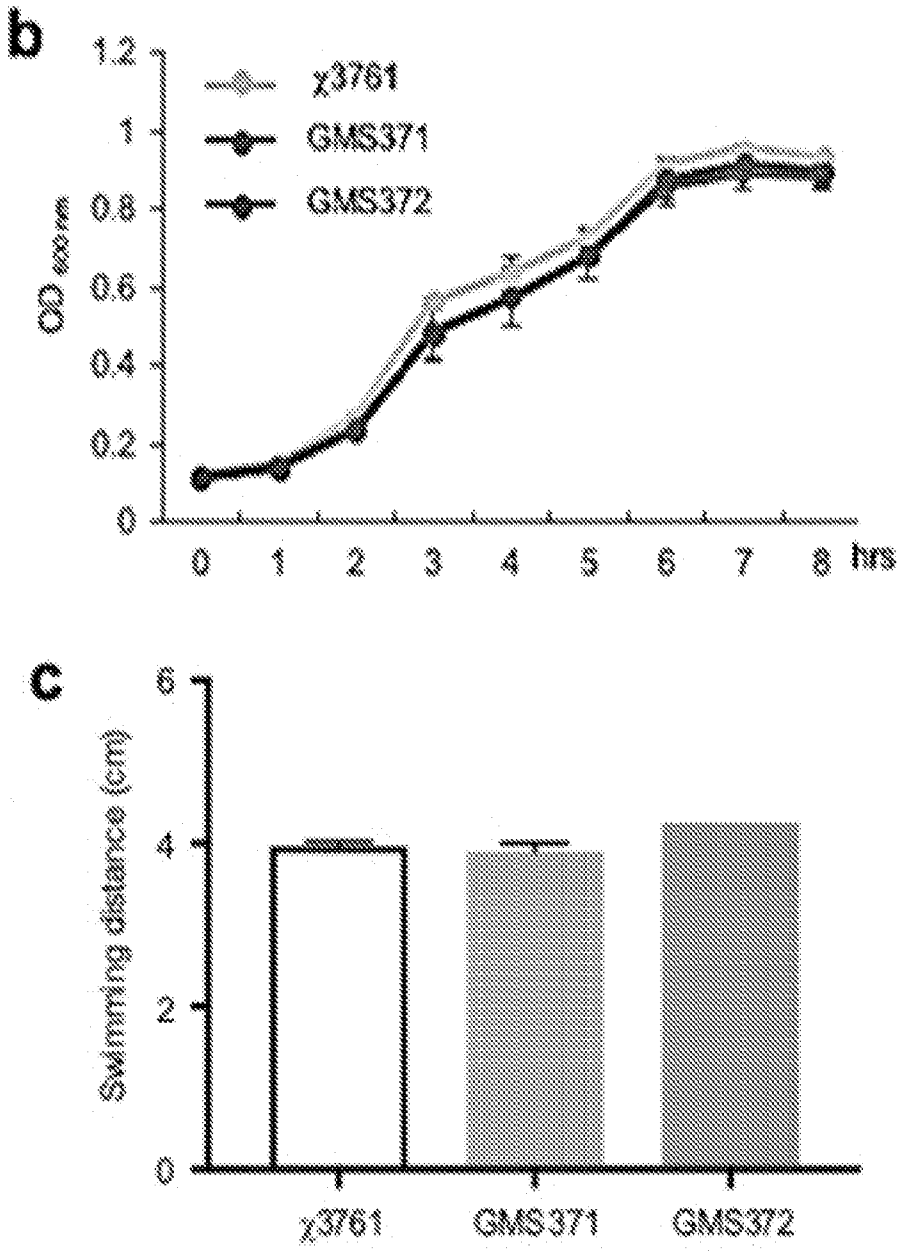
14 The method of claim 13, wherein administering comprises oral administration or intra-tumoral injection of the genetically modified *Salmonella* bacterium.

15. The method of claim 13, wherein the subject has cancer.

FIG. 1A


a
In the chromosome of χ3761
(Wild-type Salmonella)
cheW
Ptar
tar cheR
Replacing tsr
promoter with Pmutacio
In the chromosome of GMS371
χ3761
Tsr ↑
GMS371
(Tsr enhanced)
In the chromosome of χ3761
yjiY
Ptar
tsr STM4534
Replacing tsr
promoter with Pmutacio
In the chromosome of GMS372
GMS372
(Tsr enhanced)

FIGS. 1B-1C


b
1.2
1
0.8
0.6
0.4
0.2
0
χ3761
GMS371
GMS372
0 1 2 3 4 5 6 7 8 hrs
c
Swimming distance (cm)
6
4
2
0
χ3761
GMS371
GMS372

FIGS. 1D-1E

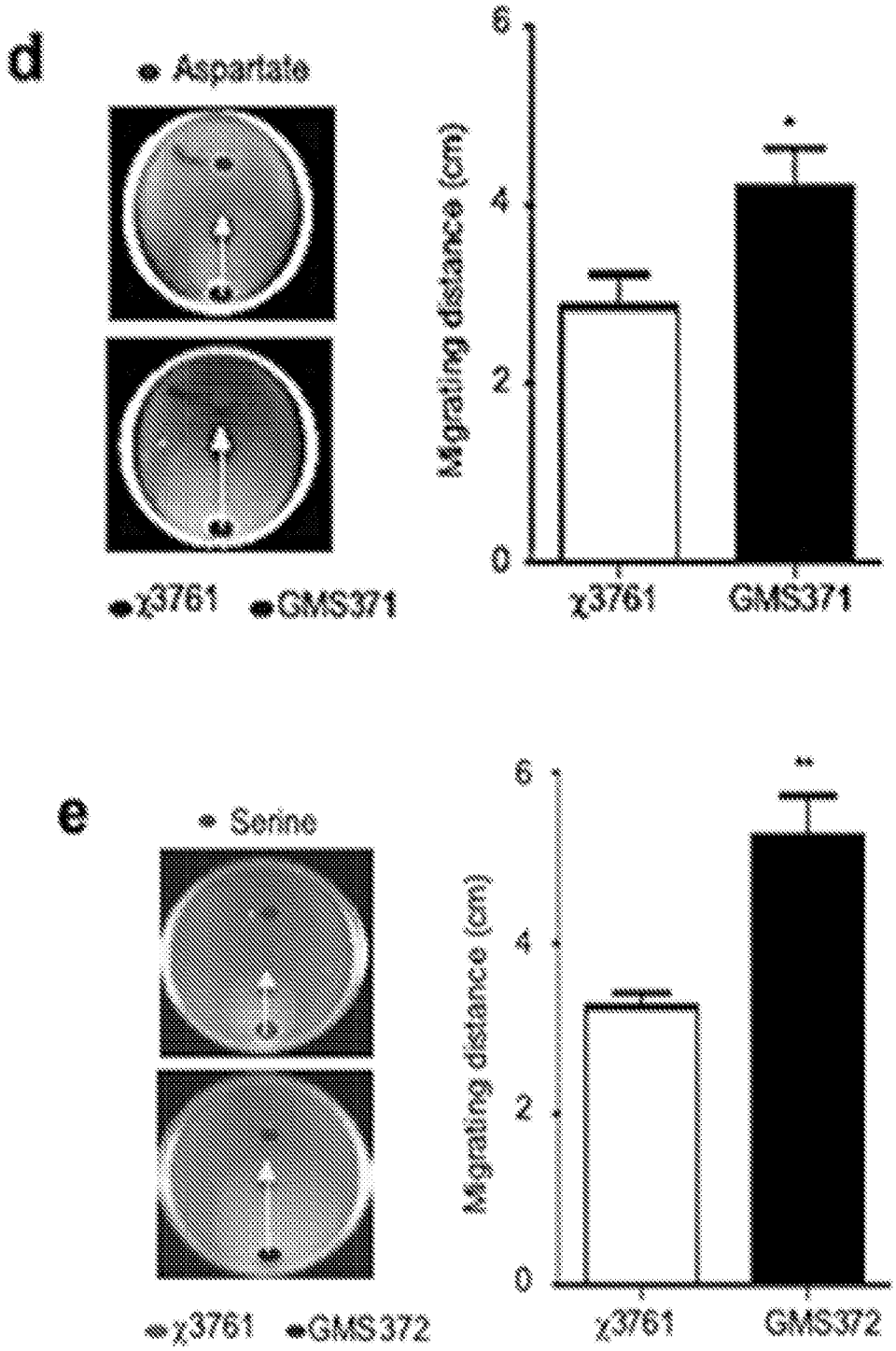

d
Aspartate
χ3761
GMS371
Migrating distance (cm)
0
2
4
6
χ3761
GMS371
e
Serine
χ3761
GMS372
Migrating distance (cm)
0
2
4
6
χ3761
GMS372

FIG. 2A

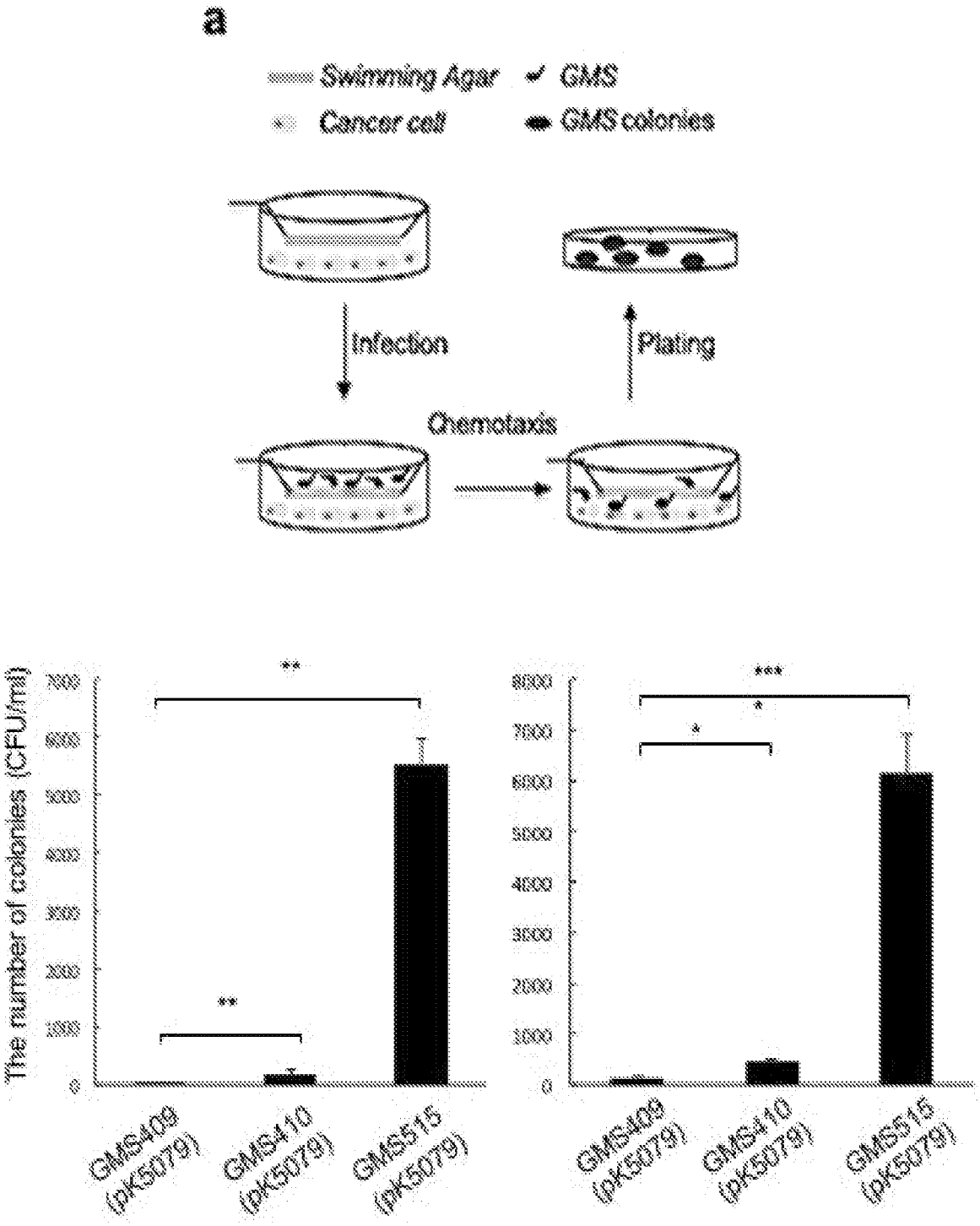

a
Swimming Agar
GMS
Cancer cell
GMS colonies
Infection
Plating
Chemotaxis
The number of colonies (CFU/ml)
GMS409 (pK5079)
GMS410 (pK5079)
GMS515 (pK5079)

FIG. 2B

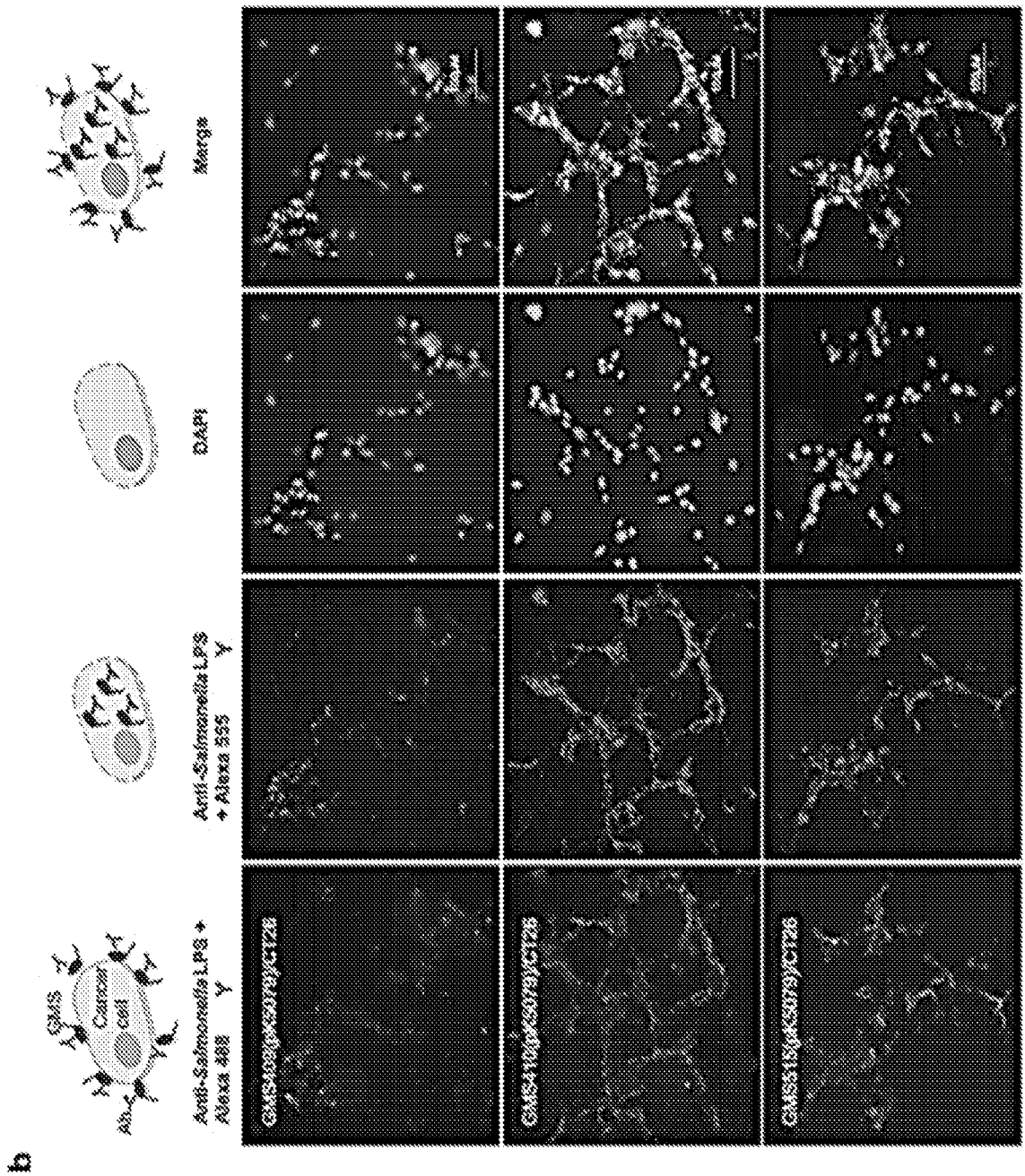

b
GMS
Cancer cell
Anti-Salmonella LPS + Alexa 488
Anti-Salmonella LPS + Alexa 555
DAPI
Merge
GMS-102/Oxaliplatin/CT26
GMS-110/Oxaliplatin/CT26
GMS-113/Oxaliplatin/CT26

FIG. 2C

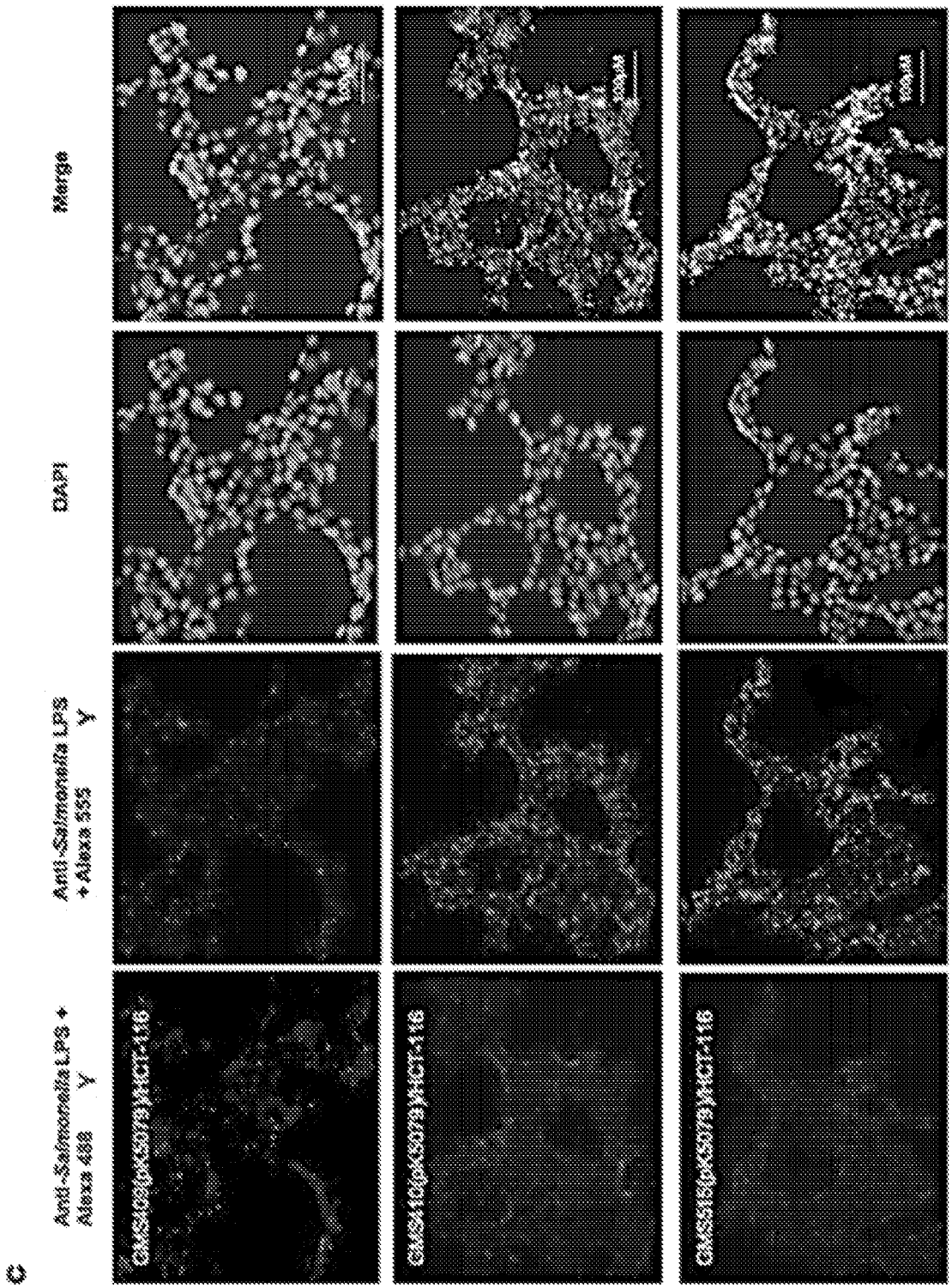

C
Anti-Salmonella LPS + Alexa 488 Y
Anti-Salmonella LPS + Alexa 555 Y
DAPI
Merge
CMSM16(pHCSTB)/HCT-116
CMSM16(pHCSTB)/HCT-116
CMSM16(pHCSTB)/HCT-116

FIG. 3A

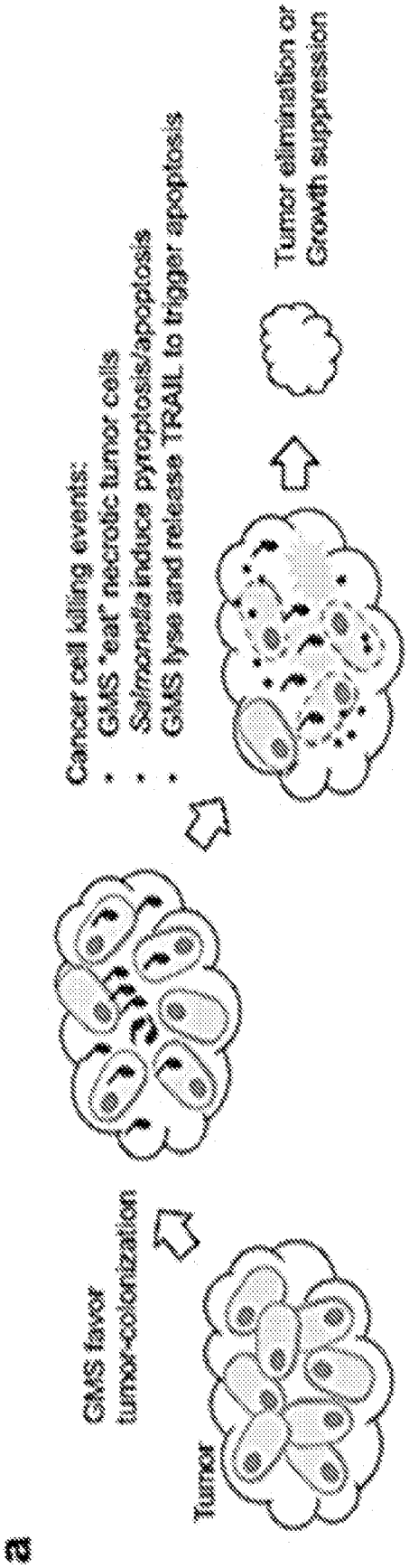
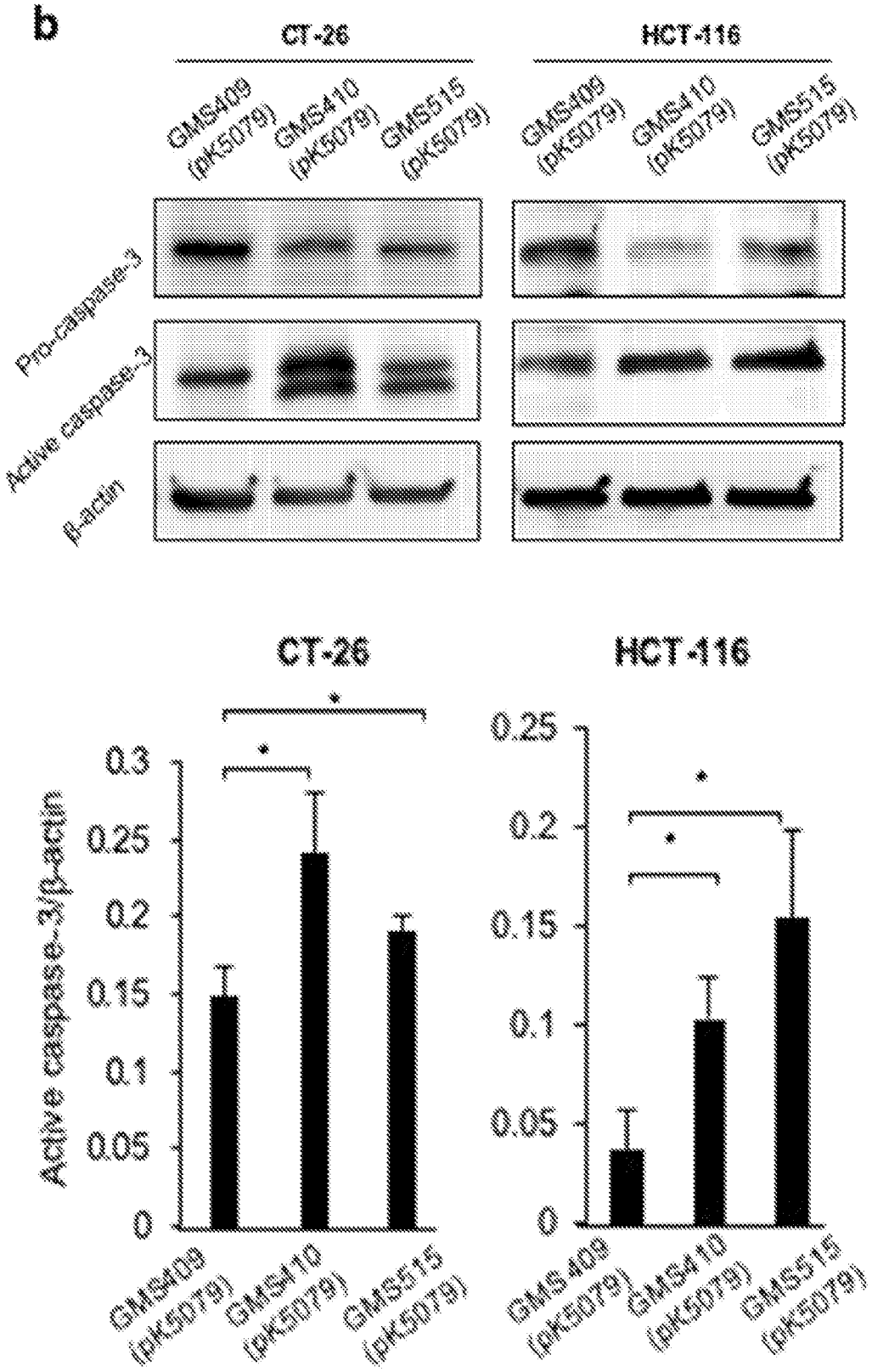
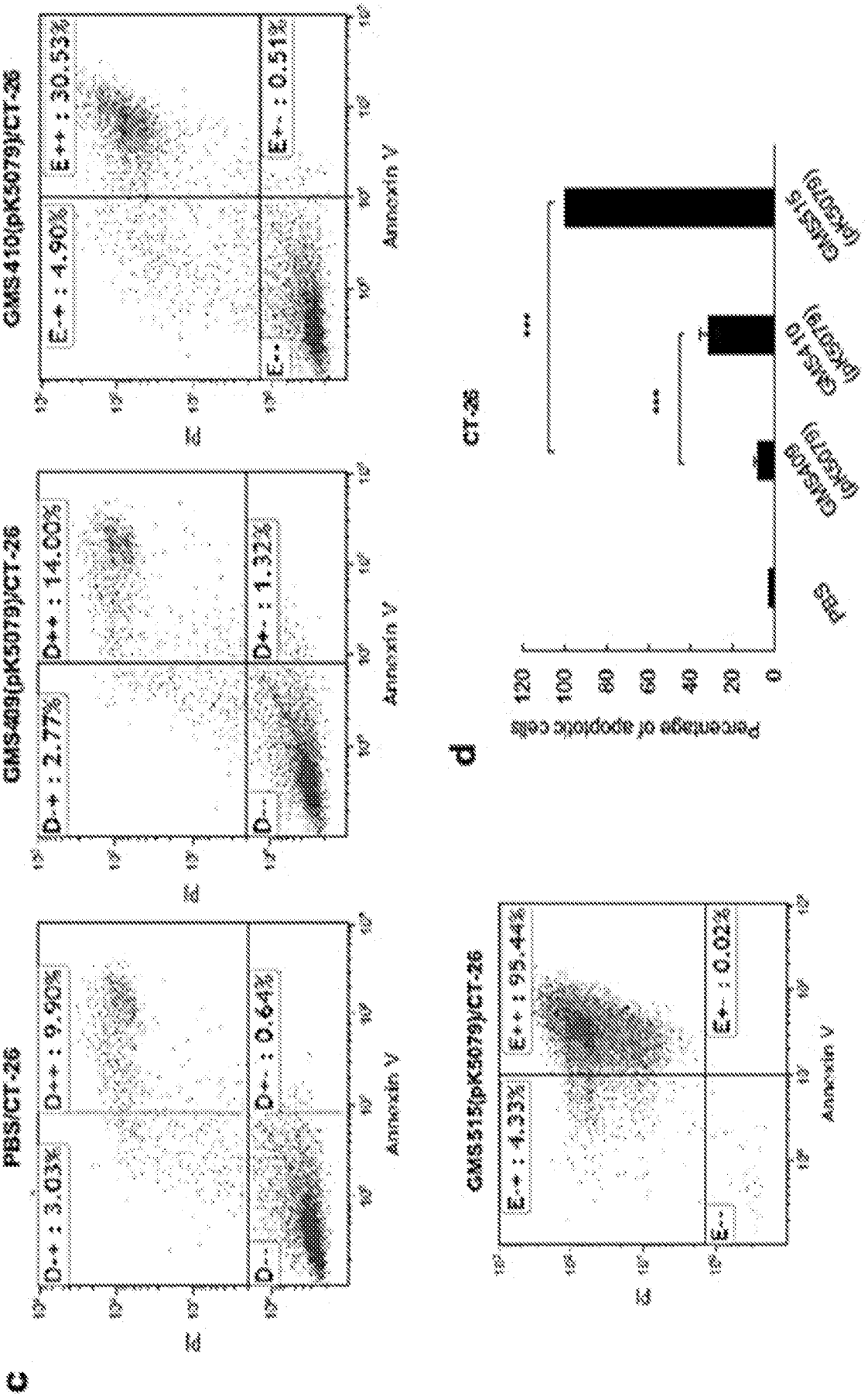

a
Tumor
GMS favor tumor-colonization
Cancer cell killing events:
* GMS "eat" necrotic tumor cells
* Salmonella induce pyroptosis/apoptosis
* GMS lyse and release TRAIL to trigger apoptosis
Tumor elimination or Growth suppression

FIG. 3B


b
CT-26
HCT-116
GMS409 (pK5079)
GMS410 (pK5079)
GMS515 (pK5079)
Pro-caspase-3
Active caspase-3
β-actin
CT-26
HCT-116
Active caspase-3/β-actin
0
0.05
0.1
0.15
0.2
0.25
0.3
*
*
*
*

FIGS. 3C-3D


C
PBS/CT-26
GMS403(pK5079)/CT-26
GMS410(pK5079)/CT-26
GMS315(pK5079)/CT-26
Q4: 3.03%
Q2: 9.90%
Q1: 2.77%
Q2: 14.00%
Q4: 1.32%
E-+ : 4.90%
E++ : 30.53%
E-- : 0.51%
E++ : 95.44%
E+- : 0.02%
E-- : 4.33%
Annexin V
d
CT-26
Percentage of apoptotic cells
PBS
GMS410 (pK5079)
GMS315 (pK5079)

FIGS. 3E-3F

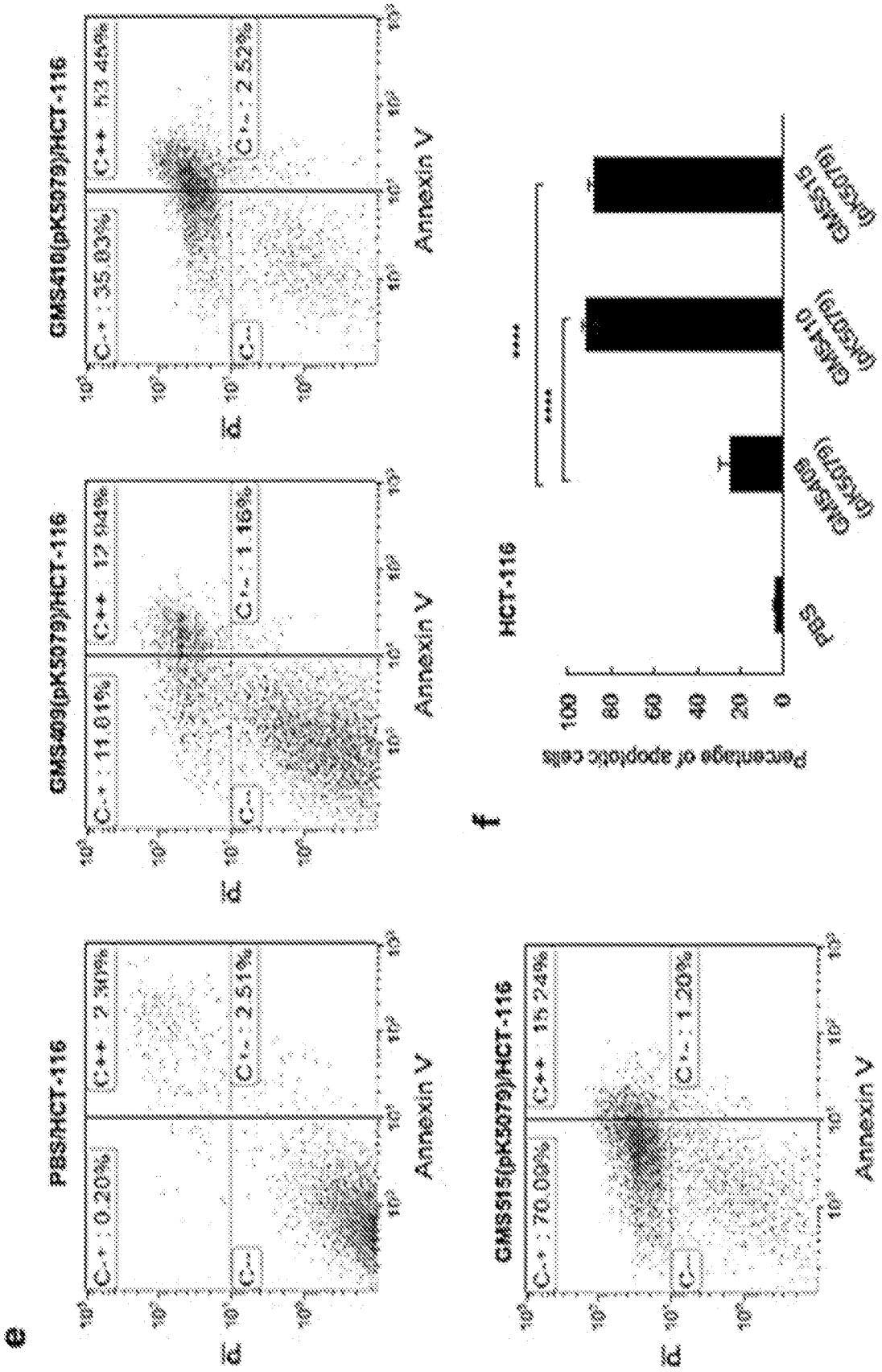

e
PBS/HCT-116
C-+ : 0.20%
C++ : 2.38%
C-- : 2.51%
C+- : 94.91%
GMS515(pKS079)/HCT-116
C-+ : 70.00%
C++ : 16.24%
C-- : 1.20%
C+- : 12.56%
GMS409(pKS079)/HCT-116
C-+ : 11.01%
C++ : 12.94%
C-- : 1.16%
C+- : 74.89%
GMS416(pKS079)/HCT-116
C-+ : 35.83%
C++ : 53.46%
C-- : 2.52%
C+- : 8.19%
Annexin V
f
HCT-116
Percentage of apoptotic cells
PBS
GMS409 (pKS079)
GMS416 (pKS079)
GMS515 (pKS079)
****
****

FIG. 4A

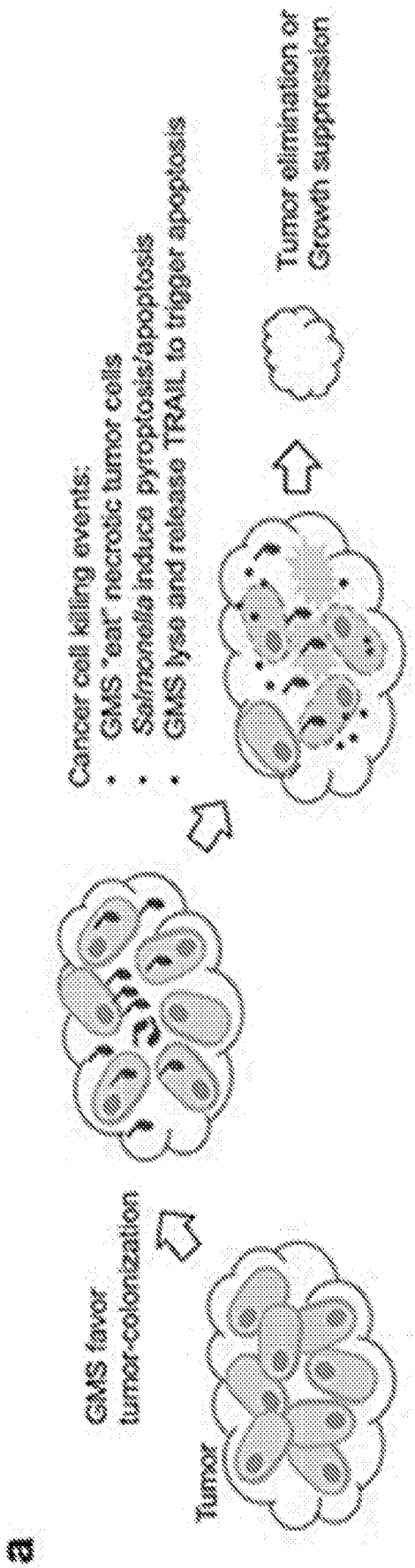

a
Tumor
GMS favor tumor-colonization
Cancer cell killing events:
GMS "eat" necrotic tumor cells
Salmonella induce pyroptosis/apoptosis
GMS lyse and release TRAIL to trigger apoptosis
Tumor elimination or Growth suppression

FIG. 4B

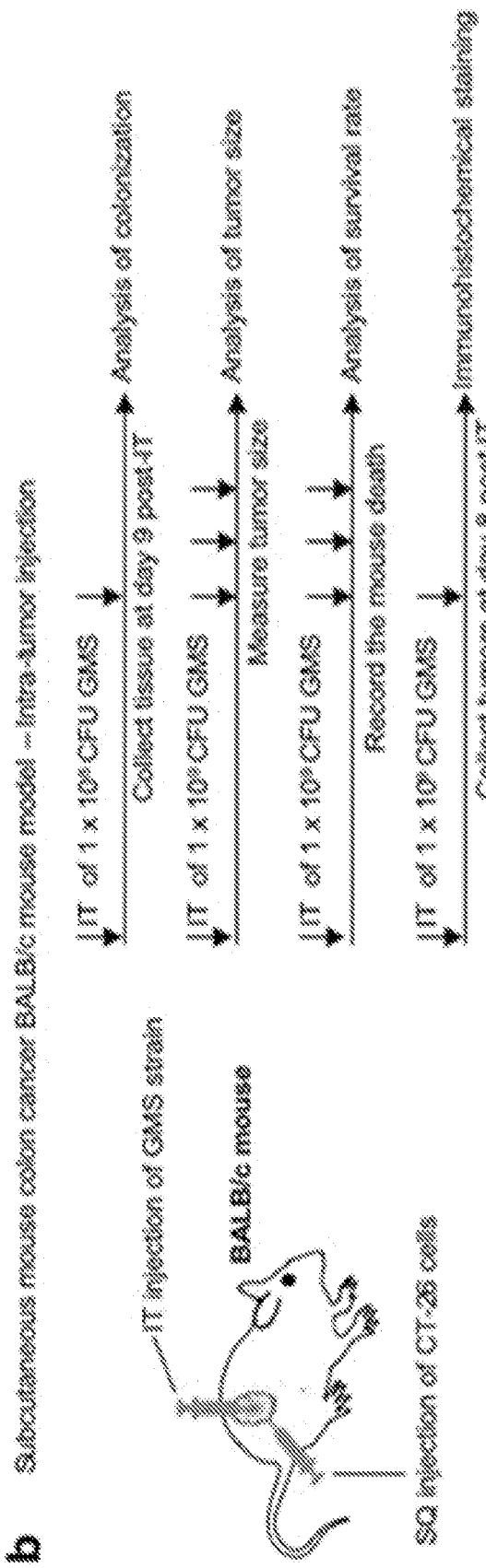
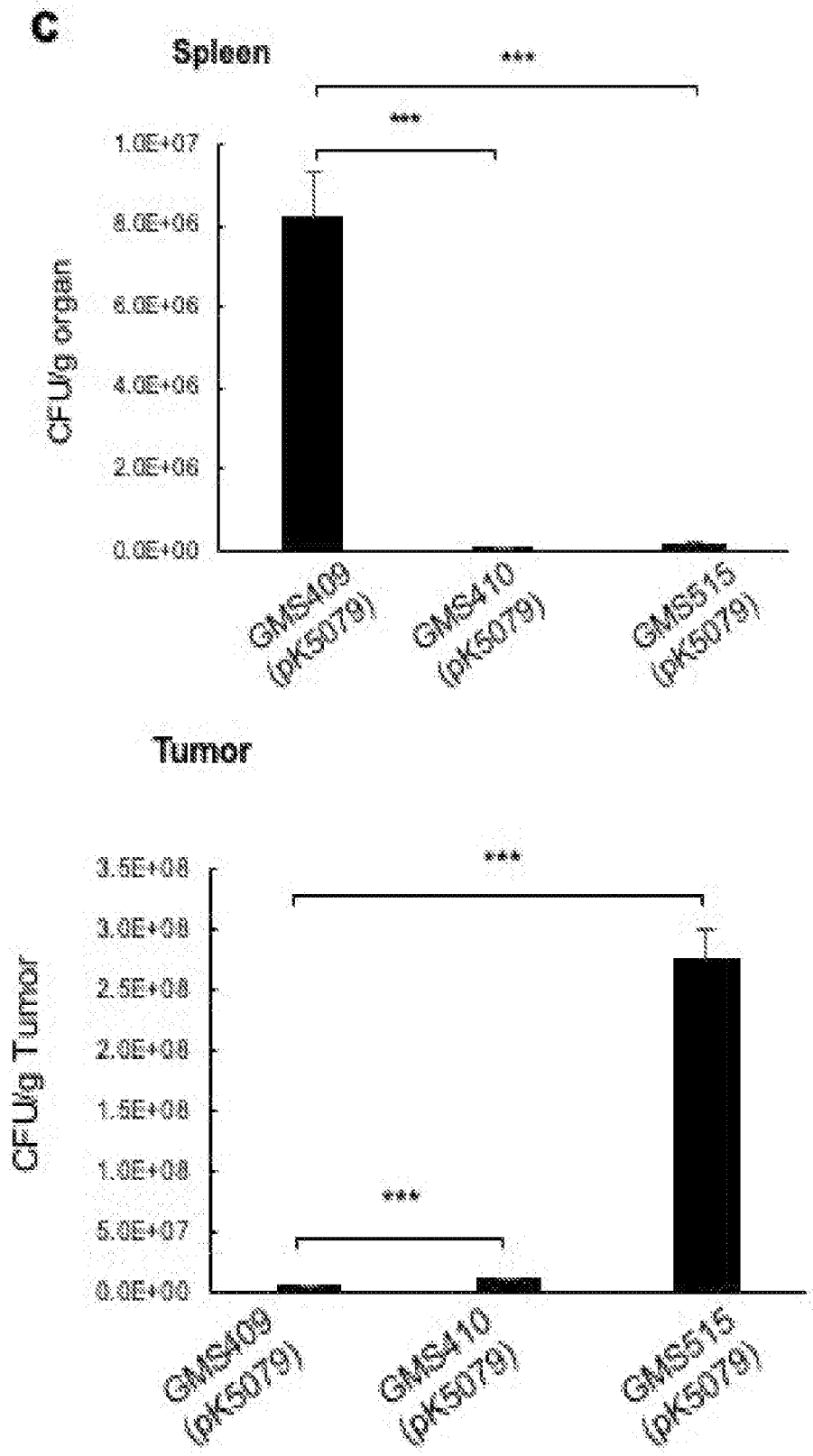

b
Subcutaneous mouse colon cancer BALB/c mouse model – Intra-tumor injection
IT of 1 x 10⁸ CFU GMS
Analysis of colonization
Collect tissue at day 9 post-IT
IT of 1 x 10⁸ CFU GMS
Analysis of tumor size
Measure tumor size
IT of 1 x 10⁸ CFU GMS
Analysis of survival rate
Record the mouse death
IT of 1 x 10⁸ CFU GMS
Immunohistochemical staining
Collect tumors at day 8 post-IT
IT injection of GMS strain
BALB/c mouse
SQ injection of CT-26 cells

FIG. 4C


C
Spleen
***
***
1.0E+07
8.0E+06
6.0E+06
4.0E+06
2.0E+06
0.0E+00
CFU/g organ
GMS409 (pK5079)
GMS410 (pK5079)
GMS515 (pK5079)
Tumor
***
***
3.5E+08
3.0E+08
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
CFU/g Tumor
GMS409 (pK5079)
GMS410 (pK5079)
GMS515 (pK5079)

FIGS. 4D-4E

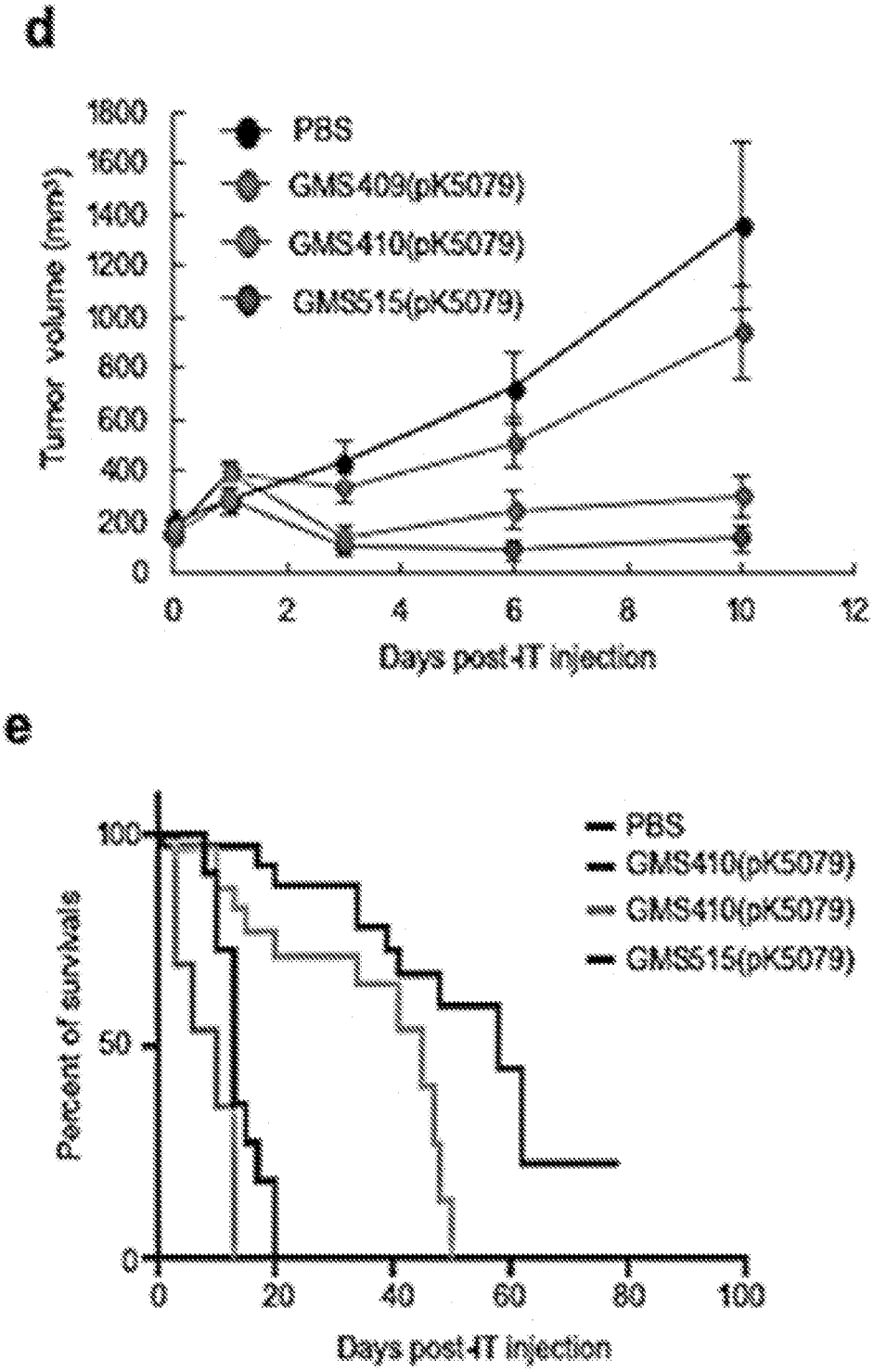

d
Tumor volume (mm³)
1800
1600
1400
1200
1000
800
600
400
200
0
PBS
GMS409(pK5079)
GMS410(pK5079)
GMS515(pK5079)
0
2
4
6
8
10
12
Days post-4T injection
e
Percent of survivals
100
50
0
PBS
GMS410(pK5079)
GMS410(pK5079)
GMS515(pK5079)
0
20
40
60
80
100
Days post-4T injection

FIG. 4F

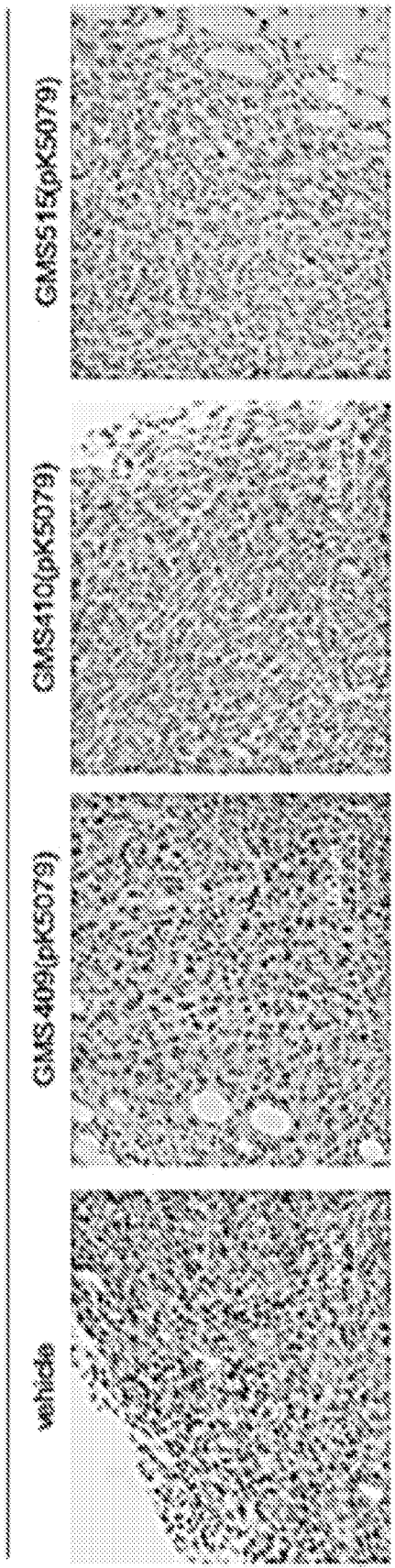

Ki67 staining
vehicle
GMS409(pK5079)
GMS410(pK5079)
GMS515(pK5079)

FIG. 4G

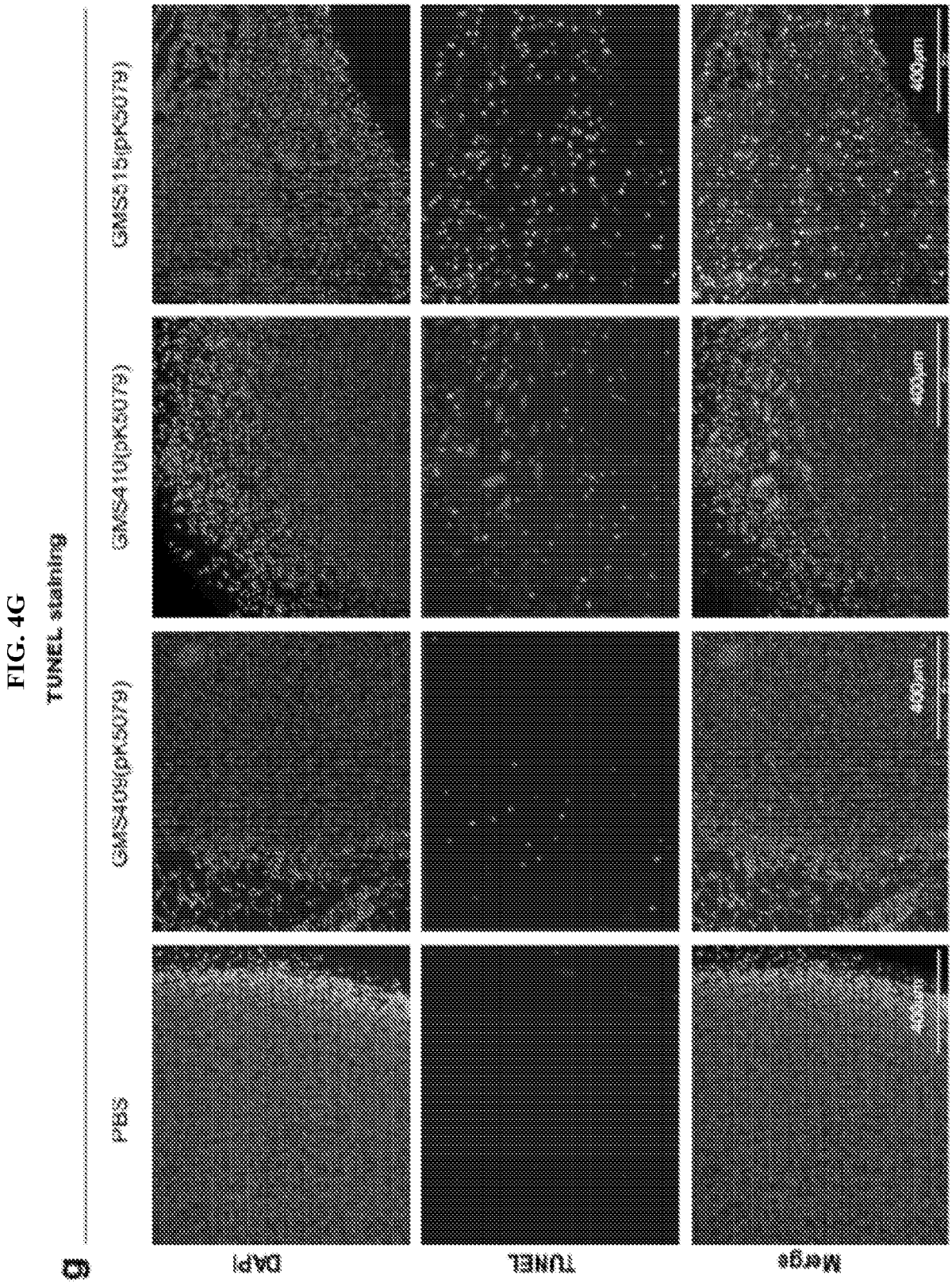

TUNEL staining
G
PBS
GMSA10pk30(7d)
GMSA10pk30(7d)
GMSA16pk30(7d)
DAPI
TUNEL
Merge
400μm

FIG. 4H

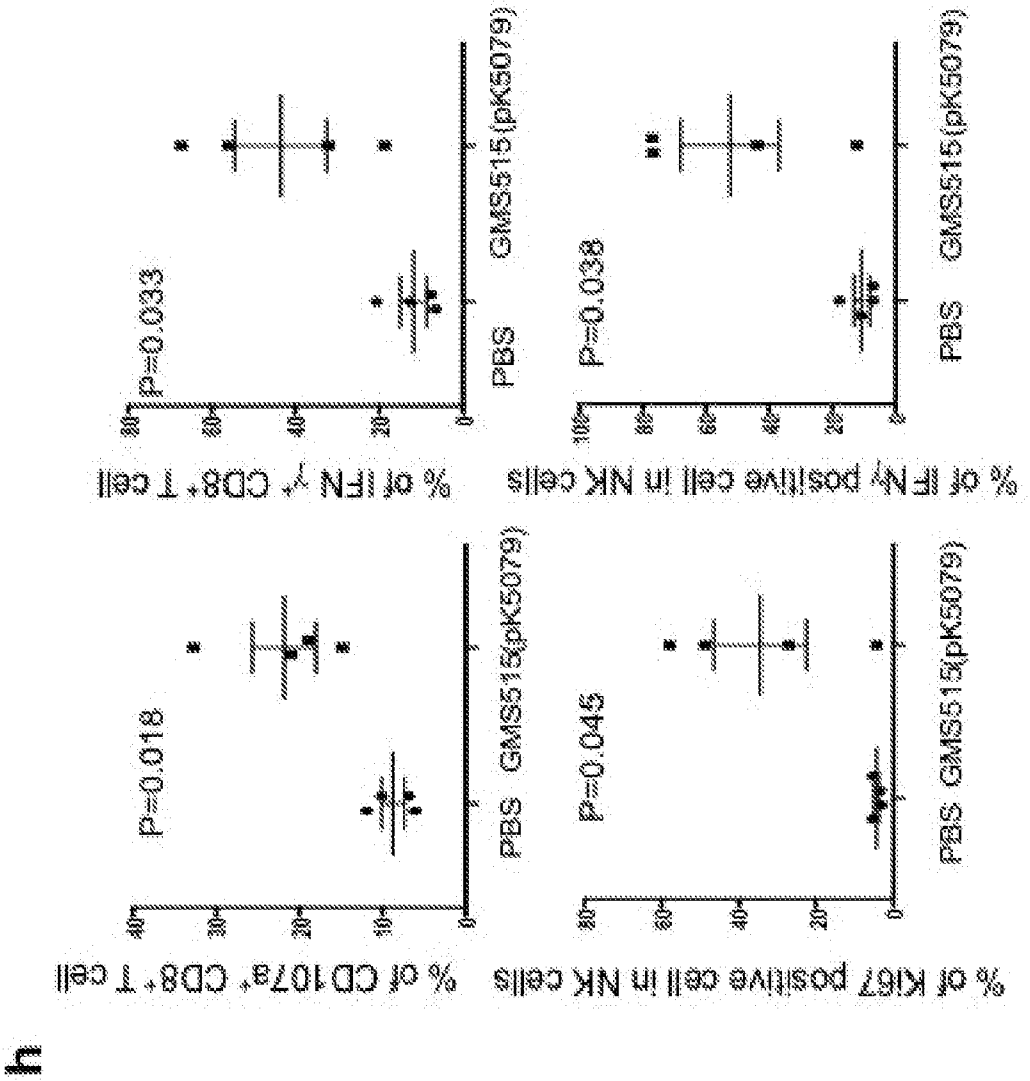

h
% of CD107a+ CD8+ T cell
P=0.018
PBS
GMS515(pK5079)
% of IFNγ+ CD8+ T cell
P=0.033
PBS
GMS515(pK5079)
% of KI67 positive cell in NK cells
P=0.045
PBS
GMS515(pK5079)
% of IFNγ positive cell in NK cells
P=0.038
PBS
GMS515(pK5079)

FIG. 5A

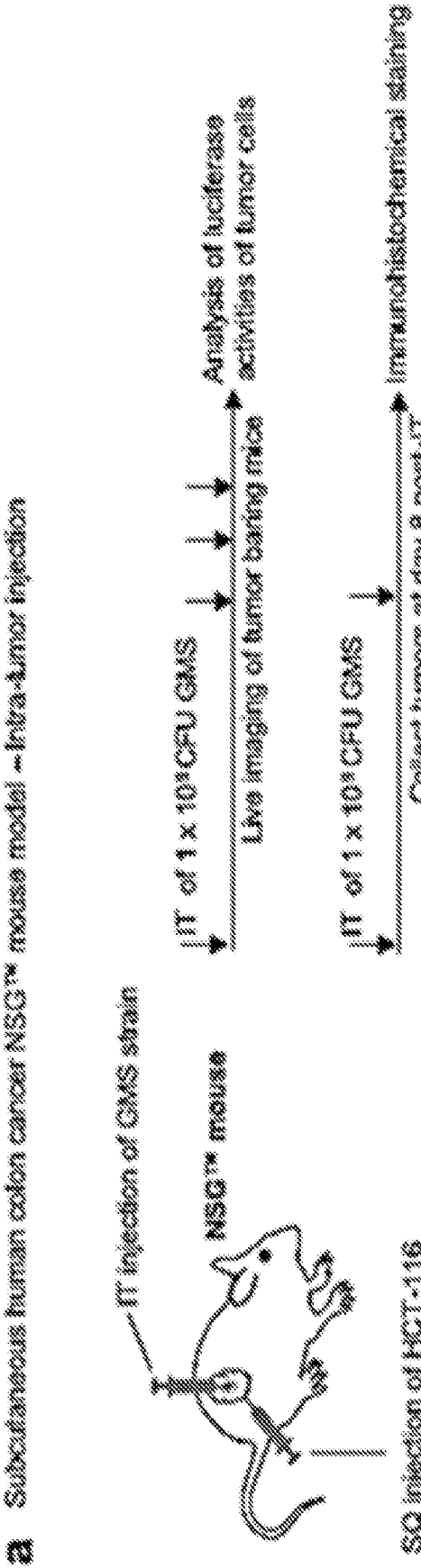

a
Subcutaneous human colon cancer NSG™ mouse model – Intra-tumor injection
IT injection of GMS strain
NSG™ mouse
SQ injection of HCT-116
luciferase expressing cells
IT of 1 x 10⁸ CFU GMS
Live imaging of tumor baring mice
Analysis of luciferase
activities of tumor cells
IT of 1 x 10⁸ CFU GMS
Collect tumors at day 8 post-IT
Immunohistochemical staining

FIG. 5B

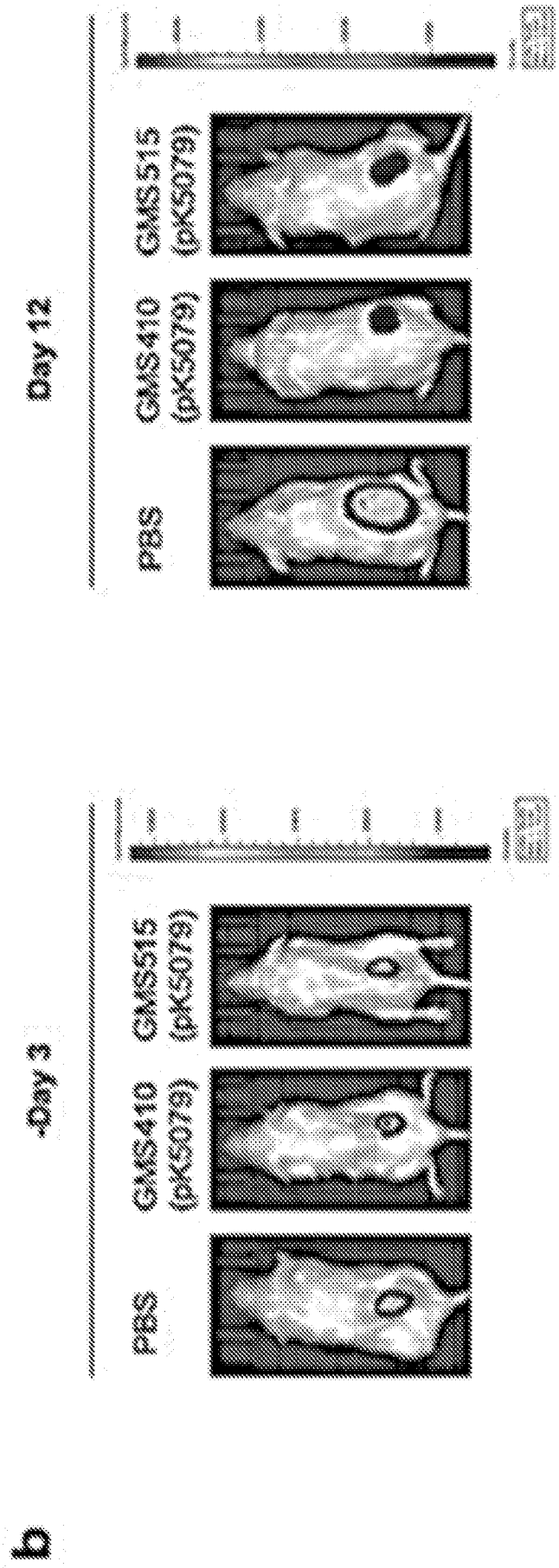


FIG. 5C

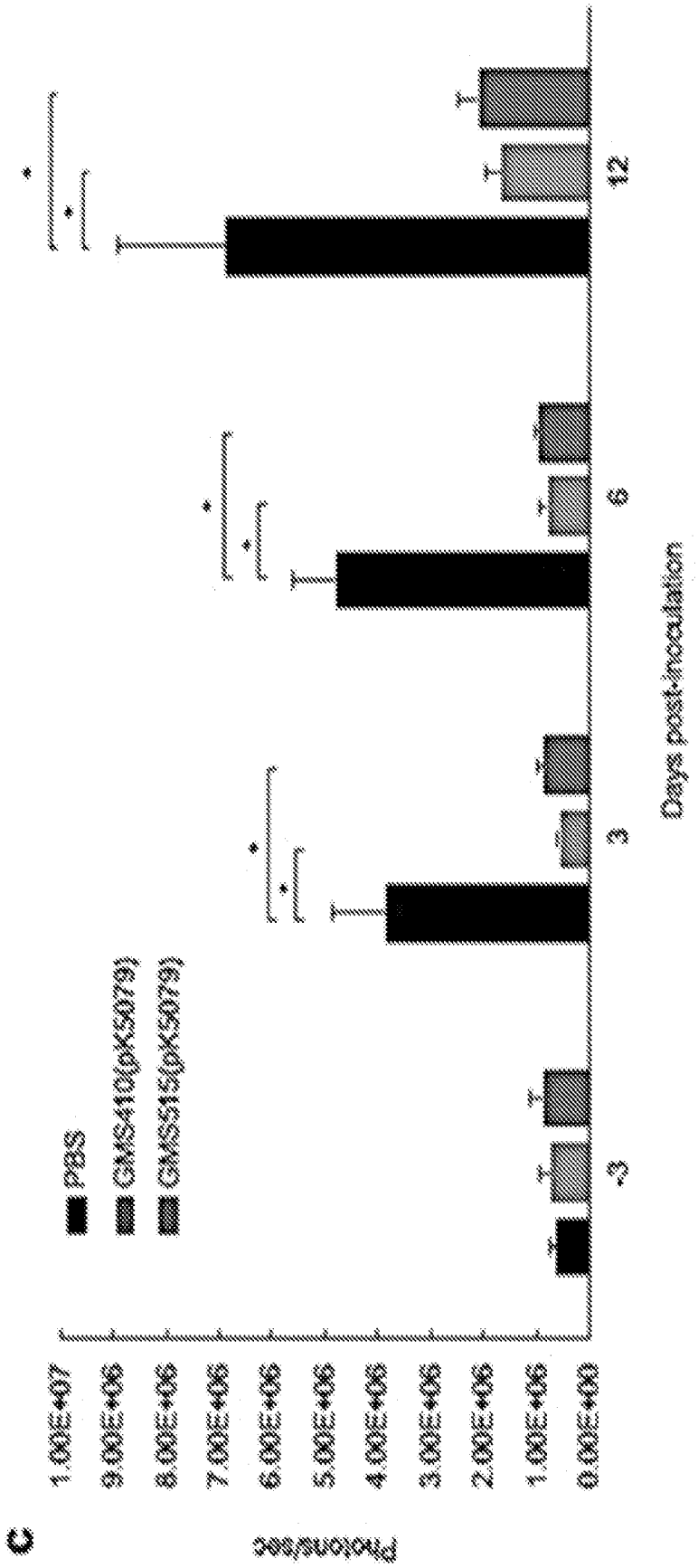

C
Photons/Sec
1.00E+07
9.00E+06
8.00E+06
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
PBS
GMS410(pKS079)
GMS515(pKS079)
-3
3
6
12
Days post-inoculation

FIG. 5D

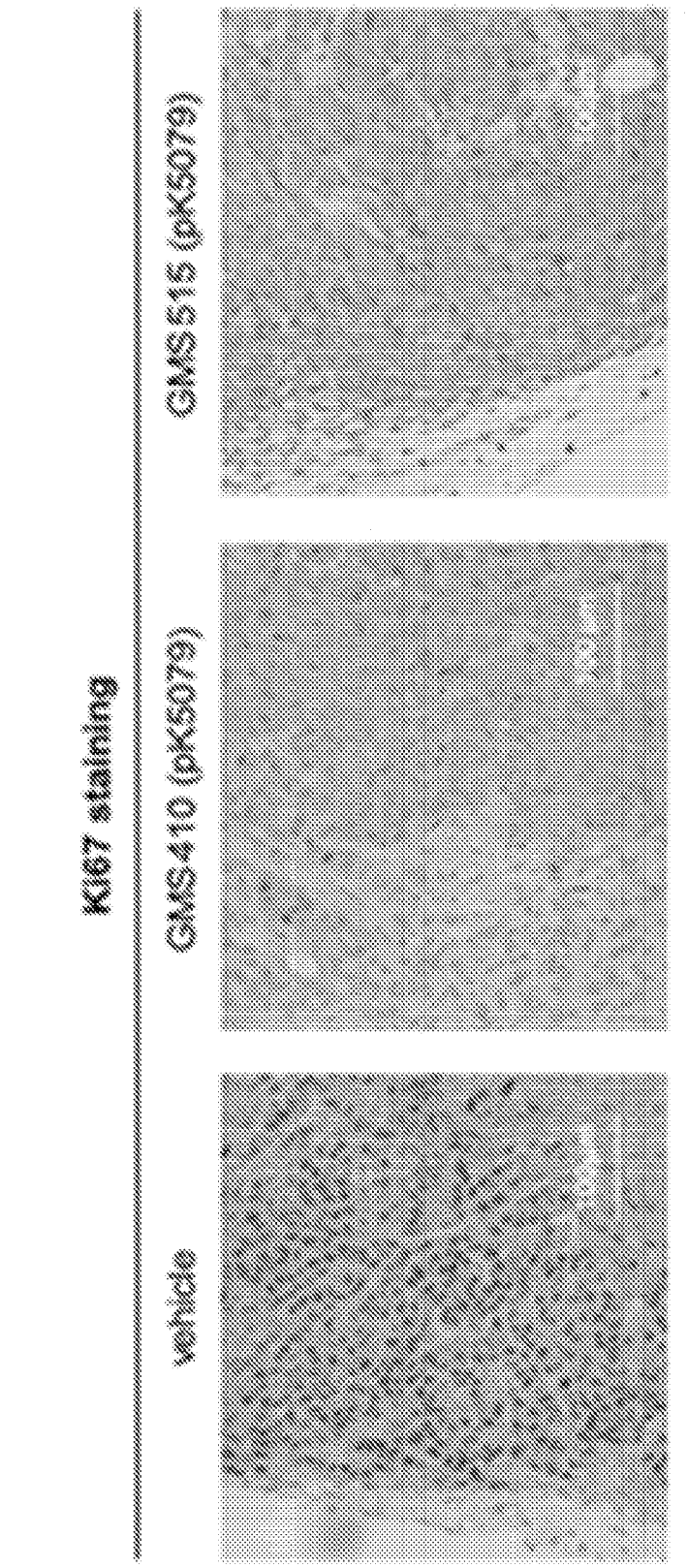


FIG. 5E

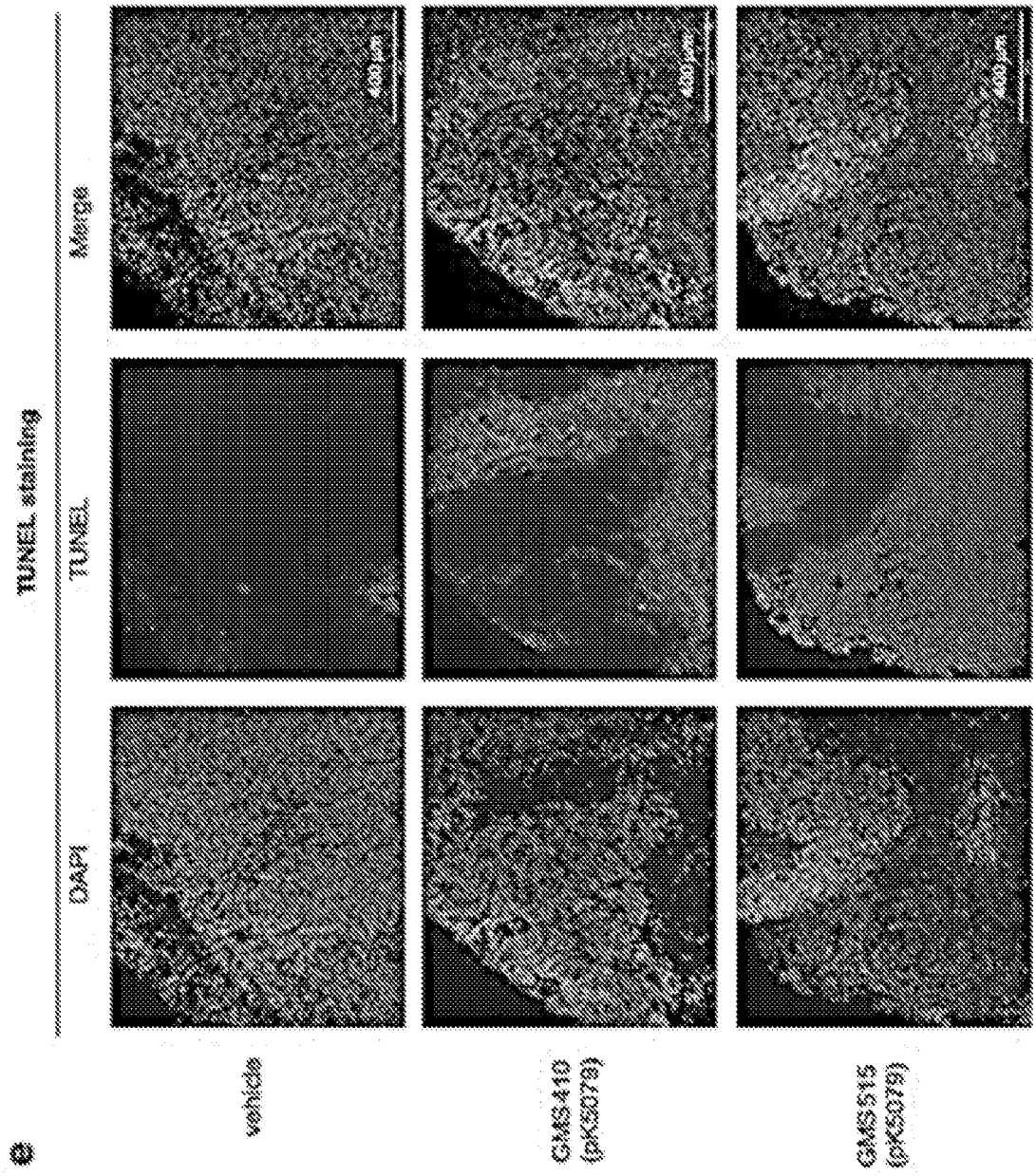

e
TUNEL staining
DAPI
TUNEL
Merge
vehicle
GMS410 (pKS078)
GMS515 (pKS079)

FIG. 6A

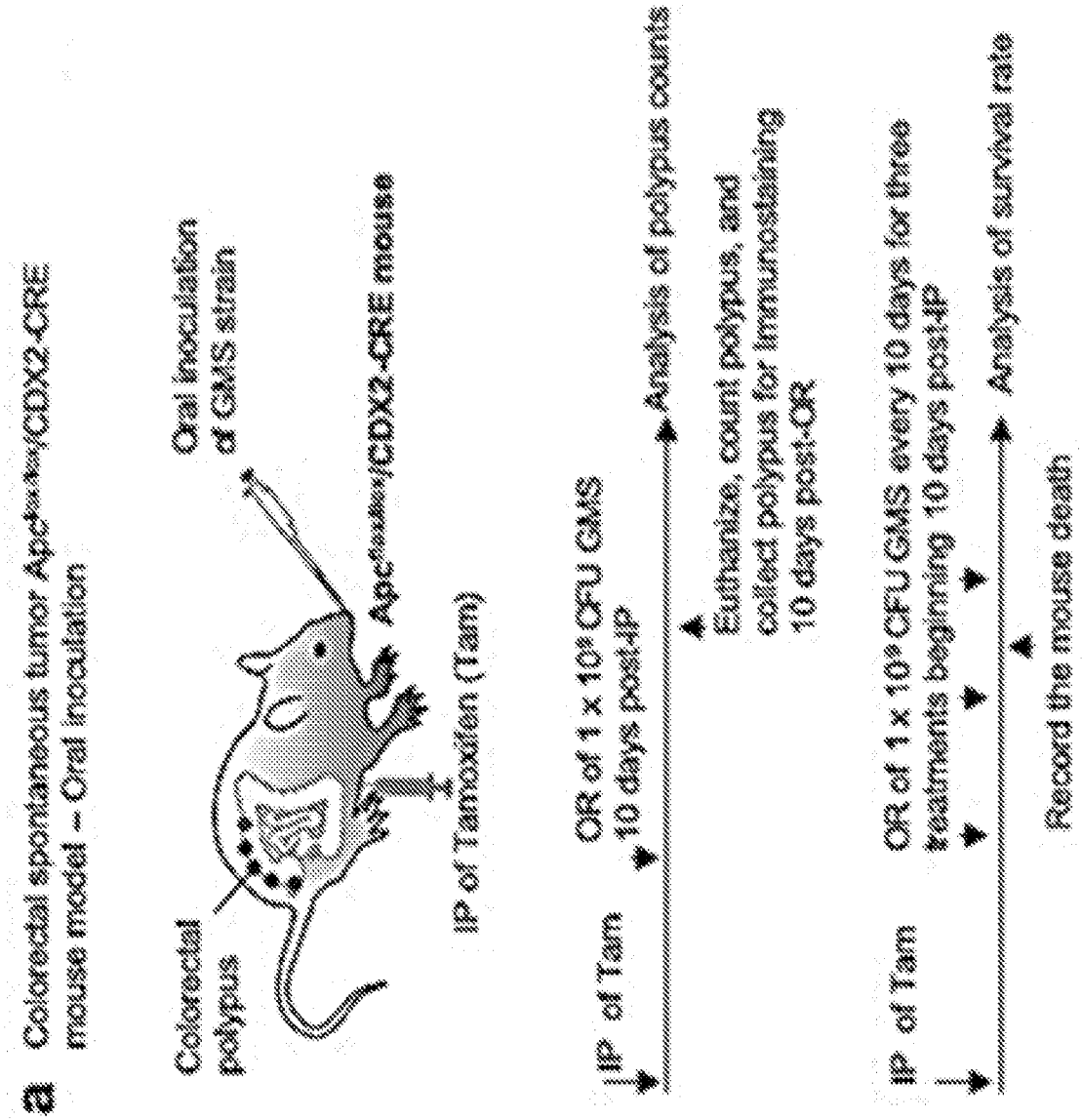
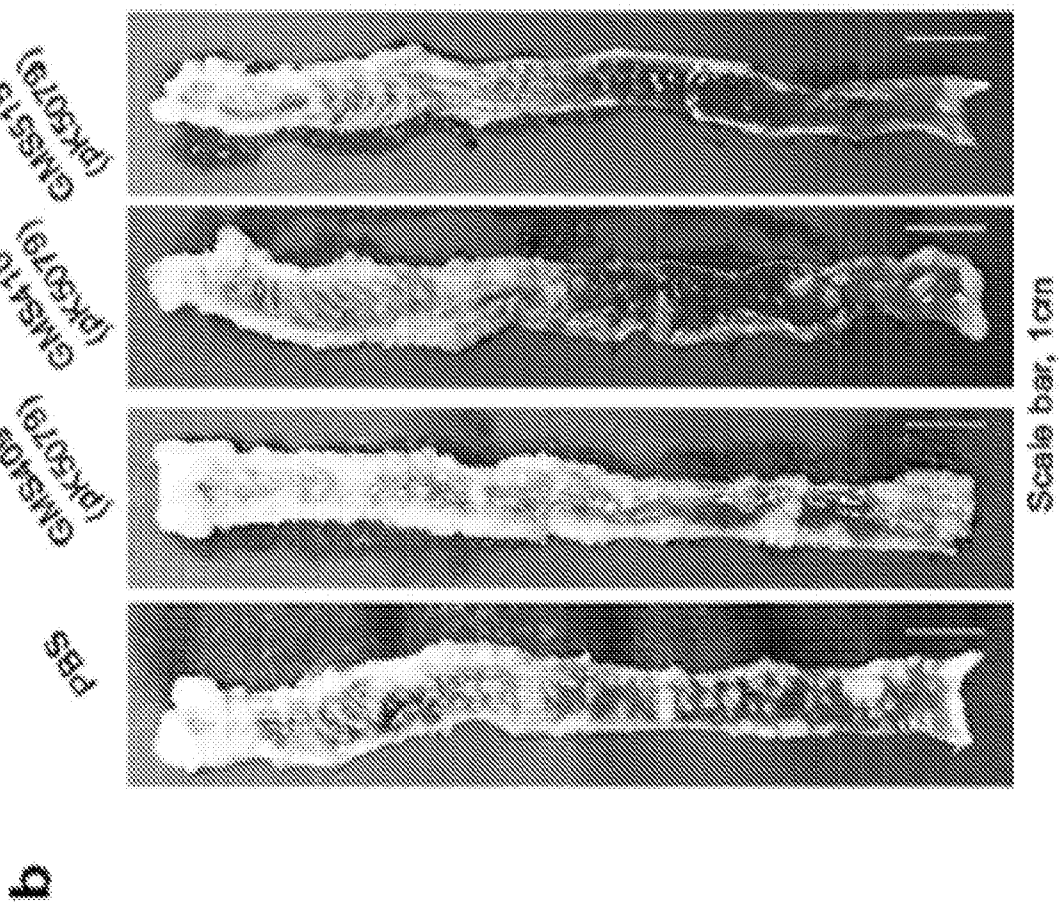

a
Colorectal spontaneous tumor Apc$^{fl/fl}$/CDX2-CRE mouse model – Oral inoculation
Colorectal polypus
Oral inoculation of GMS strain
Apc$^{fl/fl}$/CDX2-CRE mouse
IP of Tamoxifen (Tam)
IP of Tam
OR of 1 x 10^8 CFU GMS 10 days post-IP
Analysis of polypus counts
Euthanize, count polypus, and collect polypus for Immunostaining 10 days post-OR
IP of Tam
OR of 1 x 10^8 CFU GMS every 10 days for three treatments beginning 10 days post-IP
Analysis of survival rate
Record the mouse death

FIG. 6B


b
PBS
GMSA03 (pKS078)
GMSA10 (pKS078)
GMSA15 (pKS078)
Scale bar, 1cm

FIGS. 6C-6D

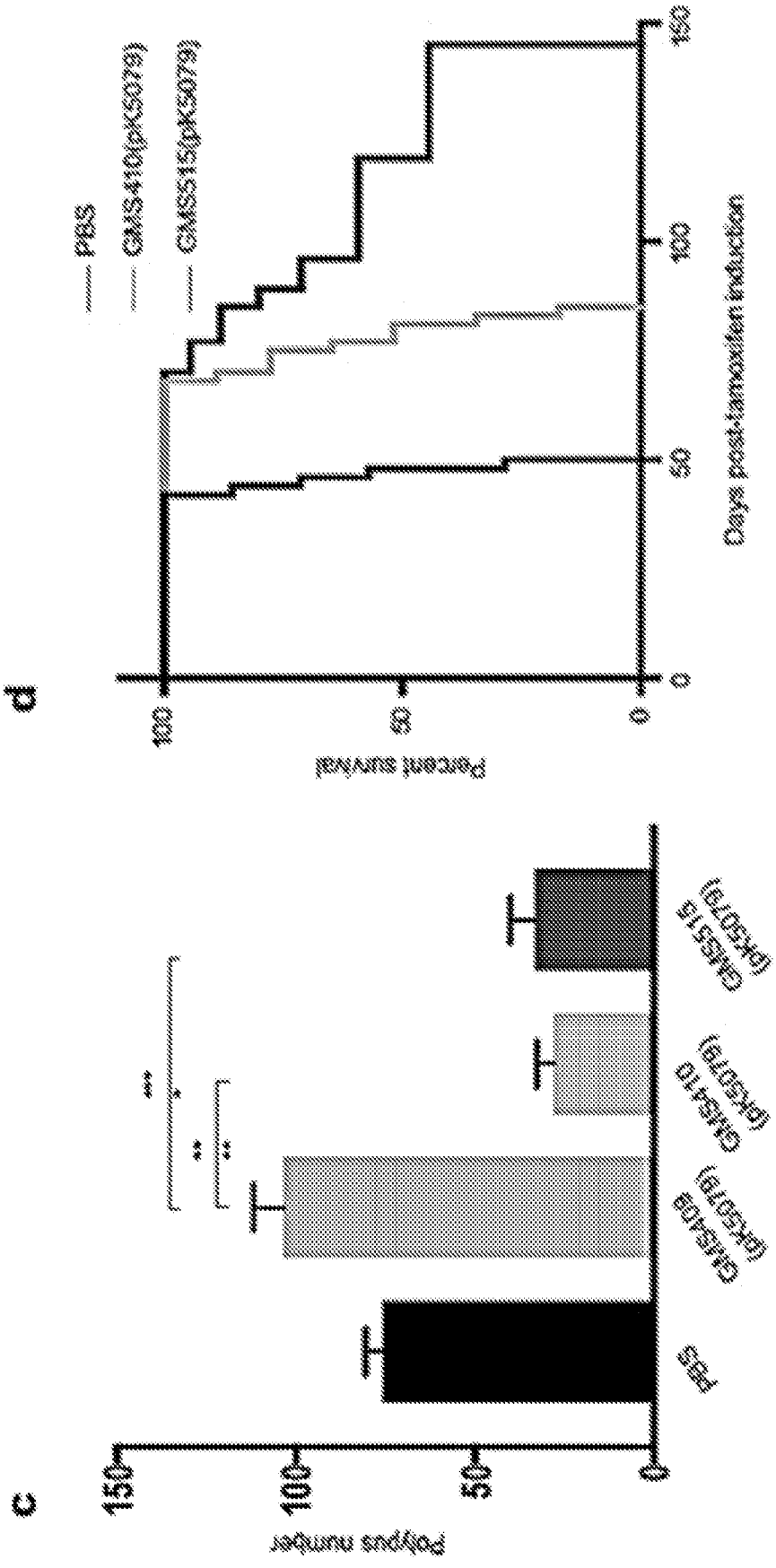

d
PBS
GMS410(pK5079)
GMS515(pK5079)
Percent survival
Days post-tamoxifen induction
c
Polyps number
PBS
GMS409 (pK5079)
GMS410 (pK5079)
GMS515 (pK5079)

FIG. 6E

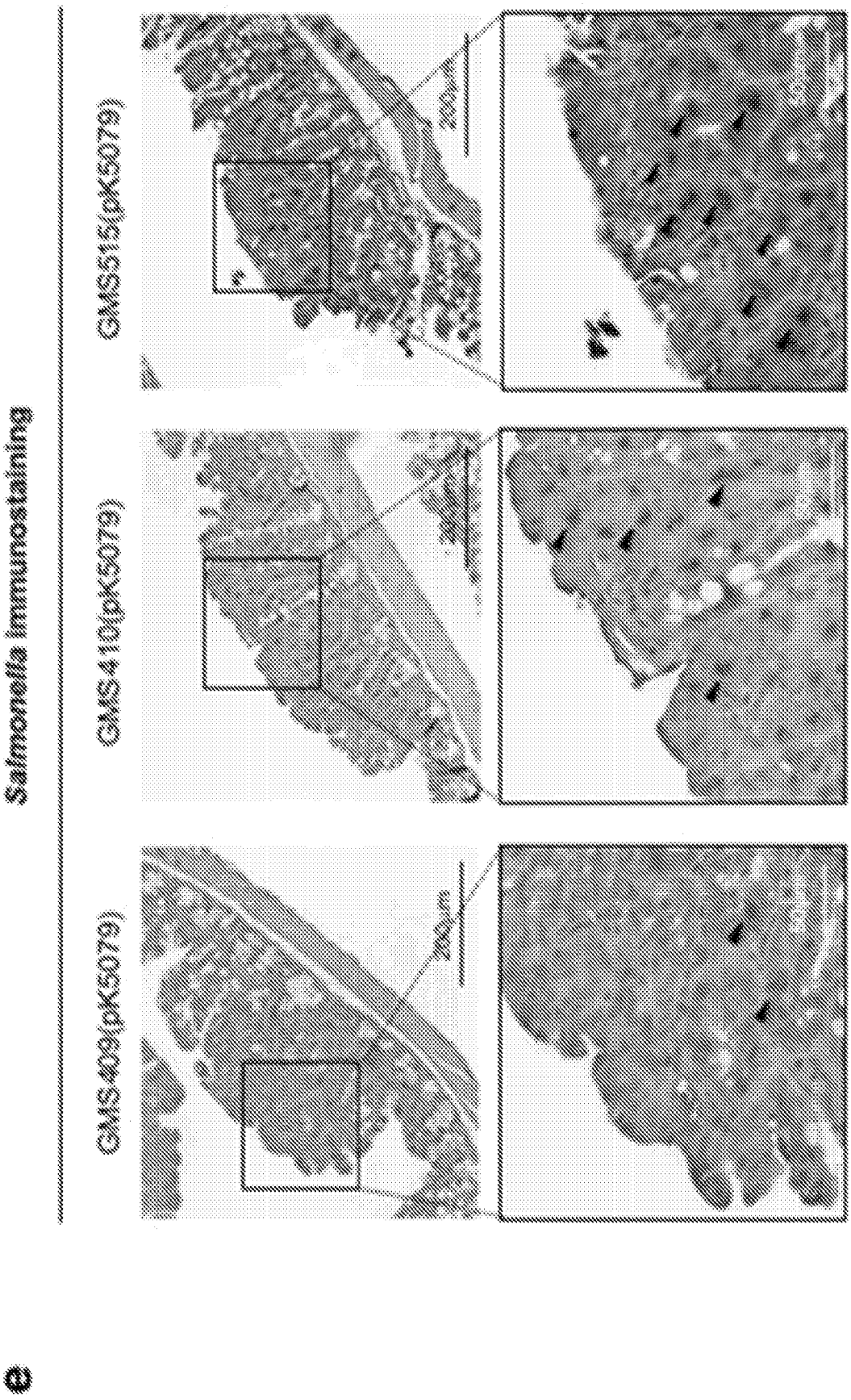

e
Salmonella immunostaining
GMS400(pK5079)
GMS410(pK5079)
GMS515(pK5079)
200μm

FIG. 6F

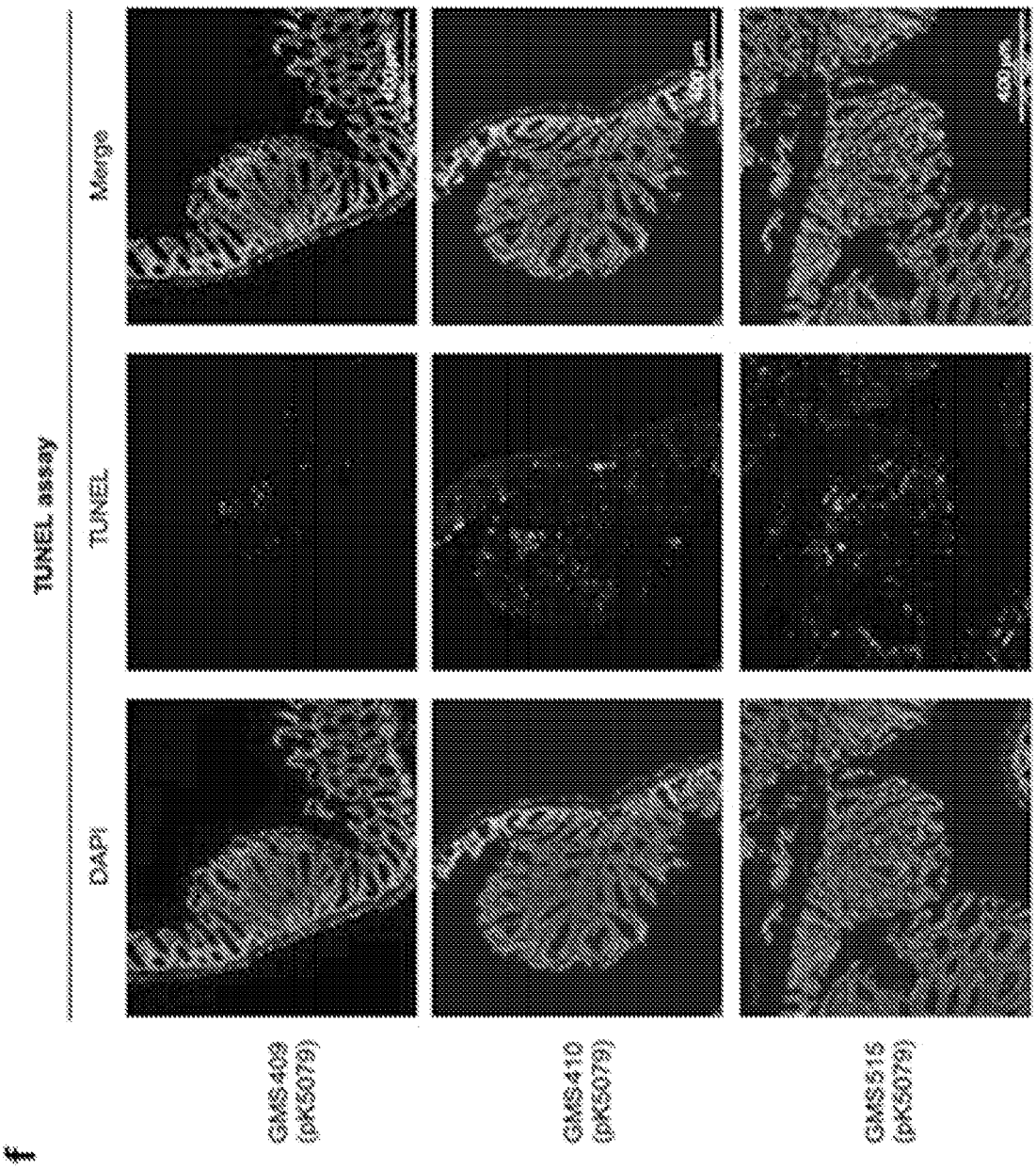

f
TUNEL assay
DAPI
TUNEL
Merge
GMS409 (dK5079)
GMS410 (dK5079)
GMS515 (dK5079)

FIG. 6G

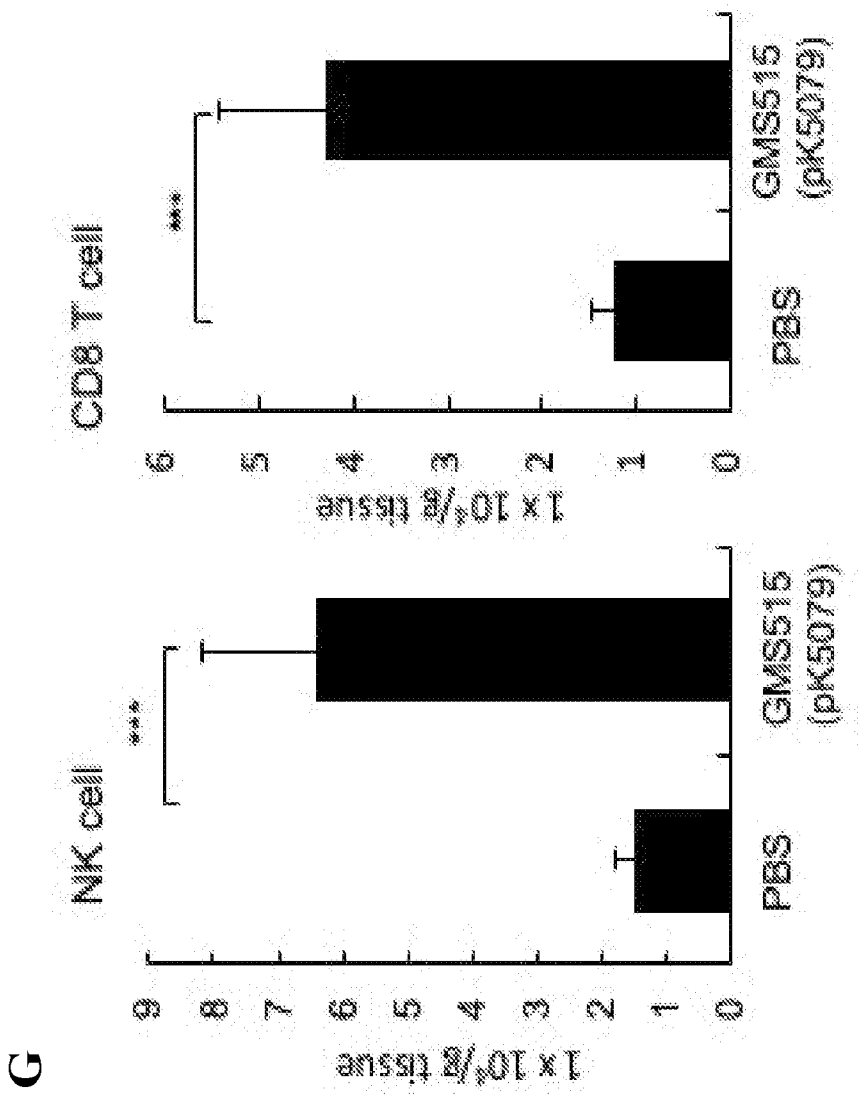

G
NK cell
***
1 x 10⁷/g tissue
0
1
2
3
4
5
6
7
8
9
PBS
GMS515 (pK5079)
CD8 T cell
***
1 x 10⁶/g tissue
0
1
2
3
4
5
6
PBS
GMS515 (pK5079)

FIG. 7A

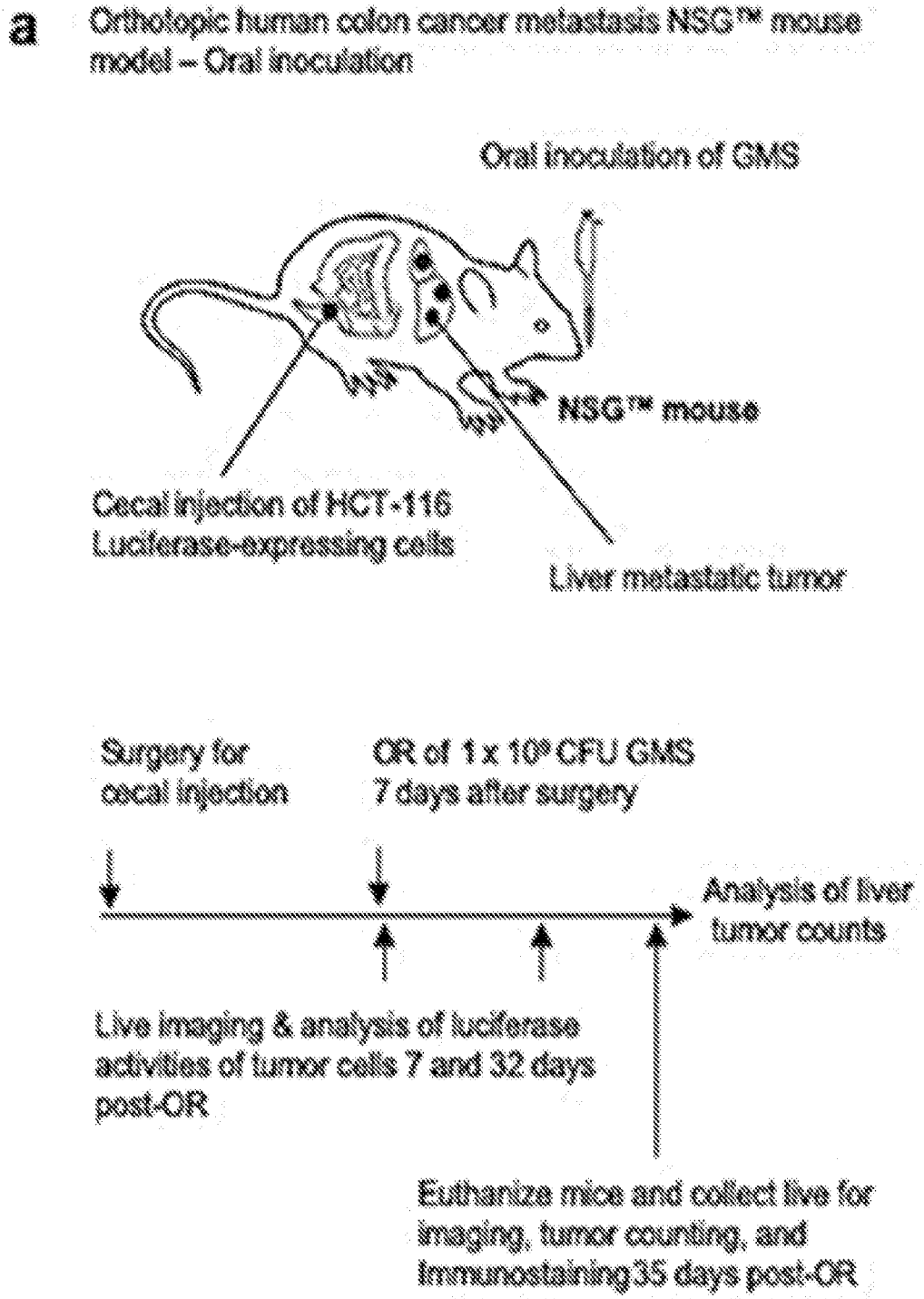

a
Orthotopic human colon cancer metastasis NSG™ mouse model – Oral inoculation
Oral inoculation of GMS
NSG™ mouse
Cecal injection of HCT-116 Luciferase-expressing cells
Liver metastatic tumor
Surgery for cecal injection
OR of 1 x 10⁸ CFU GMS 7 days after surgery
Analysis of liver tumor counts
Live imaging & analysis of luciferase activities of tumor cells 7 and 32 days post-OR
Euthanize mice and collect live for imaging, tumor counting, and Immunostaining35 days post-OR

FIG. 7B

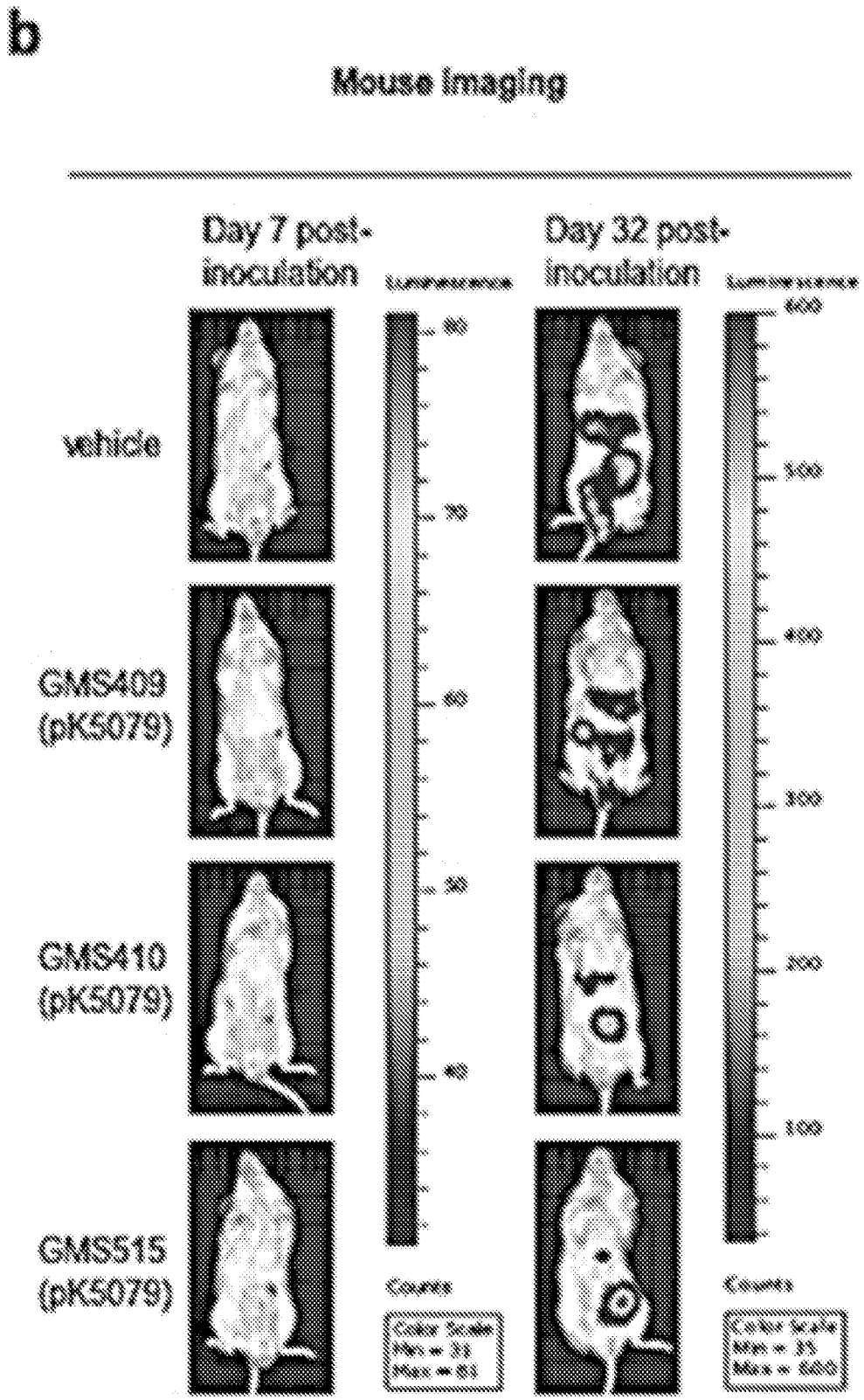

b
Mouse Imaging
Day 7 post-inoculation
Day 32 post-inoculation
vehicle
GMS409 (pK5079)
GMS410 (pK5079)
GMS515 (pK5079)

FIG. 7C

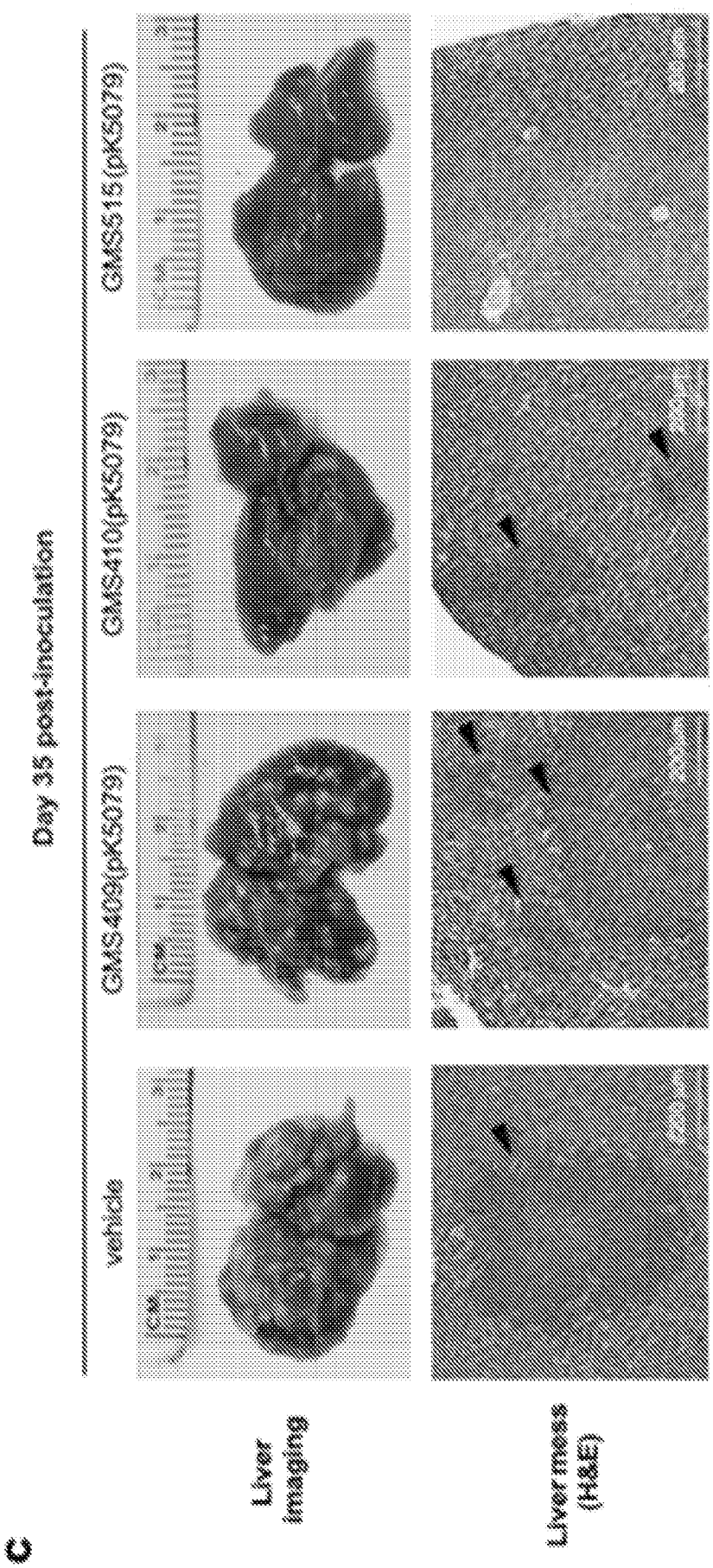
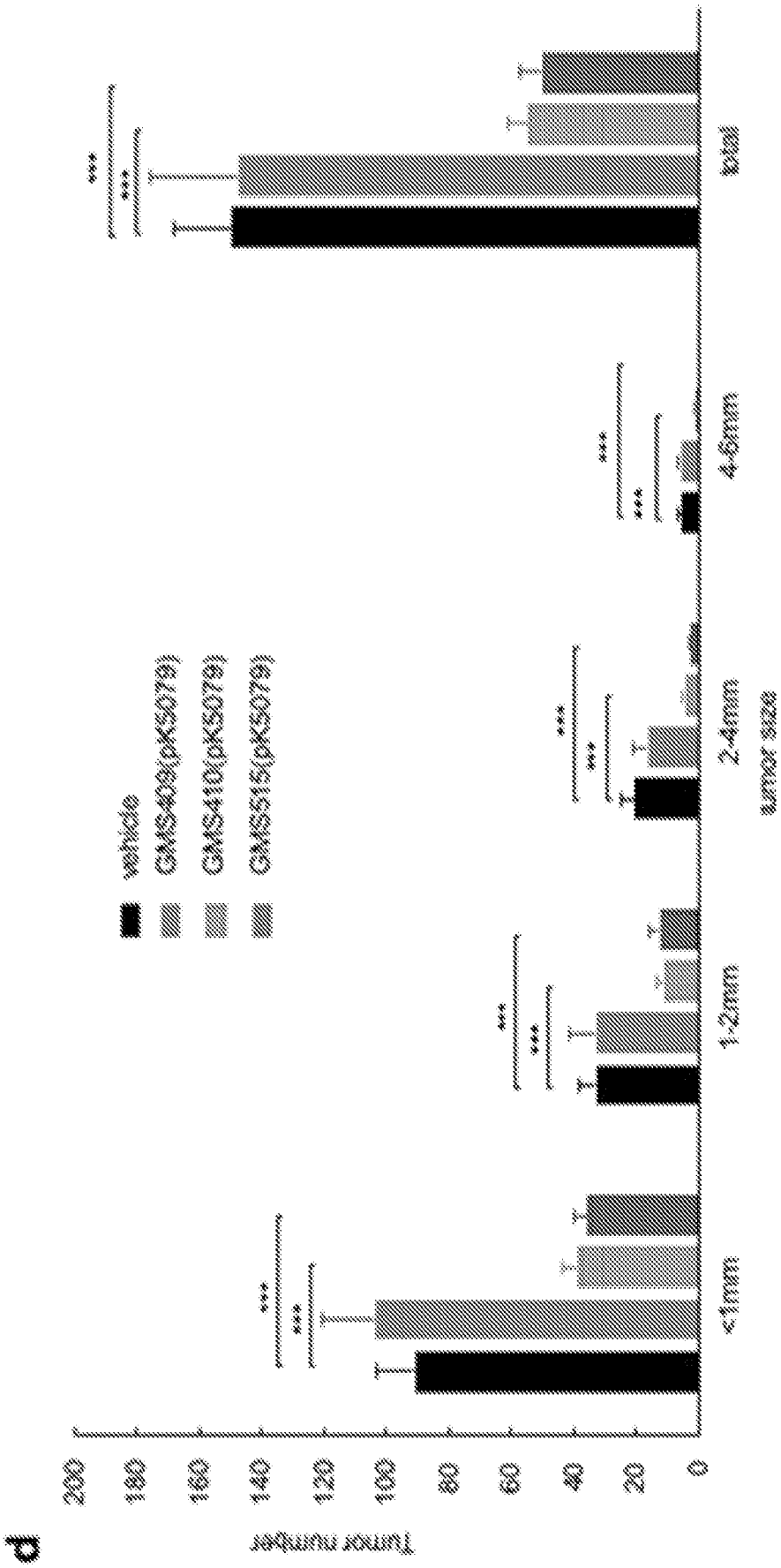
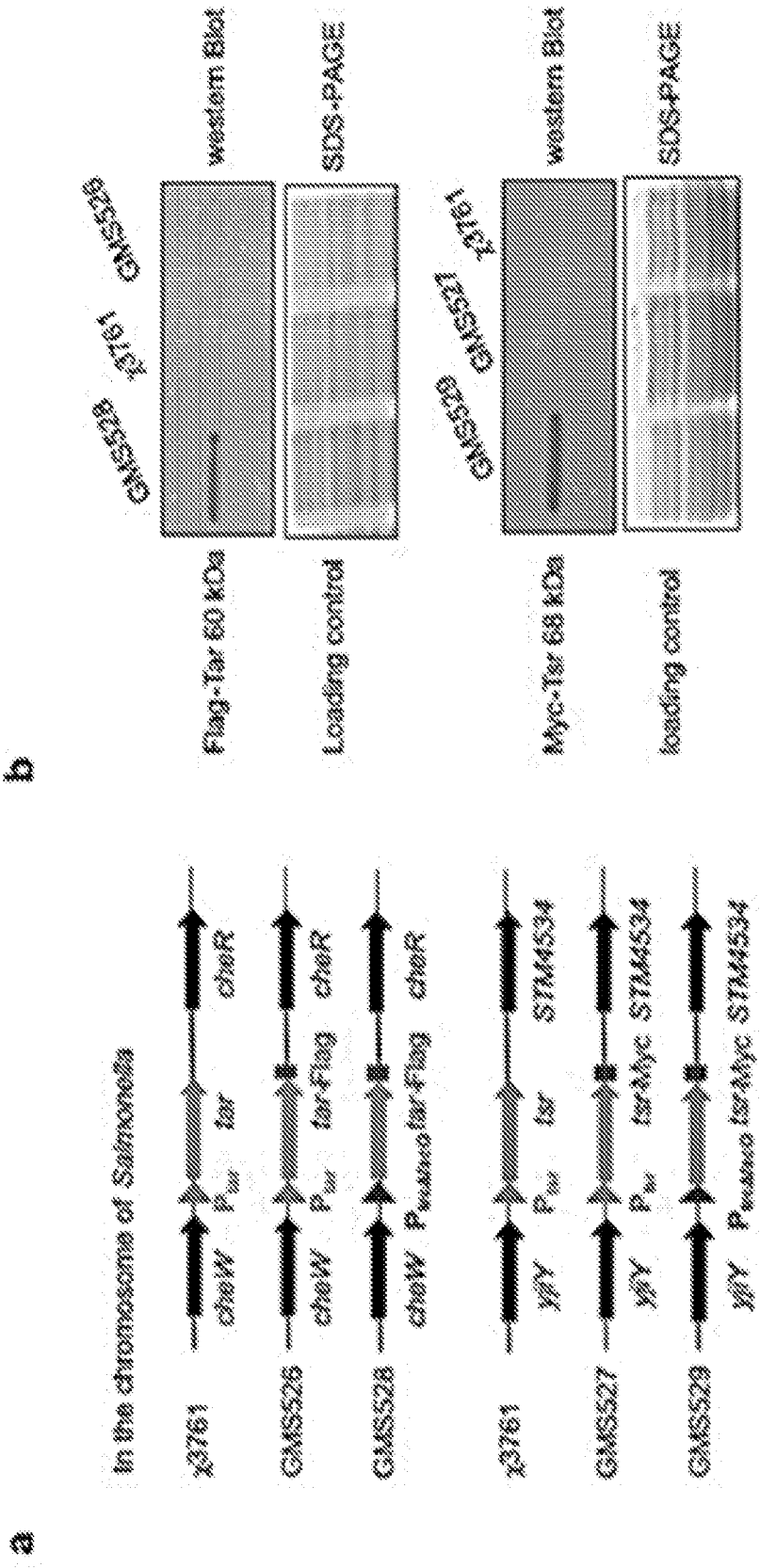

C
Day 35 post-inoculation
vehicle
GMS403(pKS079)
GMS410(pKS079)
GMS515(pKS079)
Liver imaging
Liver mass (H&E)

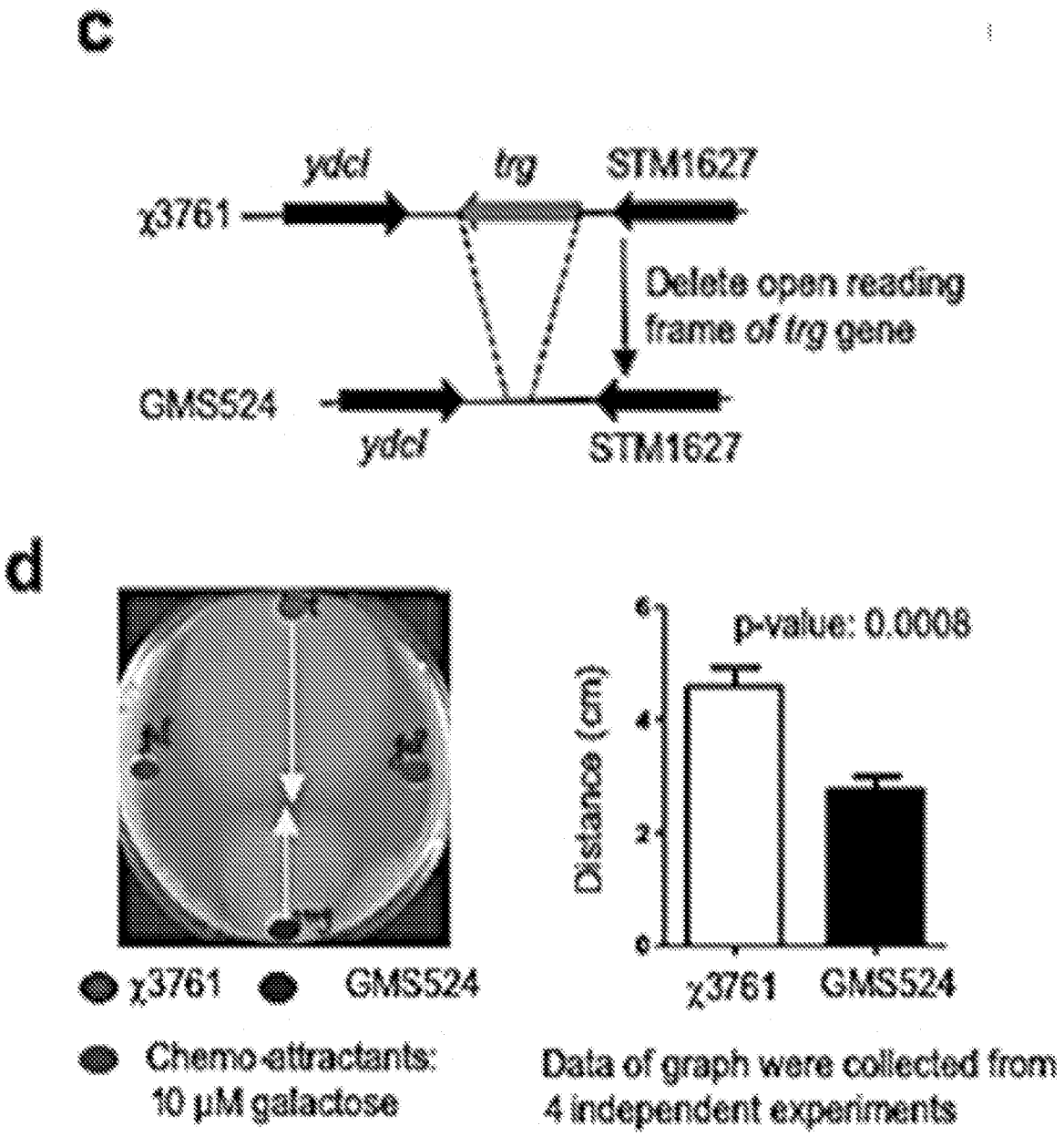
FIG. 7D


D
Tumor number
200
180
160
140
120
100
80
60
40
20
0
vehicle
GMS409(pKS079)
GMS410(pKS079)
GMS515(pKS079)
<1mm
1-2mm
2-4mm
4-6mm
total
tumor size
***

FIGS. 8A-8B


a
In the chromosome of Salmonella
χ3761
cheW
P
tar
cheR
GMS526
tar-Flag
GMS528
P
tar-Flag
χ3761
yjfY
STM4534
GMS527
tsr-Myc
GMS529
tsr-Myc
b
GMS528
χ3761
GMS526
Flag-Tar 60 kDa
Loading control
western Blot
SDS-PAGE
GMS529
GMS527
Myc-Tar 68 kDa
loading control

FIGS. 8C-8D


c
ydcI
trg
STM1627
χ3761
Delete open reading frame of trg gene
GMS524
ydcI
STM1627
d
χ3761
GMS524
Chemo-attractants: 10 μM galactose
p-value: 0.0008
Distance (cm)
χ3761
GMS524
Data of graph were collected from 4 independent experiments

FIGS. 9A-9B

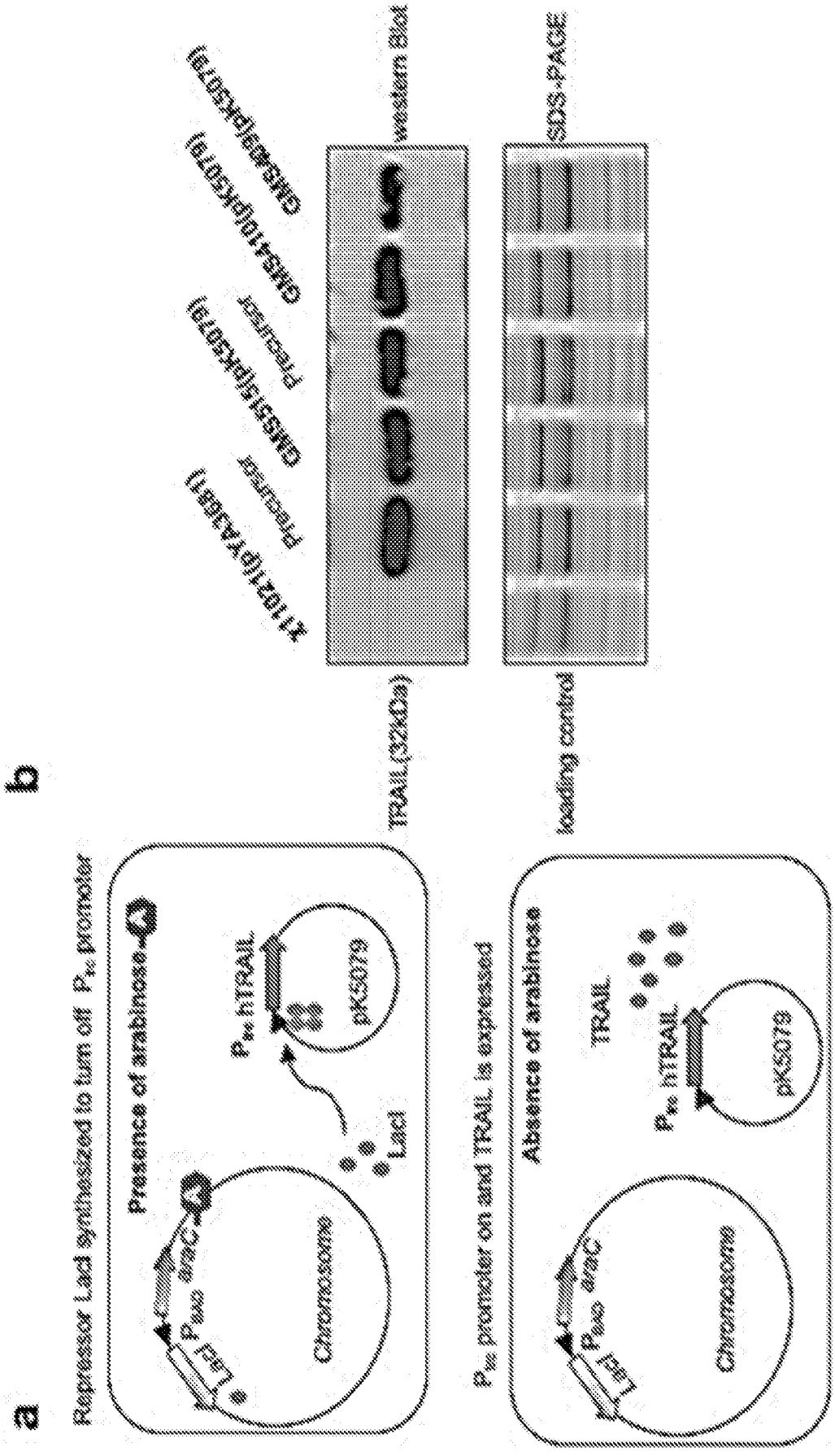

a
Repressor LacI synthesized to turn off P_{trc} promoter
Presence of arabinose
P_{BAD} araC
pBR
Chromosome
LacI
P_{trc} hTRAIL
pK5079
P_{trc} promoter on and TRAIL is expressed
Absence of arabinose
TRAIL
b
TRAIL(32kDa)
loading control
Western Blot
SDS-PAGE
1 (pBAD33)
2 (pK5079)
3 (pK5079)
4 (pK5079)

FIG. 9C

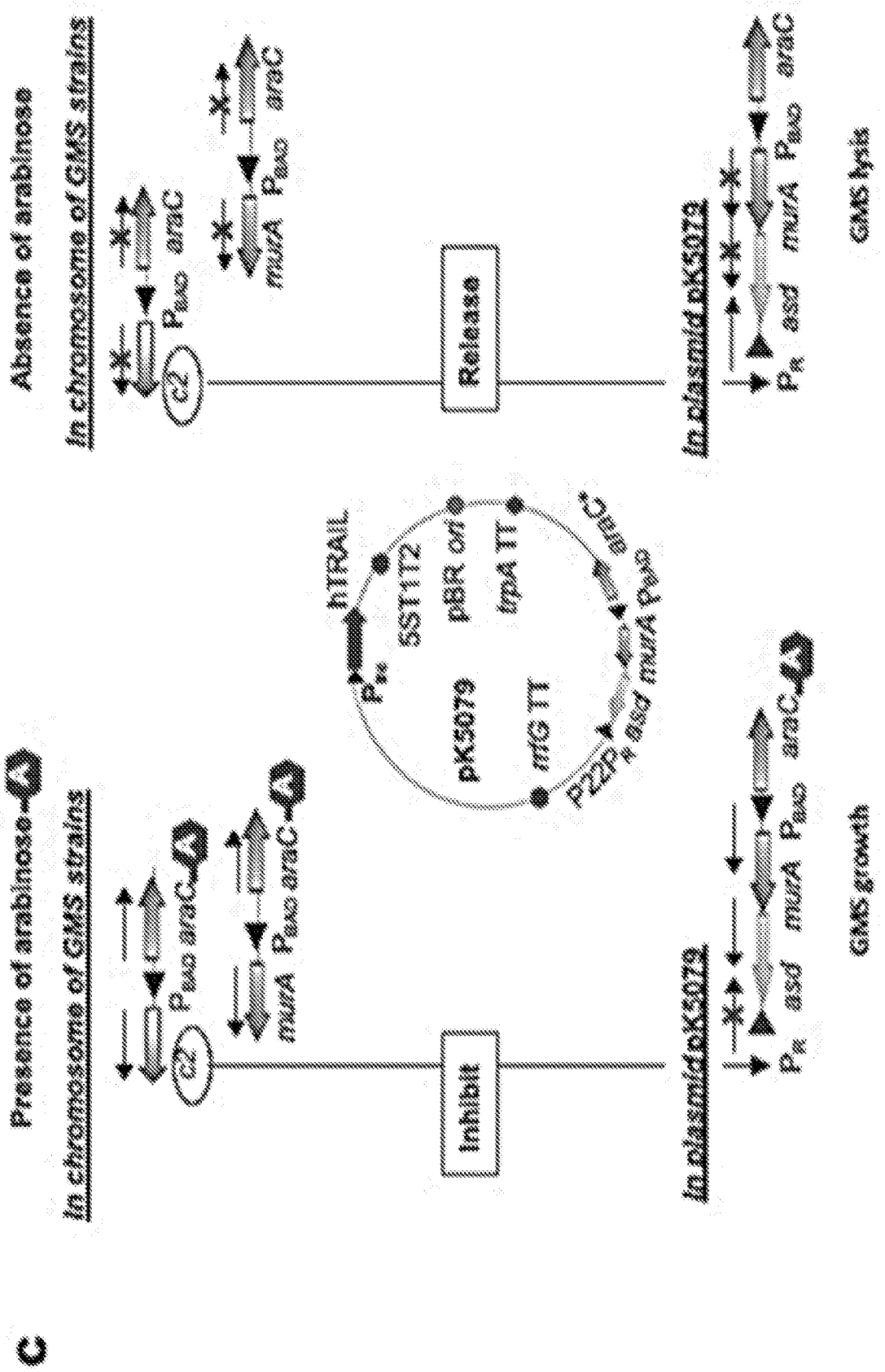
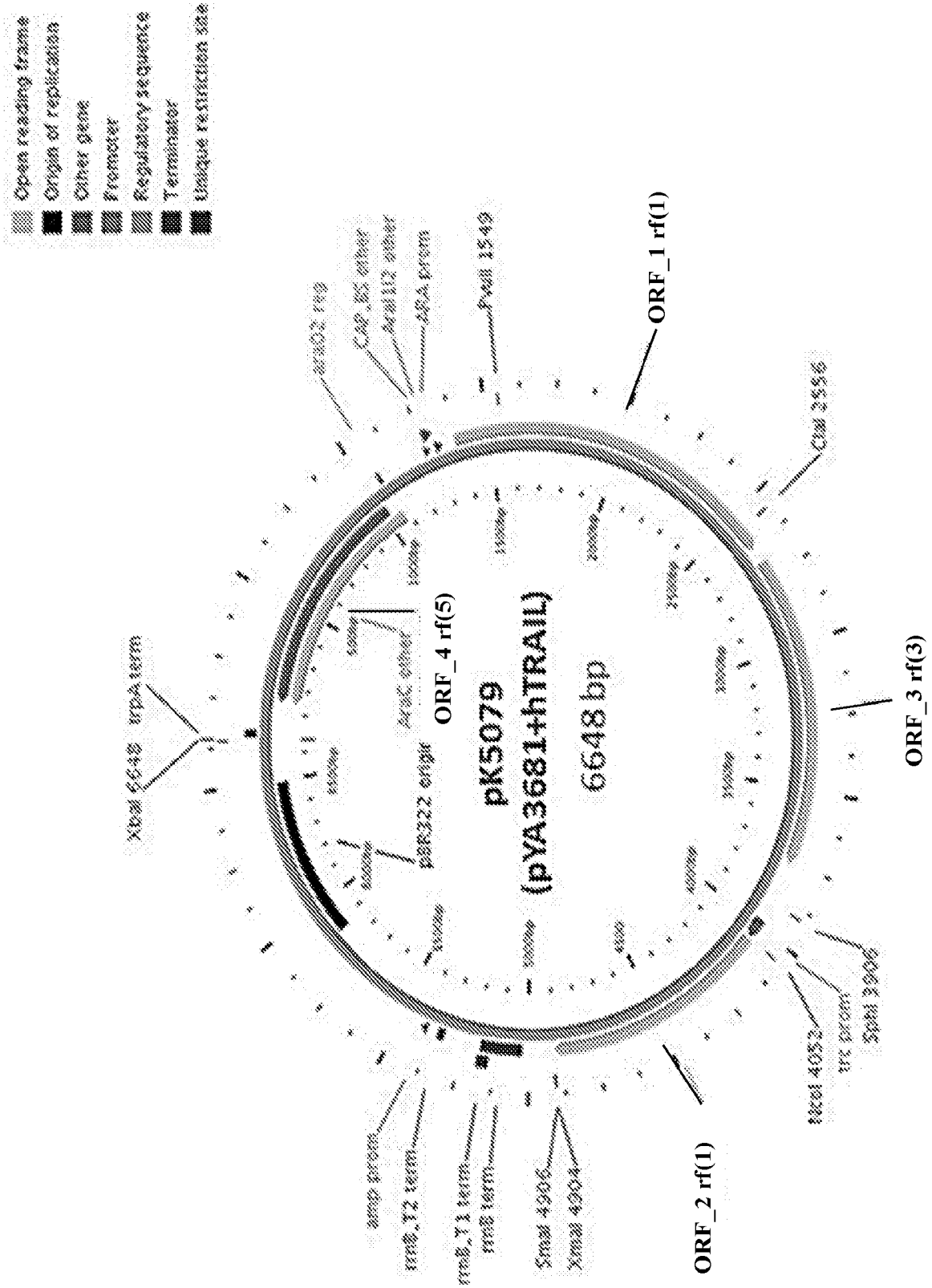

C
Presence of arabinose
In chromosome of GMS strains
Absence of arabinose
In chromosome of GMS strains
Inhibit
Release
In plasmid pK5079
In plasmid pK5079
GMS growth
GMS lysis
hTRAIL
SST1T2
pBR ori
tmA TT
mG TT
pK5079
araC
murA
asd

FIG. 10


Open reading frame
Origin of replication
Other gene
Promoter
Regulatory sequence
Terminator
Unique restriction site
pK5079
(pYA3681+hTRAIL)
6648 bp
ORF_1 rf(1)
ORF_2 rf(1)
ORF_3 rf(3)
ORF_4 rf(5)
XbaI 6648
trpA term
pBR322 origin
AraC ether
araC rep
CAP_ES ether
AraBAD ether
ara prom
PvuII 1549
Cat 3556
ttrcI 4052
trc prom
SphI 3906
amp prom
rrnB_T2 term
rrnB_T1 term
rrnB term
SmaI 4906
XmaI 4904

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/019111

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/74; C07K 14/705; C12N 15/74 (2020.01)
CPC - C07K 14/70575; C07K 14/70578; C12N 15/74; C12R 1/42 (2020.02)

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; 435/69.5 (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	1-3, 5-8, 10-15
Y	US 5,763,223 A (WILEY et al) 09 June 1998 (09.06.1998) entire document	1-3, 5-8, 10-15
Y	US 2017/0333493 A1 (OHIO STATE INNOVATION FOUNDATION) 23 November 2017 (23.11.2017) entire docum	6-8, 10
A	US 2009/0169517 A1 (BERMUDES et al) 02 July 2009 (02.07.2009) entire document	1-15
A	US 2005/0244374 A1 (GOEBEL et al) 03 November 2005 (03.11.2005) entire document	1-15
A	WO 2017/123675 A8 (SYNLOGIC, INC.) 24 August 2017 (24.08.2017) entire document	1-15

☐ 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	Date of mailing of the international search report
28 April 2020	15 MAY 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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/019111

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*.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. ☐ 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:7 was searched.