

DESCRIPTION

ENGINEERED SHIGELLA BACTERIA TO TARGET CANCER CELLS

BACKGROUND

5 [0001] This application claims the benefit of United States Provisional Patent Application No. 63/515,758, filed July 26, 2023, the entirety of which is incorporated herein by reference.

1. Field

[0002] The present invention relates generally to the field of molecular biology and medicine. More particularly, it concerns engineered bacteria that can selectively target or infect
10 cancerous cells such as glioblastoma.

2. Description of Related Art

[0003] Select microorganism-based drug delivery systems, such as viral delivery systems, can be used to target or treat solid tumors.[1-4] These approaches typically utilize a virus, such as a herpes simplex virus, to directly lyse malignant cells and/or edit the cancer
15 genome through gene therapy.[4-11]

[0004] Glioblastoma presents particular challenges for targeting with a microorganism or virus. Previous studies have used microorganisms as delivery vehicles for GBM and other brain tumors.[1-8,10,11,36] Most studies, including those advancing to clinical trials, have focused on using a virus to deliver a therapeutic to the tumor.[4] While these approaches may
20 have possible advantages over traditional chemotherapy, using a virus as a therapeutic factory presents challenges. Namely, most viruses have limited specificity for GBM cells, small cassette sizes, and safety concerns.[37]

[0005] While the use of microorganisms as drug delivery systems to treat cancer has expanded recently, including FDA approval of certain viruses as oncolytics, significant
25 limitations remain. For example, current microbial delivery systems such as AAV9 and herpes virus have limited cassette sizes, minimal cancer cell selectivity, and low innate cytotoxicity, which have limited the use of this class of therapeutics.[11] Although microorganisms have several unique benefits compared to traditional pharmacologic agents including dose independence, the ability to produce therapeutic proteins locally within the tumor, and

simplicity of administration, a limited number of bacteria have been generated that can selectively infect cancerous cells. Clearly, there is a need for new and improved methods for targeting cancerous cells with microorganisms.

SUMMARY

5 [0006] The present disclosure overcomes limitations in the prior art by providing engineered *Shigella* bacteria (*e.g.*, *Shigella flexneri*) that can be used to selectively infect or be internalized into cancerous cells including brain cancer cells (*e.g.*, glioblastoma). The *Shigella* can be used to treat a cancer, such as a brain cancer, or deliver a therapeutic gene to cancerous cells. Bacterial-based drug delivery systems, based on *Shigella flexneri*, that can selectively
10 internalize into glioblastoma (GBM) brain tumor cells are provided. As shown in the below examples, *S. flexneri* were engineered to selectively internalize into GBM cells using iterative co-cultured assays. After 50 rounds of co-culture, the engineered strain infected 95 percent of GBM cells in about 2 hours. GBM-engineered *Shigella* demonstrated greater than a 123-fold preference for internalizing in nine different GBM cell lines compared to Normal Astrocytes
15 (NA) controls. Additionally, an in-cell western was developed to identify GBM-engineered *Shigella* clones that preferentially internalize in cancer cells from patient samples without iterative co-culture. Without wishing to be bound by any theory, data supports the idea that internalization into GBM cells is mediated via a factor modified by myristoylation. Preferably the engineered *Shigella* has functional MsbB1 and MsbB2 genes. The engineered *Shigella*
20 may preferentially internalize in brain tumor cells such as GBM. This system provides benefits over current interventions and other microbial strategies for treating brain tumors such as including high selectivity, vastly expanded cassette space, and improvements to the safety profile. As shown in the below examples, co-administration of GBM-infecting *Shigella* and gentamicin restricted tumor expansion and growth *in vivo* using a mouse model of GBM (*e.g.*,
25 FIGS. 11-12). Administering an antibiotic (*e.g.*, a lytic antibiotic such as doxycycline) to the subject after administration of GBM-infecting *Shigella* can result in release of DNA into the cytoplasm of the GBM which can result in transcription of a polypeptide from the DNA (FIGS. 13-14); these results demonstrate that the *Shigella* can be genetically modified to deliver a therapeutic nucleic acid or therapeutic polypeptide to the cytoplasm of cancerous cells such as
30 GBM. The therapeutic nucleic acid may encode a therapeutic or an anti-cancer polypeptide that is transcribed in the cytoplasm of GBM cells. The GBM may comprise a nucleic acid encoding polypeptides for gene editing, such as, *e.g.*, one or more CRISPR/CAS9 polypeptides.

For example, the gene editing polypeptides may comprise a Cas9 enzyme and a guide RNA that can be used, *e.g.*, to treat a disease (*e.g.*, selectively kill cancer cells based on alteration of the genome of the cancer cells). The engineered *Shigella* may be administered directly into the brain (*e.g.*, intraventricularly, into the cerebrospinal fluid, etc.) or peripherally (*e.g.*,
5 intravenously) to treat brain cancer cells (*e.g.*, GBM cells) that are located within or outside of, respectively, the central nervous system of a mammalian subject such as a human.

[0007] An aspect of the present disclosure relates to an engineered *Shigella flexneri* bacterium, wherein the bacterium does not produce a functional Shiga toxin 1 (stx1) or Shiga toxin 2 (stx2), wherein the bacterium comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more
10 mutations from Table 1, and wherein the bacterium selectively internalizes into glioblastoma cells as compared to normal astrocytes or neurons. The bacterium preferably comprises functional MsbB1 and MsbB2 genes. In some embodiments, the genetic background of the bacterium is *S. flexneri* strain 2475 Serotype 2a. In some embodiments, the bacterium does not internalize into normal brain cells or the bacterium preferentially internalizes into cancerous
15 cells as compared to normal brain cells. In some embodiments, the bacterium does not induce an innate immune response in a mammalian subject, preferably a human. In some embodiments, the bacterium does not engage TLR4. The bacterium may further comprise a nucleic acid encoding a recombinant protein under the control of a bacterial promoter (*e.g.*, a constitutive promoter or a hypoxia-inducible promoter). The promoter may be a eukaryotic
20 promoter (*e.g.*, a human promoter, a promoter that is active in human cells, and/or a promoter that is active in GBM cells). The recombinant protein may be under the control of a eukaryotic promoter or a promoter that is active in GBM cells; for example, the recombinant protein may be a therapeutic protein or an anticancer polypeptide (*e.g.*, that may be transcribed from the nucleic acid in GBM cells after lysis of the engineered *Shigella flexneri* and release of the
25 nucleic acid into the cytoplasm of GBM cells). The recombinant protein may be an immunomodulator, a toxin, a cytotoxic factor, a TNF cytokine family member, an anti-angiogenic factor, a cytotoxic polypeptide, or a cytotoxic peptide. The bacterium may further comprise a kill switch. The bacterium may be sensitive to beta-lactams, quinolones, or tetracyclines.

30 [0008] Another aspect of the present disclosure relates to a composition comprising the bacterium described above or herein.

[0009] Yet another aspect of the present disclosure relates to a pharmaceutical formulation comprising the bacterium described above or herein. The pharmaceutical formulation may be formulated for intravenous, intracranial, intratumoral, intrathecal, or intraventricular administration.

5 [0010] Another aspect of the present disclosure relates to a method of treating a cancer in a mammalian subject, the method comprising administering a therapeutically effective amount of the pharmaceutical formulation described above or herein to the subject. The cancer may be a brain cancer (*e.g.*, a glioblastoma, glioma, meningioma, oligodendroglioma, astrocytoma, medulloblastoma, craniopharyngioma, or ependymoma). In some preferred
10 embodiments, the brain cancer is a glioblastoma. The method may further comprise administering a second anti-cancer therapy to the subject. The second anti-cancer therapy may be a chemotherapeutic agent, immunotherapy, radiotherapy, or cytotoxic factor. The method may further comprise administering an antibiotic to the subject (*e.g.*, doxycycline, ampicillin, ciprofloxacin, or gentamicin). The mammalian subject may be a human.

15 [0011] Yet another aspect of the present disclosure relates to a panel or population of *Shigella flexneri* clones, wherein each clone (i) does not produce a functional Shiga toxin 1 (stx1) or Shiga toxin 2 (stx2) and (ii) selectively internalizes into glioblastoma cells as compared to normal astrocytes. The clones may preferably comprise functional MsbB1 and MsbB2 genes. In some embodiments, each of the clones comprises, independently, at least 2,
20 3, 4, 5, 6, 7, 8, 9, 10 or more mutations to genes from Table 1. In some preferred embodiments, the genetic background of the clones is *S. flexneri* strain 2475 Serotype 2a. In some embodiments, the clones do not internalize into normal brain cells. In some embodiments, the clones do not induce an innate immune response in a mammalian subject, preferably a human. In some embodiments, the clones do not engage TLR4. The clones may further comprise a
25 nucleic acid encoding a recombinant protein under the control of a bacterial promoter or a mammalian promoter; for example, antibiotic mediated lysis of the *Shigella flexneri* clones can be used to deliver the nucleic acid to the cytosol of mammalian cells, and the promoter may enable expression in the mammalian cells of the recombinant protein. The promoter may be a constitutive promoter, a quorum sensing promoter, or a hypoxia-inducible promoter. The
30 recombinant protein may be an immunomodulator, a toxin, a cytotoxic factor, a TNF cytokine family member, an anti-angiogenic factor, a cytotoxic polypeptide, or a cytotoxic peptide. The

clones may further comprise a kill switch. In some embodiments, the clones are sensitive to one or more beta-lactams, quinolones, or tetracyclines.

[0012] Another aspect of the present disclosure relates to a method of identifying an engineered *Shigella flexneri* clone for use in treating a patient's cancer, the method comprising

5 (i) incubating each of at least two cultures of cells from the patient's cancer with a single engineered *Shigella flexneri* clone selected from a panel of *Shigella flexneri* clones to allow internalization of the *Shigella flexneri*; (ii) determining the number of *Shigella flexneri* internalized into the cells of each of the at least two cultures of cells; and (iii) selecting an engineered *Shigella flexneri* clone from a culture determined to have a number of internalized

10 *Shigella flexneri* that is at least 50%, at least 60%, at least 70%, at least 80%, or least 90% of the highest number of internalized *Shigella flexneri* from among the at least two cultures of cells. In some embodiments, the cells from the patient's cancer were obtained from a resection sample. In some embodiments, the cells from the patient's cancer have been passaged *in vitro*. In some embodiments, the cells from the patient's cancer have not been passaged *in vitro*. The

15 panel of *Shigella flexneri* clones may be the panel of bacterium described above or herein. In some embodiments, the number of cultures of cells tested equals the number of *Shigella flexneri* clones in the panel. The method may further comprise (a) incubating normal astrocytes with each engineered *Shigella flexneri* clone selected from the panel of *Shigella flexneri* clones to allow internalization of the *Shigella flexneri*; (b) determining the number of each *Shigella*

20 *flexneri* clone internalized into the normal astrocytes; and (c) selecting an engineered *Shigella flexneri* clone for which no internalization into normal astrocytes was detected. The method may further comprise (iv) administering the selected engineered *Shigella flexneri* clone to the patient. The cancer may be a brain cancer (*e.g.*, a glioblastoma, glioma, meningioma, oligodendroglioma, astrocytoma, medulloblastoma, craniopharyngioma, or ependymoma). In

25 some preferred embodiments, the brain cancer is a glioblastoma. The method may further comprise administering a second anti-cancer therapy to the subject. The second anti-cancer therapy may be a chemotherapeutic agent, immunotherapy, radiotherapy, or cytotoxic factor.

[0013] Yet another aspect of the present disclosure relates to a method of generating engineered *Shigella flexneri* for use in treating a patient's cancer, the method comprising (i)

30 incubating a first culture of cells from the patient's cancer with an engineered *Shigella flexneri* clone to allow internalization of the *Shigella flexneri*; (ii) harvesting the internalized *Shigella flexneri*; (iii) incubating a second culture of cells from the patient's cancer with the *Shigella*

flexneri harvested in step (ii) to allow internalization of the *Shigella flexneri*; (iv) repeating steps (i) to (iii) until at least 95% of cells in the culture are infected with more than one *Shigella flexneri*. In some embodiments, The cells from the patient's cancer were obtained from a resection sample. In some embodiments, the cells from the patient's cancer have been passaged

5 *in vitro*. In some embodiments, steps (i) to (iii) are repeated at least 30 times, at least 35 times, at least 40 times, at least 45 times, or at least 50 times. The method may further comprising (v) generating a panel of single engineered *Shigella flexneri* clones from the *Shigella flexneri* harvested after step (iv); (vi) incubating each of at least two cultures of cells from the patient's cancer with single engineered *Shigella flexneri* clones selected from the panel of *Shigella*

10 *flexneri* clones to allow internalization of the *Shigella flexneri*; (vii) determining the number of *Shigella flexneri* internalized into the cells of each of the at least two cultures of cells; and (viii) selecting an engineered *Shigella flexneri* clone from a culture determined to have a number of internalized *Shigella flexneri* that is at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the highest number of internalized *Shigella flexneri* from among the at least two

15 cultures of cells. In some embodiments, the number of cultures of cells tested equals the number of *Shigella flexneri* clones in the panel. The method may further comprise (a) incubating normal astrocytes with each engineered *Shigella flexneri* clone selected from the panel of *Shigella flexneri* clones to allow internalization of the *Shigella flexneri*; (b) determining the number of each *Shigella flexneri* clone internalized into the normal astrocytes;

20 and (c) selecting an engineered *Shigella flexneri* clone for which no internalization into normal astrocytes was detected. The method may further comprise administering the selected engineered *Shigella flexneri* clone to the patient. The cancer may be a brain cancer (*e.g.*, a glioblastoma, glioma, meningioma, oligodendroglioma, astrocytoma, medulloblastoma, craniopharyngioma, or ependymoma). In some preferred embodiments, the brain cancer is a

25 glioblastoma. The method may further comprise administering a second anti-cancer therapy to the subject. The second anti-cancer therapy may be a chemotherapeutic agent, immunotherapy, radiotherapy, or cytotoxic factor.

[0014] Another aspect of the present disclosure relates to a panel or population of *Shigella flexneri* clones described above or herein. In some embodiments, each clone (i) does

30 not produce a functional Shiga toxin 1 (stx1) or Shiga toxin 2 (stx2) and (ii) selectively internalizes into glioblastoma cells as compared to normal astrocytes. The clones may preferably comprise functional MsbB1 and MsbB2 genes. In some embodiments, each of the clones comprises, independently, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more mutations from genes

of Table 1. The genetic background of the clones may be *S. flexneri* strain 2475 Serotype 2a. In some embodiments, the clones do not internalize into normal brain cells. In some embodiments, the clones do not induce an innate immune response in a human. In some embodiments, the clones do not engage TLR4. The clones may further comprise a nucleic acid
5 encoding a recombinant protein under the control of a bacterial promoter (*e.g.*, a constitutive promoter, a quorum sensing promoter, or a hypoxia-inducible promoter) or a eukaryotic promoter. The recombinant protein may be an immunomodulator, a toxin, a cytotoxic factor, a TNF cytokine family member, an anti-angiogenic factor, a cytotoxic polypeptide, or a cytotoxic peptide. The clones may further comprise a kill switch. The clones may be sensitive
10 to one or more beta-lactams, quinolones, or tetracyclines.

[0015] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within
15 the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better
20 understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0017] **FIGS. 1A-B: Scheme to generate GBM-engineering *Shigella* and verification of safety.** (FIG. 1A) Scheme to select *S. flexneri* that preferentially internalize into Glioblastomas. (FIG. 1B) We sequenced the genome of GBM-engineered *Shigella* to
25 identify mutations that arose during engineering. These mutations were located inside 46 genes. Seventeen of the effected genes are related to metabolism/homeostasis, 13 related to DNA regulation, 7 related to transcriptional regulation, and 6 related to stress response.

[0018] **FIGS. 2A-D: Engineering *Shigella* to internalize into a GBM cell line.** *Shigella* is incubated in U-251 cells *in vitro*. Internalized *Shigella* are isolated and amplified.
30 This process is repeated fifty times, indicated by round number. (FIG. 2A) Fluorescent microscopy of internalized *Shigella* in U-251 cells. *Shigella* was incubated with U-251 GBM

cells, fixed, and stained with Hoechst 33342 for DNA (Blue) and Wheat Germ Agglutinin (WGA) for cell membranes (Green). White arrows indicate individual *Shigella*. The scale bar indicates 20 μ m. **(FIG. 2B)** The percentage of U-251 cells with internalized *Shigella* identified via microscopy is plotted over fifty rounds of co-culture with a minimum of three fields counted per round. **(FIG. 2C)** The mean number of *Shigella* rods per U-251 cell, with at least one *Shigella* rod inside the cell membrane, is plotted over fifty rounds of internalization. Three fields are counted for each round. **(FIG. 2D)** GBM-engineered *Shigella* were streaked, individual colonies expanded, and single colonies incubated with GSC-112 GBM cells. After incubation, the amount of internalized *Shigella* is quantified using an in-cell western. The number of *Shigella* internalized into GSC-112 and NA is plotted for 96 individual GBM-engineered *Shigella* clones. Clones exhibiting below detectable levels of the bacterial signal are represented as zero.

[0019] FIGS. 3A-C: GBM-engineered *Shigella* retain *S. Flexneri* phenotype (FIG. 3A) GBM-engineered *Shigella* or control are incubated with U-251 GBM cells. Gentamycin and washing eliminate non-internalized bacteria. Cells are fixed and stained with Hoechst 33342 for DNA (Blue), Phalloidin for F-Actin (Red), anti-*Shigella* antibody (ab65282), and counterstained with alexa-fluor 488 (green) to visualize *Shigella*. Scale bars indicate 20 μ m, and white arrows highlight *Shigella*. **(FIG. 3B)** The average signal intensity of *Shigella* fluorescence is quantified and plotted (* = $p < 0.05$ by ANOVA, $n = 3$ individual fields/group). **(FIG. 3C)** *Shigella* isolated from GBM cells are plated on TSB + congo red agar plates. Representative plates are shown. Red colonies demonstrate that GBM-engineered *Shigella* can still process congo red dye indicating retention of virulence plasmid

[0020] FIGS. 4A-C: GBM-engineered *Shigella* preferential internalization into GBM cells compared to normal astrocyte controls. Internalized GBM-engineered *Shigella* is quantified in a panel of GBM cell lines and two unique Normal Astrocyte (NA) cell preparations. **(FIG. 4A)** GBM-engineered *Shigella* are incubated with either GBM or Normal Astrocyte cells. Internalized *Shigella* is recovered and plated on TSB + congo Red plates to quantify internalized bacteria. Representative images are demonstrated in panel A. Dilutions depicted for NA and U-251 cells is 1/40. All GSC cell lines are diluted 1/200. **(FIG. 4B)** *Shigella* recovered from each cell line is plotted and compared to normal astrocytes (* = $p < 0.001$ via multi-comparison corrected ANOVA). **(FIG. 4C)** The fold change of internalization for each GBM cell line compared to NA is plotted. The average fold change

over all GBM cell lines was 123 ± 21.2 (standard error of the mean), and GSC 115 displayed the greatest fold change of 322 ± 34.3 .

[0021] FIGS. 5A-E: Characterization of GBM-engineered *Shigella*. (FIG. 5A)

GBM-engineered *Shigella*, GBM-engineered *Shigella* MsbB1 and MsbB2 knock out, or control are incubated with U-251 GBM cells. Gentamycin and washing eliminate non-internalized bacteria. Cells are fixed and stained with Hoechst 33342 for DNA (Blue), Phalloidin for F-Actin (Red), anti-*Shigella* antibody (ab65282), and counterstained with alexa-flour 488 (green) to visualize *Shigella*. (FIG. 5B) The percentage of U-251 cells with internalized *Shigella* identified via microscopy is plotted. (FIG. 5C) The mean number of *Shigella* rods per U-251 cell, with at least one *Shigella* rod inside the cell membrane. (FIG. 5D) The average signal intensity of *Shigella* fluorescence is quantified and plotted (* = $p < 0.05$ by ANOVA, $n = 3$ individual fields/group). (FIG. 5E) GBM-engineered *Shigella* or MsbB1 and MsbB2 knock out *Shigella* are incubated with GBM cells. Internalized *Shigella* is recovered and plated on TSB + congo red plates to quantify internalized bacteria.

[0022] FIG. 6: Gel Images from Stx1 and Stx2 Detection. PCR is used to determine the presence of Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) genes in the starting strain of *Shigella* used to create GBM-engineered *Shigella* (Stx1=699 bp, Stx2=627 bp, virF= 607 bp).

[0023] FIG. 7: The absence of Shiga toxin protein in GBM-engineered and non-engineered *Shigella* (R50 and R0, respectively) is measured using a Shiga toxin detection kit. Shiga toxin was tested under normal growth conditions, and under Mitomycin C (Mito) induction. Band intensity corresponding to Stx1, Stx2, or controls are plotted for each group.

[0024] FIG. 8A-C: Gel Images from MsbB Deletion: PCR is used to determine the presence of MsbB1 KO cassette (471 bp). (FIG. 8A) Gel image of MsbB1 KO. (FIG. 8B) MsbB2 KO gel comparing parental round 50 GBM-engineered *Shigella* to MsbB2+ cassette (471 bp), and (FIG. 8C) MsbB2 KO with virF (607 bp) to ensure retention of virulence plasmid.

[0025] FIG. 9: TLR4 stimulation levels from *S. flexneri* and *Shigella* lpxM deletion. The optical density from this live cell assay is measured at 620 nm to quantify stimulation of TLR4.

[0026] **FIG. 10:** Growth rate of round 50 *Shigella* compared to MsbB Dual KO. The OD₆₅₀ of each sample was measured every hour.

[0027] **FIGS. 11A-B:** Gentamicin Can Prevent Extracellular Replication of GBM-infecting *Shigella* Without Inhibiting Intracellular Replication. **FIG. 11A**, *Shigella* is incubated with GSC112 GBM cells for 45 minutes then gentamicin is added to the media. Cells and media are harvested each hour and the amount of intracellular (red) and extracellular (blue) *Shigella* is plotted. After 7 hours *Shigella* begins to replicate in extracellularly. **FIG. 11B**, The assays are repeated as in A for 24 hours with gentamicin supplemented every 7 hours to halt extracellular growth.

[0028] **FIGS. 12A-C:** Co-administration of GBM-infecting *Shigella* and Gentamicin Restricts *Shigella* Growth to the Tumor Region in Immunocompetent Mice Bearing Orthotopic GBM. **FIG. 12A**, C57BL/6 mice bearing orthotopic GL261 tumors are administered GBM-infecting *Shigella*. After 30 minutes, mice are administered gentamicin for 24 hours (50 mg/mL, 1 mL/hour) via osmotic pump. After 24 hours, the brains are harvested and the amount of *Shigella* in the tumor region, contralateral brain, and CSF is quantified. **FIG. 12B**, Quantification of plates from mice receiving GBM-infecting *Shigella* and PBS in the osmotic pump (n=3). **FIG. 12C**, Quantification of plates from mice receiving GBM-infecting *Shigella* and gentamicin (n=3).

[0029] **FIGS. 13A-G:** GBM-infecting *Shigella* Modulate GBM Cell GFP Expression by Antibiotic-Mediated Release of DNA Plasmids Directly into the Cytosol. **FIG. 13A**, GBM-infecting *Shigella* are transformed with a pSelect plasmid that drives GFP expression off a mammalian promoter. GBM-infecting *Shigella*+pSelect are infected into GSC112 GBM cells. After 30 minutes, gentamicin is added to eradicate extracellular bacteria. After 4 hours antibiotics are added that lyse internalized GBM-infected *Shigella* allowing pSelect access to the GBM cell cytosol. After 48 hours, GBM cells are imaged via confocal microscopy. **FIG. 13B**, Image of GSC112 GBM cells incubated with GBM-infecting *Shigella*+pSelect that only received gentamicin at 30 minutes. Nuclei are rendered blue (Hoechst 33342), cell membrane red (wheatgerm-rhodamine), and GFP signal green. **FIG. 13C**, Image of GSC112 GBM as in B that received gentamicin at hour 4. **FIG. 13D**, Image of GSC112 GBM as in B that received ampicillin at hour 4. **FIG. 13E**, Image of GSC112 GBM cells as in B that received doxycycline at hour 4. **FIG. 13F**, GFP signal is quantified per group. **FIG. 13G**, Number of GFP positive cells per group (*=p<0.05 via ANOVA).

[0030] **FIGS. 14A-D:** GBM-infecting *Shigella* Modulate GFP Expression in Ex Vivo Brains by Antibiotic-Mediated Release of DNA plasmids Directly Cytosol. **FIG. 14A**, GBM-infecting *Shigella* are transformed with a pSelect plasmid that drives GFP expression off a mammalian promoter. GBM-infecting *Shigella*+pSelect are mixed with one millimeter thick murine brain section containing GL261 GBM. After 30 minutes, gentamicin is added to eradicate extracellular bacteria. After 4 hours doxycycline is added to lyse internalized GBM-infected *Shigella* allowing pSelect to access the GBM cell cytosol. After 24 hours, GBM cells are imaged via confocal microscopy. **FIG. 14B**, Image of ex vivo GL261 GBM incubated with GBM-infecting *Shigella*+pSelect that only received gentamicin at 30 minutes. Nuclei are rendered blue (Hoechst 33342) and GFP signal green. **FIG. 14C**, Image of ex vivo GL261 GBM incubated with GBM-infecting *Shigella*+pSelect that received doxycycline. **FIG. 14D**, GFP signal is quantified in each group (*= $p < 0.05$ via ANOVA).

DESCRIPTION

I. Engineered *Shigella* Bacteria

[0031] Engineered *Shigella* bacteria are provided herein that can selectively infect or target cancerous cells (*e.g.*, a brain cancer, glioma, glioblastoma). GBM is the most common malignant, primary brain tumor observed in adults, and patients diagnosed with GBM demonstrate a median survival of only 15 months.[15,16] The standard of care for GBM is gross total surgical resection, if possible, followed by radiation and chemotherapy. Even with complete surgical resection of the tumor and adjuvant chemotherapy, these tumors recur, ultimately resulting in mortality.[15-19].

[0032] The engineered *Shigella* has preferably been genetically altered to knockout or not express any functional Shiga toxins. For example, molecular biology techniques can be used to remove or mutate DNA in the engineered *Shigella* that code for the Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). A variety of genetic mutations (*e.g.*, substitution mutations, deletions, etc.) can be used to generate *Shigella* that do not produce any functional Shiga toxin.

[0033] A variety of *Shigella* can be used herein. The *Shigella* is preferably *Shigella flexneri* (*e.g.*, *Shigella flexneri* 2475 Serotype 2a). Nonetheless, other *Shigella* can be used including, *e.g.*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, or *Shigella boydii*; preferably the Shiga toxins have been removed or knocked out from any engineered *Shigella* that will be administered to a mammalian or human patient.

[0034] *S. flexneri* is a gram-negative intracellular bacterium. *Shigella* has a type-3 secretion system capable of administering therapeutic proteins into the host cell cytosol.[12,20,21] Additional modifications to improve the safety of the bacterium can be made. *Shigella* has a very large cassette space to encode a very wide variety of therapeutic proteins.[22-24] Engineered *S. flexneri* provided herein can include multiple potential benefits compared to current oncolytic virus platforms. The safety profile of the parental and GBM-engineered *S. flexneri* strain, engineered *Shigella* to selectively internalize into GBM cells via iterative co-culture, was evaluated and such bacteria may be administered to a mammalian subject, such as a human. Preferably, the *S. flexneri* has not been modified to remove the capacity for myristoylation since myristoylation enzymes were observed to be important for internalization into GBM cells.

[0035] A population of *Shigella flexneri* strains capable of selectively infecting GBM cells are provided herein. The clones present in this population of *Shigella flexneri* strains have been shown to selectively infect or be internalized into GBM cells. These bacteria contain 177 mutations in 46 genes (see FIG. 1B, and Table 1). Seventeen mutations are in genes related to metabolism/homeostasis, 13 in genes involved in DNA regulation, 7 in genes involved in transcriptional regulation, 6 in genes associated with stress response, and 3 in genes with unknown function.

[0036] The engineered *Shigella flexneri* may contain mutations in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more, or all of the genes listed in Table 1. Mutations were observed in the genes listed in Table 1 in a population of *Shigella*, and may include substitutions, deletions, truncations, missense, and other mutations. The sequencing was performed on several clones that came out of FIGS. 2A-C as the population evolved.

Table 1.

Gene	Name
S_RS27100	IS3 family transposase
WP_000125617.1	PTS maltose transporter subunit IICB
WP_000129530.1	bifunctional ADP-dependent NAD(P)H-hydrate dehydratase/NAD(P)H-hydrate epimerase
WP_000130034.1	D-alanine--D-alanine ligase
WP_000156579.1	helix-turn-helix transcriptional regulator
WP_000354046.1	FAD assembly factor SdhE

WP_000454701.1	TerC family protein
WP_000570673.1	ribosome-dependent GTPase TypA
WP_000577615.1	o-succinylbenzoate--CoA ligase
WP_000695507.1	alpha-glucosidase
WP_000741619.1	phosphate ABC transporter permease PstC
WP_000844882.1	primosomal replication protein N"
WP_000847163.1	aminotransferase class I/II-fold pyridoxal phosphate-dependent enzyme
WP_000850244.1	dimethylsulfoxide reductase subunit A
WP_000883129.1	NO-inducible flavohemoprotein
WP_000937883.1	exodeoxyribonuclease VII large subunit
WP_000983717.1	iron/manganese ABC transporter ATP-binding protein SitB
WP_001162179.1	metalloprotease PmbA1
WP_001194727.1	cell division protein ZapD
WP_001207893.1	nitrate/nitrite transporter NarU
WP_001223145.1	tRNA/rRNA methyltransferase
WP_001237077.1	glucosamine-6-phosphate deaminase
WP_001250207.1	peptide ABC transporter substrate-binding protein SapA
WP_001258671.1	aspartate--tRNA ligase
WP_001278539.1	FAD-dependent oxidoreductase
WP_001289665.1	electron transport complex subunit RsxE
WP_005049126.1	IS110-like element ISSf14 family transposase
WP_011110552.1	ChiQ/YbfN family lipoprotein
WP_011110568.1	bifunctional acid phosphatase/4-phytase
WP_011110582.1	Gfo/Idh/MocA family oxidoreductase
WP_011110619.1	biosynthetic arginine decarboxylase
WP_011110635.1	IS66-like element ISSf13 family transposase
WP_024260057.1	ATP-binding cassette domain-containing protein
WP_094081518.1	IS3-like element IS600 family transposase
WP_094092384.1	IS3-like element IS600 family transposase
WP_094105670.1	IS3-like element IS911 family transposase
WP_094105677.1	transposase
WP_094105679.1	IS3-like element IS911 family transposase
WP_167389526.1	ribosomal slippage
WP_171525780.1	IS3 family transposase
WP_240532658.1	ribosomal slippage
WP_240532662.1	ribosomal slippage
WP_276282982.1	P-type conjugative transfer protein TrbL

[0037] The engineered *Shigella* may be used to target GBM. A GBM-targeting engineered *Shigella* may still replicate inside a mammalian cell, not unlike virus strategies.[14] The larger size and complexity of *Shigella* may allow for rapid identification of clones that preferentially internalize into GBM (in contrast, it typically takes hundreds of passages with a given cell type to change the tropism of a virus).[38,39] The large genome and DNA editing resources available for *Shigella* allow even very large sized cassettes to be permanently

integrated into the genome.[22,26] Thus, almost any combination of therapeutic protein(s) are available for researchers to identify the optimal combination of therapeutics for treating GBM. *Shigella* contains the equipment to ensure therapeutic proteins are delivered directly into the cytosol of infected GBM cells via a type three secretion system or antibiotic mediated bacterial cell lysis.[12,20] In terms of safety, the potential for large cassette sizes allows for improved safety controls as bacterial therapeutics factories can be programmed to self-destruct and/or suicide switches added ab lib into the platform.[22,24,26] Finally, the GBM-engineered *Shigella* may remain sensitive to beta-lactams so simple administration of penicillin, doxycycline or ciprofloxacin may serves as a master suicide switch to immediately halt GBM-engineered *Shigella* activity.

[0038] The engineered bacteria may also comprise a kill switch. Suitable kill switches are described in International Patent Application Publication WO2016/210373, the contents of which are herein incorporated by reference in their entirety. The kill switch is intended to actively kill engineered microbes in response to external stimuli. As opposed to an auxotrophic mutation where bacteria die because they lack an essential nutrient for survival, the kill switch may be triggered by a particular factor in the environment that induces the production of toxic molecules within the microbe that cause cell death.

[0039] Bacteria engineered with kill switches have been engineered for in vitro research purposes, e.g., to limit the spread of a biofuel-producing microorganism outside of a laboratory environment. Bacteria engineered for *in vivo* administration to treat a disease or disorder may also be programmed to die at a specific time after the expression and delivery of a heterologous gene or genes, for example, a therapeutic gene(s) or after the subject has experienced the therapeutic effect. For example, the kill switch may be activated to kill the bacteria after a period of time following oxygen level-dependent expression of a therapeutic payload. Alternatively, the bacteria may be engineered to die if the bacteria have spread outside of a target site (e.g., a tumor site). Specifically, it may be useful to prevent the spread of the microorganism outside the area of interest (for example, outside of the tumor site) within the subject, or spread of the microorganism outside of the subject into the environment (for example, spread to the environment through the blood or stool of the subject). Examples of such toxins that can be used in kill switches include, but are not limited to, bacteriocins, lysins, and other molecules that cause cell death by lysing cell membranes, degrading cellular DNA, or other mechanisms. Such toxins can be used individually or in combination. The switches

that control their production can be based on, for example, transcriptional activation (toggle switches), translation (riboregulators), or DNA recombination (recombinase-based switches), and can sense environmental stimuli such as anaerobiosis or reactive oxygen species. These switches can be activated by a single environmental factor or may require several activators to induce cell death. For example, a riboregulator switch may be activated by tetracycline, isopropyl β -D-1-thiogalactopyranoside (IPTG), and arabinose to induce the expression of lysins, which permeabilize the cell membrane and kill the cell. IPTG induces the expression of the endolysin and holin mRNAs, which are then derepressed by the addition of arabinose and tetracycline. All three inducers must be present to cause cell death.

10 II. Delivery of a Therapeutic or Anti-Cancer Protein or Gene

[0040] The engineered *Shigella* bacteria provided herein may be genetically engineered to deliver a heterologous gene (e.g., a therapeutic gene or a gene encoding an anti-cancer protein) to a cancerous cell (e.g., a brain cancer, a glioma, or a glioblastoma) in a mammalian subject. The engineered *Shigella* may recombinantly express a therapeutic or anti-cancer protein that may further benefit the treatment of the cancer in the subject. In some embodiments, the *Shigella* can be lysed or killed after infecting a cell, thus releasing a nucleic acid in the cell that can induce expression of a therapeutic or anti-cancer protein in the cancerous cell.

[0041] Engineered *Shigella* provided herein may be used to express a variety of anti-cancer proteins. For example, L-ASNase can be expressed under the control of the araBAD promoter (PBAD) of the *E. coli* arabinose operon, which can be induced by injection of L-arabinose, and such approaches can be used to produce anticancer effects (e.g., Kim *et al.* 2018). The bacteria may constitutively express a recombinant immunotoxin comprising a growth factor or targeting polypeptide (e.g., TGF α) and a cytotoxic moiety (e.g., a toxin, or endotoxin such as *Pseudomonas* exotoxin A (PE38)) using a constitutive exponential phase promoter (e.g., Mai *et al.*, 2023). A cytotoxic payload described in [47-49] can be expressed by an engineered *Shigella* described herein to achieve an anti-cancer effect. Other therapeutic or anti-cancer genes or proteins that can be expressed by the engineered *Shigella* include cytokines (IFN γ , IL2, IL12 or IL23), enzymes involved with gene editing (e.g., CRISPR, Zinc finger, and other gene editing systems), toxins or cytotoxic proteins that can kill or initiate apoptosis in a cell or cancerous cell (e.g., cytosolic restricted enzymatic toxins such as gelonin, shigella toxin subunit alpha, granzymes (e.g., granzyme B), caspases (e.g., caspases 3).

[0042] The heterologous gene may be expressed under the control of a promoter. The heterologous gene and operatively linked promoter may be present on a chromosome in the bacterium. The heterologous gene and operatively linked promoter may be present on a plasmid in the bacterium. A “promoter” as used herein, refers to a nucleotide sequence that is capable of controlling the expression of a coding sequence or gene. Promoters are generally located 5’ of the sequence that they regulate. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from promoters found in nature, and/or comprise synthetic nucleotide segments. Those skilled in the art will readily ascertain that different promoters may regulate expression of a coding sequence or gene in response to a particular stimulus, e.g., in a cell- or tissue-specific manner, in response to different environmental or physiological conditions, or in response to specific compounds. Prokaryotic promoters are typically classified into two classes: inducible and constitutive. Expression of a therapeutic or anti-cancer gene or protein may be driven by a quorum-sensing-controlled promoter in the engineered *Shigella*.

[0043] As used herein, “heterologous” as used in the context of a nucleic acid or polypeptide sequence, “heterologous gene”, or “heterologous sequence”, refers to a nucleotide or polypeptide sequence that is not normally found in a given cell in nature. As used herein, a heterologous sequence encompasses a nucleic acid sequence that is exogenously introduced into a given cell. “Heterologous gene” includes a native gene, or fragment thereof, that has been introduced into the host cell in a form that is different from the corresponding native gene. For example, a heterologous gene may include a native coding sequence that is a portion of a chimeric gene to include a native coding sequence that is a portion of a chimeric gene to include non-native regulatory regions that is reintroduced into the host cell. A heterologous gene may also include a native gene, or fragment thereof, introduced into a non-native host cell. Thus, a heterologous gene may be foreign or native to the recipient cell; a nucleic acid sequence that is naturally found in a given cell but expresses an unnatural amount of the nucleic acid and/or the polypeptide which it encodes; and/or two or more nucleic acid sequences that are not found in the same relationship to each other in nature. As used herein, the term “endogenous gene” refers to a native gene in its natural location in the genome of an organism. As used herein, the term “transgene” refers to a gene that has been introduced into the host organism, e.g., host bacterial cell, genome.

[0044] “Operably linked” refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. A regulatory element is operably linked with a coding sequence when it is capable of affecting the expression of the gene coding sequence, regardless of the distance between the regulatory element and the coding sequence. More specifically, operably linked refers to a nucleic acid sequence that is joined to a regulatory sequence in a manner which allows expression of the nucleic acid sequence. In other words, the regulatory sequence acts in cis. In one embodiment, a gene may be “directly linked” to a regulatory sequence in a manner which allows expression of the gene. In another embodiment, a gene may be “indirectly linked” to a regulatory sequence in a manner which allows expression of the gene. In one embodiment, two or more genes may be directly or indirectly linked to a regulatory sequence in a manner which allows expression of the two or more genes.

[0045] A constitutive promoter is a promoter that is capable of facilitating continuous transcription of a coding sequence or gene under its control and/or to which it is operably linked. Constitutive promoters and variants are well known in the art and include, but are not limited to, BBa_J23100, a constitutive *Escherichia coli* σ s promoter (e.g., an *osmY* promoter (International Genetically Engineered Machine (iGEM) Registry of Standard Biological Parts Name BBa_J45992; BBa_J45993)), a constitutive *Escherichia coli* σ 32 promoter (e.g., *htpG* heat shock promoter (BBa_J45504)), a constitutive *Escherichia coli* σ 70 promoter (e.g., *lacQ* promoter (BBa_J54200; BBa_J56015), *E. coli* CreABCD phosphate sensing operon promoter (BBa_J64951), *GlnRS* promoter (BBa_K088007), *lacZ* promoter (BBa_K119000; BBa_K119001); M13K07 gene I promoter (BBa_M13101); M13K07 gene II promoter (BBa_M13102), M13K07 gene III promoter (BBa_M13103), M13K07 gene IV promoter (BBa_M13104), M13K07 gene V promoter (BBa_M13105), M13K07 gene VI promoter (BBa_M13106), M13K07 gene VIII promoter (BBa_M13108), M13110 (BBa_M13110)), a constitutive *Bacillus subtilis* σ A promoter (e.g., promoter *veg* (BBa_K143013), promoter 43 (BBa_K143013), *PliaG* (BBa_K823000), *PiepA* (BBa_K823002), *Pveg* (BBa_K823003)), a constitutive *Bacillus subtilis* σ B promoter (e.g., promoter *ctc* (BBa_K143010), promoter *gsiB* (BBa_K143011)), a *Salmonella* promoter (e.g., *Pspv2* from *Salmonella* (BBa_K112706), *Pspv* from *Salmonella* (BBa_K112707)), a bacteriophage T7 promoter (e.g., T7 promoter (BBa_I712074; BBa_I719005; BBa_J34814; BBa_J64997; BBa_K113010; BBa_K113011; BBa_K113012; BBa_R0085; BBa_R0180; BBa_R0181; BBa_R0182; BBa_R0183;

BBa_Z0251; BBa_Z0252; BBa_Z0253)), and a bacteriophage SP6 promoter (e.g., SP6 promoter (BBa_J64998)).

[0046] An inducible promoter refers to a regulatory nucleic acid region that is operably linked to one or more genes, wherein transcription of the gene(s) is increased in response to a stimulus (e.g., an inducer) or an exogenous environmental condition. A “directly inducible promoter” refers to a regulatory region, wherein the regulatory region is operably linked to a gene encoding a protein or polypeptide, where, in the presence of an inducer of said regulatory region, the protein or polypeptide is expressed. An “indirectly inducible promoter” refers to a regulatory system comprising two or more regulatory regions, for example, a first regulatory region that is operably linked to a first gene encoding a first protein, polypeptide, or factor, e.g., a transcriptional regulator, which is capable of regulating a second regulatory region that is operably linked to a second gene, the second regulatory region may be activated or repressed, thereby activating or repressing expression of the second gene.

[0047] An inducible promoter may be induced by low-oxygen, hypoxic, or anaerobic conditions. “Hypoxia” is used to refer to reduced oxygen supply to a tissue as compared to physiological levels, thereby creating an oxygen-deficient environment. “Normoxia” refers to a physiological level of oxygen supply to a tissue. Hypoxia is a hallmark of solid tumors and characterized by regions of low oxygen and necrosis due to insufficient perfusion. Examples of oxygen level-dependent transcription factors include, but are not limited to, FNR (fumarate and nitrate reductase)-responsive promoters, ANR (anaerobic nitrate respiration)-responsive promoters, and DNR (dissimilatory nitrate respiration regulator)-responsive promoters. Multiple FNR-responsive promoters, ANR-responsive promoters, and DNR-responsive promoters which can be used in the present invention are known in the art. Examples of FNR responsive genes, promoters, and/or regulatory regions include nirB, ydfZ, pdhR, focA, ndh, hlyE, narK, narX, narG, yfiD, and tdcD. Examples of ANR responsive genes, promoters, and/or regulatory regions include arcDABC. Examples of DNR responsive genes, promoters, and/or regulatory regions include norb and norC. An exemplary hypoxia-inducible promoter is a *pflE* promoter.

[0048] An inducible promoter may be activated by inflammatory conditions, e.g., a reactive nitrogen species or RNS promoter. As used herein, “RNS-inducible regulatory region” refers to a nucleic acid sequence to which one or more RNS-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription

factor activates downstream gene expression; in the presence of RNS, the transcription factor binds to and/or activates the regulatory region. Examples of RNS-sensing transcription factors and RNS-responsive genes include NsrR (which is able to regulate norB, aniA, nsrR, hmpA, ytfE, ygbA, hcp, hcr, nrfA, aox), NorR (which is able to regulate norVW, norR), and DNR (which is able to regulate norCB, nir, nor, nos).

[0049] An inducible promoter may be activated by conditions of cellular damage, e.g., a reactive oxygen species or ROS promoter. As used herein, “ROS-inducible regulatory region” refers to a nucleic acid sequence to which one or more ROS-sensing transcription factors is capable of binding, wherein the binding and/or activation of the corresponding transcription factor activates downstream gene expression; in the presence of ROS, the transcription factor binds to and/or activates the regulatory region. In some embodiments, the ROS-inducible regulatory region comprises a promoter sequence. In some embodiments, the transcription factor senses ROS and subsequently binds to the ROS-inducible regulatory region, thereby activating downstream gene expression. In alternate embodiments, the transcription factor is bound to the ROS-inducible regulatory region in the absence of ROS; in the presence of ROS, the transcription factor undergoes a conformational change, thereby activating downstream gene expression. The ROS-inducible regulatory region may be operatively linked to a gene sequence or gene sequence, e.g., a sequence or sequences encoding one or more transporter(s). For example, in the presence of ROS, a transcription factor, e.g., OxyR, senses ROS and activates a corresponding ROS-inducible regulatory region, thereby driving expression of an operatively linked gene sequence or gene sequences. Thus, ROS induces expression of the gene or genes. Examples of ROS-sensing transcription factors and ROS-responsive genes include OxyR (which senses H₂O₂ and which is able to regulate ahpC; ahpF; dps; dsbG; fhuF; flu; fur; gor; grxA; hemH; katG; oxyS; sufA; sufB; sufC; sufD; sufE; sufS; trxC; uxuA; yaaA; yaeH; yaiA; ybjM; ydcH; ydeN; ygaQ; yljA; ytfK), PerR (which senses H₂O₂ and which is able to regulate katA; ahpCF; mrgA; zoaA; fur; hemAXCDBL; srfA), SoxR (which is able to regulate soxS), and RosR (which senses H₂O₂ and which is able to regulate rbtT; tnp16a; rluC1; tnp5a; mscL; tnp2d; phoD; tnp15b; pstA; tnp5b; xylC; gabD1; rluC2; cgtS9; azlC; narKGHJI; rosR).

III. Cancers

[0050] The engineered *Shigella* bacteria provided herein may be used to selectively target a variety of cancerous cells. The engineered *Shigella* may selectively infect or be

internalized into a brain cancer. The brain cancer may be a glioma, glioblastoma (also called glioblastoma multiforme), ependymoma, astrocytoma, protoplasmic astrocytoma, fibrillary astrocytoma, astroblastoma, oligodendroglioma, oligodendroblastoma, primitive neuroectodermal cancer, cerebellar sarcoma, ganglioneuroblastoma, neuroblastoma, or diffuse
 5 Pontine glioma (DIPG). The brain cancer may be malignant, metastatic, or non-metastatic.

[0051] While the engineered *Shigella* provided herein selectively infect brain cancer cells (*e.g.*, GBM), engineered *Shigella* may be generated using methods provided herein to selectively infect or be internalized into cancerous cells from a variety of cancers. In some embodiments, the *Shigella* can be used to deliver a nucleic acid to a non-cancerous cell, such
 10 as a cell line used for producing a therapeutic polypeptide (*e.g.*, a therapeutic antibody). The cancer can be a cancer of the bladder, blood, bone, bone marrow, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma,
 15 undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic
 20 carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; bronchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing
 25 carcinoma; adrenal cortical carcinoma; endometrioid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma;
 30 inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant;

paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; 5 liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; 10 choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic 15 fibrosarcoma; pinealoma, malignant; chordoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; hodgkin's disease; hodgkin's; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; malignant 20 histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

25 **IV. Pharmaceutical Formulations**

[0052] Provided herein are pharmaceutical compositions comprising a recombinant bacterium disclosed herein and a pharmaceutically acceptable carrier. Also provided herein are methods of treating a disease in a subject in need thereof comprising the step of administering to the subject a pharmaceutical composition comprising a recombinant bacterium disclosed 30 herein and a pharmaceutically acceptable carrier. The bacteria may be administered to a mammalian subject peripherally (*e.g.*, intravenously or intratumorally) to target brain cancer (*e.g.*, GBM) cells or tumors that have metastasized or that are located outside of the central nervous system (CNS) of the subject. The bacteria may be administered directly into the brain

(*e.g.*, intraventricularly, intrathecally, into the cerebrospinal fluid, intratumorally *etc.*) to treat brain cancer cells (*e.g.*, GBM cells) that are located within the central nervous system. “Intratumoral administration” is meant to include any and all means for microorganism delivery to the intratumoral site and is not limited to intratumoral injection means.

5 **[0053]** As used herein a “pharmaceutical composition” refers to a preparation of bacterial cells disclosed herein with other components such as a physiologically suitable carrier and/or excipient. The phrase “pharmaceutically acceptable carrier” refers to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered bacteria. The term “excipient” refers to
10 an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples include, but are not limited to, calcium bicarbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and surfactants, including, for example, polysorbate 20.

[0054] The dose to be injected is derived from the type and size of the tumor. The dose
15 of the genetically engineered bacteria for local administration is typically lower, *e.g.*, orders of magnitude lower, than a dose for systemic intravenous administration.

[0055] The volume injected into each lesion is based on the size of the tumor. To obtain the tumor volume, a measurement of the largest plane can be conducted. The estimated tumor volume can then inform the determination of the injection volume as a percentage of the total
20 volume. For example, an injection volume of approximately 20-40% of the total tumor volume can be used.

[0056] For example, as is for example described in WO/2014/036412, for tumors larger than 5 cm in their largest dimension, up to 4 mL can be injected. For tumors between 2.5 and 5 cm in their largest dimension, up to 2 mL can be injected. For tumors between 2.5 and 5 cm
25 in their largest dimension, up to 2 mL can be injected. For tumors between 1.5 and 2.5 cm in their largest dimension, up to 1 mL can be injected. For tumors between 0.5 and 1.5 cm in their largest dimension, up to 0.5 mL can be injected. For tumors equal or smaller than 0.5 cm in their largest dimension, up to 0.1 mL can be injected. Alternatively, ultrasound scan can be used to determine the injection volume that can be taken up by the tumor without leakage into
30 surrounding tissue.

[0057] The treatment regimen may include one or more administrations. A treatment regimen may include an initial dose, which is followed by at least one subsequent dose. One or more doses can be administered sequentially in two or more cycles. For example, a first dose may be administered at day 1, and a second dose may be administered after 1, 2, 3, 4, 5, 6, days or 1, 2, 3, or 4 weeks or after a longer interval. Additional doses may be administered after 1, 2, 3, 4, 5, 6, days or after 1, 2, 3, or 4 weeks or longer intervals. The first and subsequent administrations may have the same dosage or different dosages.

[0058] The routes of administration and dosages described are intended only as a guide. The optimum route of administration and dosage can be readily determined by a skilled practitioner. The dosage may be determined according to various parameters, especially according to the location of the tumor, the size of the tumor, the age, weight and condition of the patient to be treated and the route and method of administration.

V. Combination Therapies

[0059] In order to increase the effectiveness of the engineered bacterial therapeutics provided herein, it may be desirable to combine these compositions with other agents effective in the treatment of cancer.

[0060] As a non-limiting example, the treatment of cancer may be implemented with an engineered bacterial composition, as provided herein, along with other anti-cancer agents. An “anti-cancer” agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer.

[0061] Various standard therapies, as well as surgical intervention, may be applied in combination with the provided engineered bacterial compositions. For example, the engineered bacterial therapeutics may be provided in combination with a chemotherapy, temozolomide (the imidazotetrazine derivative of the alkylating agent dacarbazine), etoposide, doxorubicin, cisplatin, paclitaxel, carmustine, lomustine, ceramide, phosphorylcholine, immunotherapy, radiation therapy, and/or surgical therapy.

VI. Definitions

[0062] As used herein the specification, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising,” the words “a” or “an” may mean one or more than one.

5 [0063] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

10 [0064] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the inherent variation in the method being employed to determine the value, the variation that exists among the study subjects, or a value that is within 10% of a stated value.

15 [0065] As used herein, “essentially free,” in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%, preferably below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

20 [0066] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

25 [0067] As used herein, the term “plasmid” or “vector” refers to an extrachromosomal nucleic acid, e.g., DNA, construct that is not integrated into a bacterial cell’s genome. Plasmids are usually circular and capable of autonomous replication. Plasmids may be low-copy, medium-copy, or high-copy, as is well known in the art. Plasmids may optionally comprise a selectable marker, such as an antibiotic resistance gene, which helps select for bacterial cells containing the plasmid and which ensures that the plasmid is retained in the bacterial cell.

[0068] The terms “subject,” “host,” “patient,” and “individual” are used interchangeably herein to refer to any mammalian subject for whom therapy is desired, particularly humans. Other subjects may include cattle, dogs, cats, guinea pigs, rabbits, rats, mice, horses, and so on.

5 [0069] The term “unit dose” when used in reference to a therapeutic composition refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, *i.e.*, carrier, or vehicle.

10 [0070] The term “effective amount” is an amount sufficient to effect beneficial or desired clinical results. An effective amount can be administered in one or more administrations. For purposes of this application, an effective amount is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease. Treatment of cancer may involve, for example, a reduction in the
15 invasiveness of a tumor, reduction in the growth rate of the cancer, or prevention of metastasis. Treatment of cancer may also refer to prolonging survival of a subject with cancer.

[0071] An effective response of a patient or a patient’s “responsiveness” to treatment refers to the clinical or therapeutic benefit imparted to a patient at risk for, or suffering from, a disease or disorder. Such benefit may include cellular or biological responses, a complete
20 response, a partial response, a stable disease (without progression or relapse), or a response with a later relapse. For example, an effective response can be reduced tumor size or progression-free survival in a patient diagnosed with cancer.

[0072] “Treatment” and “treating” refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject for the
25 purpose of obtaining a therapeutic benefit of a disease or health-related condition.

VII. Examples

[0073] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well
30 in the practice of the invention, and thus can be considered to constitute preferred modes for

its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5

EXAMPLE 1*Engineered Shigella Internalize into Glioblastoma Cells*

[0074] Overall Scheme for Engineering *Shigella* to Internalize in Brain Tumor Cells: As described in FIG. 1A, a scheme to identify *Shigella* clones that preferentially internalize into brain tumors using iterative co-culture assays was developed. Briefly, *S. flexneri*, an intracellular bacterium, was streaked onto agar plates. Clones containing the virulence factor needed to survive inside mammalian cells were expanded and incubated with a brain tumor cell line, U251 glioblastoma (GBM). Bacteria that internalize into GBM cells were harvested and used for additional rounds of co-culture.

[0075] Identification of an *S. flexneri* Strain Suitable for Engineering: In order to identify a *Shigella* strain that is safe to use as a therapeutic platform, the inventors identified a strain that does not contain shiga toxins. *S. flexneri* strain 2475 Serotype 2a was probed for DNA regions encoding shiga toxins using previously described primers, and did not result in a band at the expected size for the PCR product (FIG. 6).[25,30,31] Quantification of this region demonstrated a 15 and 21-fold less intense signal compared to positive control PCR for virulence factor F (virF). Levels of shiga toxin 1 and 2 protein were quantified in both round 0 and after 50 rounds of engineering using a rapid test commercially available detecting kit (FIG. 7). Both round 0 and round 50 *Shigella* were negative for both Stx1 and Stx2 relative to the positive control under normal conditions as well as when treated with Mitomycin-c to induce the lytic cycle of potential stx-producing prophage. Additionally, whole genome sequencing on GBM-engineered *Shigella* was performed and the data compared to the parental strain. 177 mutations in 46 genes (FIG. 1B, and Table 1) were identified. Seventeen mutations are in genes related to metabolism/homeostasis, 13 in genes involved in DNA regulation, 7 in genes involved in transcriptional regulation, 6 in genes associated with stress response, and 3 in genes with unknown function. Importantly, no mutations were identified that would be predicted to adversely affect the virulence or safety of the engineered bacterium.

[0076] Engineering *Shigella* to Internalize Into a GBM Cell Line: As shown in FIGS. 1A-B, *Shigella* clones were incubated with U-251 GBM cells. Internalized clones were selected and carried forward to the next round of co-culture. Thus, *Shigella* clones that internalized in GBM cells were enriched iteratively in the engineered population. To monitor the degree of enrichment of *Shigella* clones that internalized in GBM cells during the selection process, fluorescent microscopy was performed and the number of *Shigella* internalized in GBM cells was counted as the co-culture experiments progressed. As shown in FIG. 2A, internalized *Shigella* appeared as rod-like structures within the boundaries of the U251 GBM cell membrane (demarcated by wheat germ agglutinin) when stained with the DNA intercalating dye Hoechst 33342 (rendered blue). The number of GBM cells demonstrating an internalized bacterium increased after each round of *Shigella* co-culture, starting with $5\% \pm 2.5$ in round 10 and enriching to $95\% \pm 1$ by round 50 of co-culture. The percentage of GBM cells with an internalized bacterium was calculated by taking three randomly selected fields and dividing the total number of infected cells by the total number of U-251 cells present (FIG. 2B). The number of individual *Shigella* bacteria present inside each infected cell also increased as the rounds of co-culture progressed (FIG. 2C). In round 5, typically only 1 to 2 bacteria were present in an infected cell. By round 50, an average of 20 ± 5.5 bacteria were present within each infected GBM cell.

[0077] As demonstrated in FIG. 2D, individual GBM-engineered *Shigella* clones were expanded, and then an internalization assay was performed using patient-derived GSC 112 GBM cells and NA cells. Cells were fixed, permeabilized and subjected to a quantitative in-cell western using an anti-*Shigella* antibody and an IR₈₀₀ signal as a readout. A *Shigella* standard curve was used to convert the fluorescent signal to the amount of *Shigella* in each well. Out of 96 total clones, 85 clones internalized below detectable levels in NA cells. All 96 clones demonstrated increased internalization in GSC 112 cells compared to NA cells. The average internalization for the 96 clones into GSC 112 was 5,200 bacteria/2,000 GSC 112 cells with a range of 20,734 bacteria to 117 bacteria internalized/2,000 GSC 112 cells.

[0078] GBM-Internalizing Bacteria Retain *Shigella* Virulence Plasmid After Engineering: U-251 cells were infected with round 50 GBM-engineered *Shigella*. Cells were fixed and then stained with an anti-*Shigella* antibody targeting the LPS, phalloidin-texas red, and Hoechst 33342, and then imaged using confocal microscopy. The DNA rod-like structures observed inside GBM cells throughout this study co-localized with an anti-*Shigella* antibody

(FIG. 3A). Quantification of these images are presented in FIG. 3B; greater than a thousand-fold increase in *Shigella* fluorescent signal in round 50 GBM-engineered *Shigella* (0.909 ± 0.110 r.f.u) compared to non-engineered or other negative controls (non-engineered (R0) = 0.002 ± 0.001 *E. coli* 0.002 ± 0.001 no bacteria 0.001 ± 0.0) was observed. In GBM-engineered *Shigella* plated on TSB + 0.01% congo red agar plates, uptake of congo red (indicated by red colonies) was similar to non-engineered *Shigella* starting bacteria (positive control). A closely related *E. coli* (negative control) did not take up the red dye, as expected (FIG. 3C).

[0079] GBM-Engineered *Shigella* Preferentially Internalizes in GBM Cells

10 Compared to Normal Astrocyte Controls: Next, GBM-engineered *Shigella* were incubated with a panel of patient-derived GBM cell lines, as well as normal astrocytes, to quantify both the breadth and specificity of this platform to internalize in adult malignant brain tumors. Nine patient-derived glioblastoma stem cell (GSC) lines were incubated with GBM-engineered *Shigella*. Internalized *Shigella* were harvested and plated to count the number of internalized
15 bacteria/groups. GSC 115 showed the highest internalization at $123,800$ bacteria/ 5×10^5 GBM cells. To demonstrate the specificity of the platform, GBM-engineered *Shigella* were incubated with normal astrocytes and internalized bacteria counted. The inventors observed ~ 383 bacteria internalized/ ~ 300 NA cells (two separate patient cell lines). FIG. 4A shows the colony counts recovered after internalization for each GSC or NA cell line. A one-way ANOVA of
20 the colony counts indicated 7 out of the 9 GSC cell lines demonstrated significantly higher internalization ($p < 0.01$) compared to NA. FIG. 4C depicts the fold change between each GSC cell line relative to NA. The average fold increase was 123 ± 110 ; GSC 115 demonstrated the highest internalization with a 373 ± 59.5 -fold increase compared to NA.

[0080] Membrane anchored factor in engineered *Shigella* mediates internalization

25 in GBM cells: MsbB1 and MsbB2 myristoylation factors were removed from the round 50 GBM-engineered *Shigella* using recombineering (FIG. 8). Consistent with previous reports,[32-35] removal of the myristoylation enzymes lowered the TLR4 detection relative to unmodified *Shigella* ($p < 0.05$ via ANOVA, FIG. 9). Interestingly, removal of MsbB1 also decreased the recovery of *Shigella* that internalized into GBM cells from an average of
30 2.82×10^4 to 320 bacteria per group ($p < 0.05$ via ANOVA). Removal of both MsbB1 and MsbB2 completely abrogated internalization down to an average of < 1 bacterium recovered. Confocal imaging of GBM cells treated with either R50, MsbB1 KO, or MsbB1 and MsbB2

KO demonstrated significant decrease in cells internalizing *Shigella*. After the removal of MsbB1, the percentage of *Shigella* positive cells dropped from 95% to 25% ($p < 0.05$ via ANOVA). Quantification of the fluorescent *Shigella* signal indicated that MsbB1 removal reduced the mean signal to 0.425 rfu compared to 6.16 rfu for R50 (~14.5 fold decrease in signal). Removal of both MsbB1 and MsbB2 resulted in 0% of cells with a *Shigella* internalized and a fluorescent signal of 0.009 rfu (~684 fold decrease). GBM cells that do demonstrate *Shigella* internalization after removal of myristoylation factor MsbB1, have similar numbers of internalized *Shigella* compared to R50. The growth curves of R50, MsbB1 KO, and MsbB1 and MsbB2 KO-*Shigella* are presented in FIG. 10.

[0081] As shown above, *S. flexneri* clones were generated that selectively internalize into GBM cell lines. A strain of *Shigella* that does not express shiga toxins was selected to improve the safety profile of the platform.[12] After only 50 rounds of co-culture with GBM cells, *Shigella* clones demonstrated a high propensity for internalizing into GBM cells. The inventors observed less than one cell per field with internalized *Shigella* after 5 rounds of co-culture compared to 95% of cells infected with multiple bacteria after 50 rounds of co-culture. GBM-engineered *Shigella* retained LPS surface markers and the virulence plasmid throughout the engineering process. Finally, to improve patient specificity of the platform without further co-culture assays, an in-cell western screen was developed to complement the co-culture assays. In-cell western screening provides the opportunity to personalize this platform quickly for each patient tumor or to identify an optimal GBM-engineered *Shigella* clone for GBM cell lines that cannot be reliably cultured for 50 rounds of co-culture assays.

[0082] The data provided herein indicate that a myristoylated factor mediates selective internalization of GBM-engineered *Shigella* into GBM cells. Removal of MsbB1 and MsbB2 myristylation enzymes completely abrogate internalization of GBM-engineered *Shigella* in GBM cells. Previous reports indicate that removal of MsbB genes does not impair internalization of *Shigella* in intestinal cells, the typical target of wild-type *Shigella*. [40,41] A group reported removal of a single MsbB does not impair *Shigella* internalization in HeLa cells, and double knock out only reduces internalization by 50%. [42] The data provided herein indicates ~75% reduction in internalization with single MsbB and 100% reduction (complete) of internalization with double MsbB knockout, indicating this mechanism may be more important for internalization in GBM cells compared to other cell types. [42] Without wishing to be bound by any theory, these results are congruent with

previous studies which remove MsbB genes to “detoxify” LPS that indicate these enzymes facilitate the late acyltransferases on LPS and are associated with innate immunogenicity via TLR4 engagement.[33,43-46] Reduced TLR4 activity was observed in MsbB knock outs and the sequencing data indicates removal of only those two enzymes. GBM cells that do
5 internalize MsbB1 KO *Shigella* did so at a similar rate to R50 GBM-engineered *Shigella*, supporting the idea that this effect is not a function of reduced growth rate observed with MsbB knock outs. These data support the idea that myristylation (presumably to anchor a factor to the bacterial membrane) is essential for GBM-engineered *Shigella* to internalize into GBM cells. However, these data do not indicate what that factor is. Identification of this factor may
10 allow for deletion of enzymes upstream of MsbB that still modulate LPS TLR4 engagement without abrogating internalization in GBM cells. Controlling innate immunogenicity could complement typical anti-cancer strategies such as arming GBM-engineered *Shigella* with immunomodulators, toxins, or other cytotoxic factors to increase the selective cytotoxic potential of this platform. These approaches may be coupled with cytotoxic payloads and
15 safety measures.[47-49]

[0083] GBM-engineered clones that preferentially internalize into a given patient’s GBM can be generated based on the approaches described above. As shown in FIG. 2D, an in-cell western was developed to rapidly identify personalized GBM-engineered *Shigella* clones for a given patient tumor without further rounds of co-culture. This can allow for
20 identification of clones that internalize into patient cell lines that cannot undergo multiple rounds of passaging or samples directly resected from the operating room. These approaches can thus be used to generate a personalized GBM-engineered *Shigella* therapy for each patient afflicted with a brain tumor. It is anticipated that weaponizing GBM-engineered *Shigella* with multiple modalities and safety controls will provide benefits of this platform compared to other
25 microorganism strategies for treating brain tumors.[47,48] The myristoylated factor(s) driving internalization of GBM-engineered *Shigella* into brain tumor cells may be further identified to further improve the efficacy, specificity, and safety of these approaches.

[0084] GBM-engineered *Shigella* can selectively internalize into brain tumors compared to normal brain tissue. These engineered *Shigella* can provide numerous benefits
30 compared to existing microorganism platforms including high selectivity, vastly expanded cassette space, and possible improvements to the safety profile. These results support the use of the engineered *Shigella* to treat patients suffering from malignant brain tumors.

[0085] Gentamicin Can Prevent Extracellular Replication of GBM-infecting Shigella Without Inhibiting Intracellular Replication: Gentamicin prevents extracellular replication of GBM-infecting Shigella without inhibiting intracellular replication in GBM cells. A single dose of gentamicin is effective for ~7 hours (FIGS. 11A-B), and repeat dosing
5 facilitates exclusive intracellular replication for up to 24 hours (FIGS. 11B).

[0086] Co-administration of GBM-infecting Shigella and Gentamicin Restricts Shigella Growth to the Tumor Region in Immunocompetent Mice Bearing Orthotopic GBM: C57BL/6 immunocompetent mice bearing orthotopic, syngeneic GL261 tumors were administered GBM-infecting Shigella intratumorally. Next, mice received intratumoral
10 gentamicin via an osmotic pump (50 mg/mL at 1 mL/hour) for 24 hours (FIG. 12A). Mice administered PBS via an osmotic pump (control group) demonstrate high amounts of Shigella throughout the brain and cerebral spinal fluid (FIG. 12B), whereas mice that received gentamicin via an osmotic pump demonstrate Shigella only in the tumor region (FIG. 12C).

[0087] GBM-infecting Shigella Modulate GBM Cell GFP Expression by Antibiotic-Mediated Release of DNA Plasmids Directly into the Cytosol: GBM-infecting
15 Shigella were transfected with a pSelect GFP Plasmid that encodes GFP under a mammalian-specific promoter (EF1a/HTLV) so that only mammalian cells transfected with the plasmid produce GFP. GBM-infecting Shigella were incubated with GBM cells and allowed to internalize before gentamicin was added to eradicate extracellular bacteria. After 4 hours,
20 doxycycline or penicillin was added to cause antibiotic-mediated lysis of internalized Shigella. As the bacteria lyse, pSelect GFP plasmid was released into the cytosol of GBM cells and then migrated to the nucleus to mediate transient expression of GFP. GFP signal was quantified by confocal microscopy at 48 hours post-antibiotic lysis (FIG. 13A-G). Greater than 95% of GBM cells expressed GFP and demonstrated uniform expression across the fields. GFP signal is only
25 observed with doxycycline and penicillin, which penetrate the mammalian cell membrane, and not gentamicin. This experiment demonstrates a proof of concept for administering large biologic therapies directly into the cytosol of pathologic cells using GBM-infecting Shigella.

[0088] GBM-infecting Shigella Modulate GFP Expression in Ex Vivo Brains by Antibiotic-Mediated Release of DNA plasmids Directly Cytosol: Freshly harvested one-
30 millimeter thick brain sections bearing GL261 tumors were incubated with GBM-infecting Shigella containing pSelect GFP as described above. At the end of the experiment, the brain

sections are fixed, frozen, sectioned, and imaged. GL261 tumor cells demonstrated robust, significant expression of GFP (FIGS. 14A-D), indicating this procedure works in tissues.

EXAMPLE 2

Materials and Methods

5 **[0089]** The following materials and methods were used for the experiments in Example 1.

[0090] Shiga Toxin Protein Detection:

[0091] 24 hours prior to testing, *Shigella* were streaked out from the frozen stock onto Tryptic Soy Broth (TSB) (30g/L) + Congo Red (0.01% w/v) plates and incubated overnight
10 (O.N) at 37°C. Colonies were picked and grown for approximately 2 hours (O.D650=0.6).[13] Samples were tested according to the manufacturer's protocol for ImmunoCard STAT!® EHEC (Cat #:751630). Band intensity was measured using ImageJ.

[0092] Detecting Shiga Toxin DNA:

[0093] A single *Shigella* colony was picked using a pipet tip and resuspended in 20 µL
15 nuclease-free water. A colony was incubated for 10 min at 95°C, then centrifuged at 1000 rpm for one minute to remove membrane components.

[0094] Five µL of supernatant was used as the template for a PCR assay to quantify the presence of DNA encoding for shiga toxin(s). Potential DNA regions were amplified with the Phusion taq polymerase kit (HF buffer, 20 cycles) using previously described primers that were
20 specific for shiga toxin 1 (Stx1) shiga toxin 2 (Stx2), or positive control virulence factor F (virF).[25] Bands were identified by staining gel with Sybr Green dye (1:10,000 dilution) and imaged using a blue light transilluminator. Band intensity was quantified using the publicly available Image J software analysis suite.

[0095] MsbB Gene Deletions:

25 **[0096]** Genes were deleted as previously described using standard lambda red recombineering methods with minor modifications.[22,26] *S. flexneri* were streaked onto TSB agar plates with 0.01% congo red and grown at 37°C overnight. The next day, 96 red colonies of *Shigella* were picked using a filtered p200 pipet tip. Each colony was added into 400mL of

TSB in a deep-well block plate, and incubated at 37°C, 250 rpm until an O.D₆₅₀ of 0.6 was reached (approximately 2.5 to 3 hours). *Shigella* suspensions were combined and pelleted into a 50 mL conical tube. After washing three times with 25 mL of 10 percent glycerol, *Shigella* was resuspended in 5 mL of 10 percent glycerol. *Shigella* was electroporated with 200 ng of pRedTKI plasmid DNA (Addgene Plasmid #51628) and recovered in TSB for one hour at 30°C, followed by plating on TSB+ 50 mg/mL Kan agar plates with 0.01% congo red overnight. The pRedTKI plasmid introduced the Lambda red genes necessary for homologous recombination along with conferring Kan resistance.

[0097] *Shigella*+ pRedTKI colonies were picked and incubated at 30°C, 250 rpm until an OD₆₅₀ of 0.6 was reached (approximately 3 hours); during the last 30 minutes of incubation, l-arabinose was added at 10 mmol/liter to induce the Lambda red recombination genes along with 50 µg/ml kanamycin to maintain transmission of the pRedTKI plasmid. Bacteria were pelleted in a 50 mL conical tube by centrifugation at 4000 x g, transferred to a 1.5 mL eppendorf tube, washed three times with 500 µL of 10 percent glycerol, and resuspend in 50 µL of 10 percent glycerol. Using an electroporation cuvette with a 2 mm gap, *Shigella* were transfected via electroporation with 200 ng of I-SceI-flanking resistance cassette with 70bp homology adjacent to the gene to be deleted. Electroporated *Shigella* were allowed to recover in SOC for three hours at 30°C and plated on TSB+ 50 mg/mL Kan+ 12.5 mg/mL chloramphenicol (Cam) agar plates with 0.01% congo red overnight.

[0098] To complete the gene deletion protocol, a single colony of *Shigella*+ pRedTKI+ Cam resistance cassette was incubated at 30°C 250 rpm until an O.D.₆₅₀ of 0.6 (approximately 3 hours) in 3 mL of TSB+ 50 µg/ml kan+ 20 mmol/liter IPTG to induce I-SceI expression which excises the Cam resistance gene. A sample of liquid culture was seeded onto TSB+ 50 mg/mL Kan+ agar plates with 0.01% congo red and incubated overnight. Modifications were verified by PCR.

[0099] Internalization Assay:

[0100] *Tissue Culture*: GBM cell lines from Dr. John Kuo or ATCC were grown in low glucose DMEM w/ 10% FBS, glutamax, and 1% sodium pyruvate (without pen/strep).[27-29] Normal Astrocytes were purchased from Lonza (Cat #:CC-2565) and grown according to the manufacturer's protocol without *antibiotics*. All mammalian cells were cultured at 37°C, 5% CO₂ unless otherwise noted.

[0101] *Co-Culture Assay*: Mammalian cells were cultured as described above. 24 hours prior to co-culture assays, media was changed. Cells grown to 80% confluency in a T25 flask were used for each round of co-culture.

[0102] *S. flexneri* were streaked on TSB agar plates with 0.01% congo red, and grown at 37°C overnight. The next day, 96 red colonies of *Shigella* were picked using a filtered p200 pipet tip. Each colony was added into 400 µL of TSB in a deep-well block plate and incubated at 37°C, 250 rpm until an OD₆₅₀ of 0.6 was reached (approximately 2.5 to 3 hours). A control tube was used to estimate the *Shigella* growth rate. After reaching mid-log phase (0.6 OD₆₅₀), *Shigella* were concentrated to 2×10^9 cfu/mL (O.D₆₅₀ of 1.0 = 8.0×10^8). 250 µL of *Shigella* concentrate was added to ~5 mL of GBM or NA media in the T25 cell culture flask described above. The T25 flask was centrifuged at 200 x g for 10 min, and the culture incubated at 37°C, 5%CO₂ for 30 min. Non-internalized *Shigella* and mammalian culture media were removed by aspiration. Mammalian cells were washed four times using 5 mL of PBS for 1 minute with gentle agitation. Approximately five milliliters of mammalian cell media w/ 20 µg/mL gentamicin was added, and incubated at 37°C, 5%CO₂ for 90 min, to kill any extracellular bacteria. Media was aspirated and cells washed with PBS 4 times, one minute per wash, with gentle agitation.

[0103] After the final wash, 1 mL of accutase was added to the T25 flask and incubated at 37°C, 5% CO₂ until GBM or NA cells detached from the plate. Mammalian cell membranes were mechanically disrupted by pulling cell suspensions through a 20ga needle ten times. Mammalian cell lysates were centrifuged at 1,000 x g for 2 min to pellet any membrane-associated (but not internalized) *Shigella*. 20 µL of mammalian cell lysate were plated onto TSB+0.01% congo red agar plates and grown at 37°C overnight. These colonies can be expanded and frozen in 25% glycerol, or used directly for the next round of co-culture assays.

25 **[0104] Fluorescence Microscopy:**

[0105] *Tissue Culture*: 24 hours prior to internalization, GBM cell cultures were grown to ~80% confluence according to the steps above on an appropriate glass-bottom microscopy plate.

[0106] A *Shigella* clone was picked, grown, and co-cultured with mammalian cells in a microscopy dish as described above. Cells were fixed with 4% PFA for 10 min and then washed three times with PBS.

[0107] *For membrane + DNA imaging:* One mL of wheatgerm agglutinin-fluorescein (WGA, 1:2000) and Hoechst 33342 (1:800) in PBS was added to fixed cells and incubated for 15 min. The cells were washed three times with PBS, after which 1 mL of PBS was left in the dish for imaging. Cells were imaged using a 60x objective on a confocal microscope. Images were processed and analyzed using the publicly available Image J software package.

[0108] *For Shigella antibody staining:* The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Blocking buffer (PBS+30 mg/mL BSA + 5% donkey serum + 0.1% Triton X-100) was added and incubated at 4°C overnight. The next day, the cells were washed three times with PBS. The cells were incubated with anti-*Shigella* antibody (abcam cat# ab65282)(1:200) in PBS+30 mg/mL BSA + 0.1% Triton X-100 (total volume of one milliliter) for 90 min at room temperature, and then washed three times with PBS. The cells were then incubated with anti-rabbit alexafluor 488 secondary (1:1000) in PBS+30mg/mL BSA + 5% donkey serum + 0.1% Triton X-100 (one-milliliter total volume) for 60 min, and then washed three times with PBS. Finally, the cells were incubated with phalloidin (1:1000) and Hoechst 33342 (1:800) in PBS (one-milliliter total volume) for 15 min. The cells were imaged using a 60x objective with a confocal microscope. Images were processed and analyzed using the publicly available Image J software package.

[0109] In-Cell Western:

[0110] *Cell Culture:* 24 hours prior to internalization, GBM or NA were grown to 80% confluence using the steps described above on a black well, clear bottom 96 well tissue culture plate.

[0111] *Co-Culture:* *Shigella* were picked and grown according to the steps above in a deep block 96 well plate. 40 µL of non-concentrated single *Shigella* culture was added to 200 µL of GBM or NA mammalian cells in each well. The 96 well plate was centrifuged at 200 x g for 10 min and then incubated at 37°C, 5% CO₂ for 30 min. Non-internalized *Shigella* and mammalian culture media were removed by dumping supernatant into a sterile glass dish containing bleach. The plate was washed four times with PBS using a similar method to remove the supernatant. 200 µL of mammalian cell media containing 20 µg/mL gentamicin was added to each well and incubated at 37°C, 5% CO₂ for 90 min. The media was removed and the cells washed four times with PBS. The cells were fixed by incubating with 4% PFA for 10 min, followed by washing three times with PBS. Mammalian cells were permeabilized with 0.1%

Triton X-100 in PBS for 5 min. Blocking buffer was added and incubated at 4°C overnight. The plate was washed three times with PBS and incubated with anti-*Shigella* antibody in PBS+30mg/mL BSA + 0.1% Triton X-100 (100 µL per well) for 90 min. The plate was again washed with PBS three times. Finally, the plate was incubated with IR₈₀₀ anti-rabbit secondary
5 (1:10,000) in PBS+30mg/mL BSA + 5% donkey serum + 0.1% Triton X-100 for 60 minutes. The plate was again washed with PBS three times and imaged on a Licor Fc scanner using a 2-minute medium resolution scan. A standard curve of *Shigella* was used on each plate to convert IR₈₀₀ signal to the number of bacteria per well. It is important to ensure the moles of anti-*Shigella* antibody greatly exceed the moles of *Shigella* to ensure the saturated binding
10 assumption is valid in order to quantify the number of bacteria per well.

* * *

[0112] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be
15 apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar
20 substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. An engineered *Shigella flexneri* bacterium, wherein the bacterium does not produce a functional Shiga toxin 1 (stx1) or Shiga toxin 2 (stx2), wherein the bacterium comprises at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more mutations from Table 1, and wherein the bacterium selectively internalizes into glioblastoma cells as compared to normal astrocytes or neurons.
2. The bacterium of claim 1, wherein the bacterium comprises functional MsbB1 and MsbB2 genes.
3. The bacterium of claim 1 or 2, wherein the genetic background of the bacterium is *S. flexneri* strain 2475 Serotype 2a.
4. The bacterium of any one of claims 1-3, wherein the bacterium does not internalize into normal brain cells.
5. The bacterium of any one of claims 1-4, wherein the bacterium does not induce an innate immune response in a mammalian subject, preferably a human.
6. The bacterium of any one of claims 1-5, wherein the bacterium does not engage TLR4.
7. The bacterium of any one of claims 1-6, wherein the bacterium further comprises a nucleic acid encoding a recombinant protein under the control of a bacterial promoter or a eukaryotic promoter.
8. The bacterium of claim 7, wherein the bacterial promoter is a constitutive promoter.
9. The bacterium of claim 7, wherein the bacterial promoter is a hypoxia-inducible promoter.
10. The bacterium of claim 7, wherein the eukaryotic promoter is a human promoter.
11. The bacterium of any one of claims 7-9, wherein the recombinant protein is an immunomodulator, a toxin, a cytotoxic factor, a TNF cytokine family member, an anti-angiogenic factor, a cytotoxic polypeptide, or a cytotoxic peptide.
12. The bacterium of any one of claims 1-11, wherein the bacterium further comprises a kill switch.

13. The bacterium of any one of claims 1-12, wherein the bacterium is sensitive to one or more beta-lactams, quinolones, or tetracyclines.
14. A composition comprising the bacterium of any one of claims 1-13.
15. A pharmaceutical formulation comprising the bacterium of any one of claims 1-13.
16. The pharmaceutical formulation of claim 15, wherein the pharmaceutical formulation is formulated for intravenous, intracranial, intratumoral, intrathecal, or intraventricular administration.
17. A method of treating a cancer in a mammalian subject, the method comprising administering a therapeutically effective amount of the pharmaceutical formulation of claim 15 or 16 to the subject.
18. The method of claim 17, wherein the cancer is a brain cancer.
19. The method of claim 18, wherein the brain cancer is a glioblastoma, glioma, meningioma, oligodendroglioma, astrocytoma, medulloblastoma, craniopharyngioma, or ependymoma.
20. The method of claim 19, wherein the brain cancer is a glioblastoma.
21. The method of any one of claims 17-20, wherein the method further comprises administering a second anti-cancer therapy to the subject.
22. The method of claim 21, wherein the second anti-cancer therapy is a chemotherapeutic agent, immunotherapy, radiotherapy, or cytotoxic factor.
23. The method of any one of claims 17-22, wherein the method further comprises administering an antibiotic to the subject.
24. The method of claim 24, wherein the lytic antibiotic is doxycycline, ampicillin, ciprofloxacin, or gentamicin.
25. The method of any one of claims 17-24, wherein the mammalian subject is a human.

26. A panel or population of *Shigella flexneri* clones, wherein each clone (i) does not produce a functional Shiga toxin 1 (stx1) or Shiga toxin 2 (stx2) and (ii) selectively internalizes into glioblastoma cells as compared to normal astrocytes.
27. The panel or population of clones of claim 26, wherein the clones comprise functional MsbB1 and MsbB2 genes.
28. The panel or population of clones of claim 26 or 27, wherein each of the clones comprises, independently, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more mutations to genes from Table 1.
29. The panel or population of clones of any one of claims 26-28, wherein the genetic background of the clones is *S. flexneri* strain 2475 Serotype 2a.
30. The panel or population of clones of any one of claims 26-29, wherein the clones do not internalize into normal brain cells.
31. The panel or population of clones of any one of claims 26-30, wherein the clones do not induce an innate immune response in a mammalian subject, preferably a human.
32. The panel or population of clones of any one of claims 26-31, wherein the clones do not engage TLR4.
33. The panel or population of clones of any one of claims 26-32, wherein the clones further comprise a nucleic acid encoding a recombinant protein under the control of a bacterial promoter or a mammalian promoter.
34. The panel or population of clones of claim 33, wherein the promoter is a constitutive promoter or a quorum sensing promoter.
35. The panel or population of clones of claim 33, wherein the promoter is a hypoxia-inducible promoter.
36. The panel or population of clones of any one of claims 33-35, wherein the recombinant protein is an immunomodulator, a toxin, a cytotoxic factor, a TNF cytokine family member, an anti-angiogenic factor, a cytotoxic polypeptide, or a cytotoxic peptide.

37. The panel or population of clones of any one of claims 26-36, wherein the clones further comprise a kill switch.
38. The panel or population of clones of any one of claims 26-37, wherein the clones are sensitive to one or more beta-lactams, quinolones, or tetracyclines.
39. A method of identifying an engineered *Shigella flexneri* clone for use in treating a patient's cancer, the method comprising (i) incubating each of at least two cultures of cells from the patient's cancer with a single engineered *Shigella flexneri* clone selected from a panel of *Shigella flexneri* clones to allow internalization of the *Shigella flexneri*; (ii) determining the number of *Shigella flexneri* internalized into the cells of each of the at least two cultures of cells; and (iii) selecting an engineered *Shigella flexneri* clone from a culture determined to have a number of internalized *Shigella flexneri* that is at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the highest number of internalized *Shigella flexneri* from among the at least two cultures of cells.
40. The method of claim 39, wherein the cells from the patient's cancer were obtained from a resection sample.
41. The method of claim 39, wherein the cells from the patient's cancer have been passaged *in vitro*.
42. The method of claim 39 wherein the cells from the patient's cancer have not been passaged *in vitro*.
43. The method of any one of claims 39-42, wherein the panel of *Shigella flexneri* clones is the panel of any one of claims 26-38.
44. The method of claim 43, wherein the number of cultures of cells tested equals the number of *Shigella flexneri* clones in the panel.
45. The method of any one of claims 39-44, further comprising (a) incubating normal astrocytes with each engineered *Shigella flexneri* clone selected from the panel of *Shigella flexneri* clones to allow internalization of the *Shigella flexneri*; (b) determining the number of each *Shigella flexneri* clone internalized into the normal astrocytes; and (c) selecting an engineered *Shigella flexneri* clone for which no internalization into normal astrocytes was detected.

46. The method of any one of claims 39-45, further comprising (iv) administering the selected engineered *Shigella flexneri* clone to the patient.

47. The method of any one of claims 39-46, wherein the cancer is a brain cancer.

48. The method of claim 47, wherein the brain cancer is a glioblastoma, glioma, meningioma, oligodendroglioma, astrocytoma, medulloblastoma, craniopharyngioma, or ependymoma.

49. The method of claim 48, wherein the brain cancer is a glioblastoma.

50. The method of any one of claims 46-49, wherein the method further comprises administering a second anti-cancer therapy to the subject.

51. The method of claim 50, wherein the second anti-cancer therapy is a chemotherapeutic agent, immunotherapy, radiotherapy, or cytotoxic factor.

52. A method of generating engineered *Shigella flexneri* for use in treating a patient's cancer, the method comprising (i) incubating a first culture of cells from the patient's cancer with an engineered *Shigella flexneri* clone to allow internalization of the *Shigella flexneri*; (ii) harvesting the internalized *Shigella flexneri*; (iii) incubating a second culture of cells from the patient's cancer with the *Shigella flexneri* harvested in step (ii) to allow internalization of the *Shigella flexneri*; (iv) repeating steps (i) to (iii) until at least 95% of cells in the culture are infected with more than one *Shigella flexneri*.

53. The method of claim 52, wherein the cells from the patient's cancer were obtained from a resection sample.

54. The method of claim 52, wherein the cells from the patient's cancer have been passaged *in vitro*.

55. The method of any one of claims 52-54, wherein steps (i) to (iii) are repeated at least 30 times, at least 35 times, at least 40 times, at least 45 times, or at least 50 times.

56. The method of any one of claims 52-55, further comprising (v) generating a panel of single engineered *Shigella flexneri* clones from the *Shigella flexneri* harvested after step (iv); (vi) incubating each of at least two cultures of cells from the patient's cancer with single engineered *Shigella flexneri* clones selected from the panel of *Shigella flexneri* clones to allow

internalization of the *Shigella flexneri*; (vii) determining the number of *Shigella flexneri* internalized into the cells of each of the at least two cultures of cells; and (viii) selecting an engineered *Shigella flexneri* clone from a culture determined to have a number of internalized *Shigella flexneri* that is at least 50%, at least 60%, at least 70%, at least 80%, or least 90% of the highest number of internalized *Shigella flexneri* from among the at least two cultures of cells.

57. The method of claim 56, wherein the number of cultures of cells tested equals the number of *Shigella flexneri* clones in the panel.

58. The method of any one of claims 52-57, further comprising (a) incubating normal astrocytes with each engineered *Shigella flexneri* clone selected from the panel of *Shigella flexneri* clones to allow internalization of the *Shigella flexneri*; (b) determining the number of each *Shigella flexneri* clone internalized into the normal astrocytes; and (c) selecting an engineered *Shigella flexneri* clone for which no internalization into normal astrocytes was detected.

59. The method of any one of claims 52-58, further comprising administering the selected engineered *Shigella flexneri* clone to the patient.

60. The method of any one of claims 52-59, wherein the cancer is a brain cancer.

61. The method of claim 60, wherein the brain cancer is a glioblastoma, glioma, meningioma, oligodendroglioma, astrocytoma, medulloblastoma, craniopharyngioma, or ependymoma.

62. The method of claim 61, wherein the brain cancer is a glioblastoma.

63. The method of any one of claims 59-62, wherein the method further comprises administering a second anti-cancer therapy to the subject.

64. The method of claim 63, wherein the second anti-cancer therapy is a chemotherapeutic agent, immunotherapy, radiotherapy, or cytotoxic factor.

65. A panel or population of *Shigella flexneri* clones of any one of claims 52-58.

66. The panel or population of claim 65, wherein each clone (i) does not produce a functional Shiga toxin 1 (stx1) or Shiga toxin 2 (stx2) and (ii) selectively internalizes into glioblastoma cells as compared to normal astrocytes.
67. The panel or population of claim 65 or 66, wherein the clones comprise functional MsbB1 and MsbB2 genes.
68. The panel or population of any one of claims 65-67, wherein each of the clones comprises, independently, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more mutations from genes of Table 1.
69. The panel or population of any one of claims 65-68, wherein the genetic background of the clones is *S. flexneri* strain 2475 Serotype 2a.
70. The panel or population of any one of claims 65-69, wherein the clones do not internalize into normal brain cells.
71. The panel or population of any one of claims 65-70, wherein the clones do not induce an innate immune response in a human.
72. The panel or population of any one of claims 65-71, wherein the clones do not engage TLR4.
73. The panel or population of any one of claims 65-72, wherein the clones further comprise a nucleic acid encoding a recombinant protein under the control of a bacterial promoter or a eukaryotic promoter.
74. The panel or population of claim 73, wherein the promoter is a constitutive promoter or a quorum sensing promoter.
75. The panel or population of claim 73, wherein the promoter is a hypoxia-inducible promoter.
76. The panel or population of any one of claims 73-75, wherein the recombinant protein is an immunomodulator, a toxin, a cytotoxic factor, a TNF cytokine family member, an anti-angiogenic factor, a cytotoxic polypeptide, or a cytotoxic peptide.

77. The panel or population of any one of claims 65-76, wherein the clones further comprise a kill switch.

78. The panel or population of any one of claims 65-77, wherein the clones are sensitive to one or more beta-lactams, quinolones, or tetracyclines.