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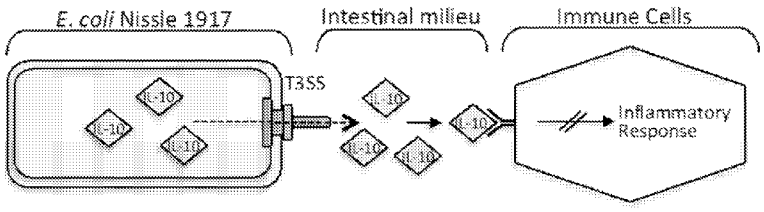

Fig. 1

(57) Abstract: Described herein are compositions and methods relating to engineered bacteria which have a modified Type 3 Secretion System (T3SS) which permits them to deliver proteins to the extracellular space (e.g., as opposed to the intracellular space of a target cell as done with a wild-type T3SS). In some embodiments, the engineered bacteria comprise a transgenic T3SS. In some embodiments, the delivered protein is non-native or transgenic with respect to the engineered bacteria.

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METHODS AND COMPOSITIONS RELATING TO ENGINEERED MICROBIAL CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/292,961 filed February 9, 2016, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant Nos. R01GM094941 and R21AI103882 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 8, 2017, is named 030258-086071_SL.txt and is 194,046 bytes in size.

TECHNICAL FIELD

[0004] The technology described herein relates generally to engineered microbial cells, e.g., non-pathogenic bacterial cells that secrete payload polypeptides into the extracellular space by means of a secretion system engineered from a type 3 secretion system (T3SS) or non-pathogenic bacterial cells that have been engineered to carry an engineered type 3 secretion system (T3SS) to translocate payloads into cells.

BACKGROUND

[0005] As part of the disease process, a number of pathogenic bacteria deliver toxins to their host cells. Some of bacteria have evolved a structure referred to as a Type 3 Secretion System (T3SS). The T3SS is essentially a needle that the bacterium uses to penetrate the host cell. The bacterium then injects the toxins through the T3SS needle into the host cell.

[0006] We have previously described engineered non-pathogenic cells that use this system for delivering some kinds of payloads into cells (see, e.g. WO/2014/138324).

SUMMARY

[0007] The T3SS's can be utilized in engineered bacteria such that instead of delivering toxins, the bacteria are delivering therapeutic molecules. However, such a direct adaptation of the T3SS machinery means that the therapeutic molecules will necessarily be delivered to

the inside of the host cell (the intracellular space). Large amount of signaling activity occurs on the exterior surface of host cells (in the extracellular space) and thus, a number of therapeutic targets are not accessible with such engineered bacteria. The inventors have surprisingly found that by specifically modifying the secretion systems, i.e., by engineering the T3SS, delivery of therapeutic molecules to the extracellular space can be achieved.

[0008] The inventors have also surprisingly found that non-pathogenic bacteria, e.g., bacteria that do not naturally express or comprise T3SS (e.g., commensal bacteria) but have been engineered to express a modified T3SS can be used to translocate the variable domains of single-domain antibodies, such as VHH or nanobodies.

[0009] In one aspect of any of the embodiments, described herein is an engineered, non-pathogenic, gram negative microbial cell comprising: a first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS); wherein the TDESS comprises at least virB; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiD; mxiA; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40; and a second nucleic acid sequence encoding an T3SS-compatible payload polypeptide. In some embodiments, the cell does not comprise or express at least one of: IpaB; IpaD; and MxiC. In some embodiments, the cell does not comprise or express at least one of: IpaB and IpaD; and MxiC. In some embodiments, the cell does not comprise or express IpaB; IpaD; and MxiC. In some embodiments, the cell does not comprise or express IpaB; IpaD; IpaC; and MxiC. In some embodiments, the cell has a mutated MxiH. In some embodiments, the cell has a D73A mutation in MxiH.

[0010] In some embodiments, the second nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding an T3SS-compatible payload polypeptide. In some embodiments, the inducible promoter sequence is regulated by a master T3SS regulator (i.e., master T3SS transcriptional regulator). In some embodiments, the inducible promoter sequence comprises a (T3SS)-associated promoter or promoter element. In some embodiments, the T3SS-associated promoter or promoter element is a MxiE recognition sequence. In some embodiments, the cell comprises a third nucleic acid sequence encoding a master T3SS regulator (i.e.., master T3SS transcriptional regulator). In some embodiments, the master T3SS regulator (i.e., master T3SS transcriptional regulator) is selected from the group consisting of: VirB and VirF. In some embodiments, the third nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding a master T3SS regulator (i.e., master T3SS transcriptional regulator).

In some embodiments, the inducible promoter is selected from the group consisting of: an arabinose-inducible promoter; pBAD arabinose-inducible promoter; tumor-induced promoters; ansB promoter; pflE promoter; napF promoter; and an inflammation-induced promoter.

[0011] In some embodiments, the TDESS comprises at least: virB; acp; ipaA; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiE; mxiD; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40. In some embodiments, the TDESS comprises polypeptides endogenous to a bacterium selected from the group consisting of: *Shigella* spp; *Salmonella* spp; enteropathogenic *E. coli*; and *Yersinia* spp. In some embodiments, the first nucleic acid sequence is located on a plasmid. In some embodiments, the first nucleic acid sequence is located on a chromosome. In some embodiments, the second nucleic acid sequence is located on a plasmid. In some embodiments, the second nucleic acid sequence is located on a chromosome.

[0012] In some embodiments, the T3SS-compatible payload polypeptide comprises a T3SS secretion sequence. In some embodiments, the T3SS-compatible payload polypeptide comprises an N-terminal T3SS secretion sequence. In some embodiments, the T3SS-compatible payload polypeptide comprises a OspC3 T3SS secretion sequence.

[0013] In some embodiments, the T3SS-compatible payload polypeptide comprises an anti-inflammatory polypeptide. In some embodiments, the anti-inflammatory polypeptide is IL-10 or IL-27.

[0014] In some embodiments, the T3SS-compatible payload polypeptide comprises an antibody reagent. In some embodiments, the antibody reagent is selected from the group consisting of: a nanobody; a VNA (VHH-based neutralizing agent); a multimeric VHH reagent; and a VHH. In some embodiments, the cell comprises at least one further nucleic acid sequence encoding an additional T3SS-compatible payload polypeptide comprising an antibody reagent. In some embodiments, the one or more antibody reagents form a multimeric complex. In some embodiments, the multimeric complex is multispecific.

[0015] In some embodiments, the antibody reagent specifically binds to a cancer cell marker. In some embodiments, the antibody reagent specifically binds to a cancer checkpoint polypeptide. In some embodiments, the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 reagent. In some embodiments, the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 VNA or VHH.

[0016] In some embodiments, the antibody reagent specifically binds to an inflammatory cytokine receptor or an inflammatory cytokine. In some embodiments, the antibody reagent binds to a molecule selected from the group consisting of: TNFα, IL-8; IL-6, IL-18, IL-21, Il-33 and IL-13. In some embodiments, the antibody reagent specifically binds to a bacterial toxin. In some embodiments, the bacterial toxin is an *E. coli* or *C. difficile* toxin. In some embodiments, the bacterial toxin is selected from the group consisting of: shiga toxin; *C. difficile* toxin A (TcdA); *C. difficile* toxin B (TcdB); cholera toxin; anthrax toxin; and botulinum toxin.

[0017] In some embodiments, the T3SS-compatible payload polypeptide comprises a toxin. In some embodiments, the T3SS-compatible payload polypeptide comprises an antigen.

[0018] In some embodiments, the microbial cell is engineered from a microbial cell selected from the group consisting of: *E. coli* NISSLE 1917 (EcN); *E. coli* K12; MP; HS; and derivative strains thereof. In some embodiments, the strain which is derivative of *E. coli* K12 is selected from the group consisting of: *E. coli* DH10β and *E. coli* DH5α. In some embodiments, the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN). In some embodiments, the microbial cell is engineered from a commensal intestinal microbial cell. In some embodiments, the commensal intestinal microbial cell is *E. coli* NISSLE 1917 (EcN). In some embodiments, the non-pathogenic microbial cell is engineered from a pathogenic microbial cell organism by deletion or mutation of one or more T3SS components. In some embodiments, the one or more T3SS components is selected from the group consisting of: a toxin; a T3SS effector; a structural T3SS polypeptide; and a master regulator of T3SS components (i.e. master T3SS transcriptional regulator). In some embodiments, the pathogenic microbial cell is selected from the group consisting of: *Salmonella* spp.; *Shigella* Spp; and*Yersinia* spp. In some embodiments, the pathogenic microbial cell is selected from the group consisting of *Salmonella typhimurium* SPI1and *Shigella felxneri* mxi-spa.

[0019] In some embodiments, the microbial cell has been: contacted with a mutagenic treatment; and selected for increased secretion. In some embodiments, the cell has been: contacted with a mutagenic treatment; and selected for increased secretion of the T3SS-compatible payload polypeptide.

[0020] In some embodiments, the cell further comprises a nucleic acid sequence encoding one or more polypeptides that increase adhesion to a target cell. In some embodiments, the polypeptides that increase adhesion to the target cell comprise Tir and intimin. In some

embodiments, the polypeptide that increases adhesion to the target cell is selected from a group consisting of: a bacterial adhesion; Afa1; AIDA; invasion; an antibody reagent specific for an extracellular epitope of a target cell polypeptide; and a single chain antibody specific for an extracellular epitope of a target cell polypeptide.

[0021] In some aspects of any of the embodiments, described herein is a method of introducing a polypeptide into a target tissue or organism, the method comprising contacting the target tissue or organism with a microbial cell as described herein.

[0022] In some aspects of any of the embodiments, described herein is a method of reducing inflammation in a subject, the method comprising administering a microbial cell as described herein to a subject in need thereof. In some embodiments, the inflammation is inflammation of the gastrointestinal tract. In some embodiments, the subject is in need of treatment for a condition selected from the group consisting of asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis. In some embodiments, the subject is a subject in need of treatment for inflammatory bowel disease. In some embodiments, the microbial cell is administered orally.

[0023] In some aspects of any of the embodiments, described herein is a method of treating cancer in a subject, the method comprising administering an microbial cell as described herein to a subject in need thereof. In some embodiments, the microbial cell is administered systemically. In some embodiments, the microbial cell is administered intratumorally. In some embodiments, the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally. In some embodiments, the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).

[0024] In some aspects of any of the embodiments, described herein is a method of treating an intestinal infection in a subject, the method comprising administering a microbial cell as described herein to a subject in need thereof. In some embodiments, the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin. In some embodiments, the intestinal infection is a *C. difficile* infection and/or the subject has *C. difficile* colitis and the toxin is TcdA and/or TcdB. In some embodiments, the intestinal infection is cholera and the toxin is cholera toxin. In some embodiments, the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin. In some embodiments, the intestinal infection is botulism and the toxin is botulinum toxin. In some embodiments, the microbial cell is administered orally.

[0025] In some embodiments, secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master regulator. In some embodiments, the compound is arabinose.

[0026] In some aspects of any of the embodiments, described herein is a method for delivering a polypeptide into a) the extracellular milieu of a subject's gastrointestinal tract or b) the extracellular milieu of a subject's tumor , the method comprising contacting administering a microbial cell as described herein to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Fig. 1 depicts a schematic of the use of a commensal bacteria-based T3SS to deliver IL-10 into the intestines, leading to suppression of the inflammatory response in IBD patients.

[0028] Figs. 2A-2B demonstrate that modified alleles of MyoD are recognized as type 3 secreted substrates and directly delivered into mammalian cells. Fig. 2A depicts a schematic of MyoD fused to a 50 amino acid secretion sequence and separated by a flexible glycine linker. Fig. 2B depicts delivery of MyoD into MEFs exposed to T3-*E. coli* expressing each of the designated MyoD alleles. After 1 hr, MEF cell lysates were collected and probed with anti-MyoD and anti-actin antibodies. SS, secretion sequence. S, supernatant. L, whole cell lysate. See Fig. 7 for a secretion assay of the secretion sequence library of 50 amino acid-MyoD fusion proteins in T3-*E.coli*.

[0029] Fig. 3 demonstrates that T3-E. *coli* express and secrete a variety of target proteins modified by a Shigella type 3 secretion sequence and FLAG tag. S, supernatant.

[0030] Figs. 4A-4B depict a model of the different T3-EcN strains and how they work. Fig. 4A depictd a schematic demonstrating that wild type T3SS are held in an off position by the tip complex and MxiC. Loss of either the tip complex or MxiC causes consitutive secretion in to the outside of the bacterial cell. Fig. 4B demonstrates that secretion profiles of the different Shigella strains are displayed on a commassie gel. SepA, loading control.

[0031] Fig. 5 depicts a schematic of strategy to capture and transfer T3SS operons from the *Shigella* virulence plasmid onto an autonomously replicating plasmid that can be introduced carried in *E. coli* as a plasmid or integrated into its chromosome.

[0032] Fig. 6 demonstrates regulatable expression of T3SS in E. *coli* (T3Ec). Immunoblots of designated strains grown under conditions that induce type 3 secretion. IPTG or arabinose added for strains that carry regulatable type 3 transcriptional regulators (VirF or

VirB). Plots probed with antibodies that recognize two secreted components of the type 3 secretion apparatus, IpaB and IpaD.

[0033] Fig. 7 demonstrates a screening platform to identify sequences sufficient to define heterologous proteins as secreted substrates. Immunoblots of T3Ec_VirFend expressing MyoD fused each of the designated secretion signals grown under conditions that induce type 3 secretion. Plots probed with designated antibodies. Supernatant (S) and whole cell lysates (L) shown.

[0034] Fig. 8 depicts a schematic of a tunable bacterial protein delivery system composed of 3 parts: (1) The delivery apparatus, a region of DNA that encodes all the genes required to assemble a functional type 3 secretion system (T3SS). (2) the activator, VirF the master Shigella transcription activator, controls expression of the T3SS and (3) The type 3 secreted substrates, target therapeutic payloads fused to a type 3 secretion sequence (SS) at their N-termini.

[0035] Fig. 9 demonstrates that T3EcN can recognize VHH as secreted substrates. Immunoblots of the type 3 secreted substrates of T3EcN grown under conditions that induce type 3 secretion are shown. (top panel) Fusion of 4 different type 3 secretion sequences to the N-terminus of a representative VHH (VHH1) identified the OspC3 sequence as sufficient for secretion in a standard secretion assay. (bottom panel) Fusion of the OspC3 secretion sequence to 4 different VHH results in all of their secretion from T3EcN.

[0036] Fig. 10 depicts the plate based secretion assay distinguishes between substrates secreted at low, intermediate, and high levels, as demonstrated by the secretion of 4 FLAG-tagged native effectors from Shigella. This assay can be easily automated and scaled up in genetic screens using robots.

[0037] Fig. 11 demonstrates that mT3sec E. *coli* constitutively secrete type III secreted substrates. The designated bacterial strains were grown under conditions that induce expression of the type III secretion. The bacteria were then transferred to PBS +/- Congo red (CR), a dye that is normally require for the secretion of effectors.

[0038] Fig. 12 demonstrates secretion of a single variable-domain heavy-chain region (VHH or nanobody) and heterometric VHH-neutralizing agents (VNA)s. Both wild type Shigella flexneri and mT3 E. coli, but not wild type E. coli, recognize VHH and VNAs as secreted substrates. Bacterial strains were grown under conditions that induce type III secretion. The heteromeric VHH (VNAs) are fused to the OspC3 secretion sequence at their N-terminus and FLAG-tagged at the C-terminus in this standard secretion assay.

Immunoblots are probed with anti-FLAG antibody. S, supernatant. P, pellet of whole cell lysate.

DETAILED DESCRIPTION

[0039] In some aspects, described herein are engineered microbial cells that comprise an altered or modified type 3 secretion system (T3SS) that, instead of injecting proteins into a target cell, surprisingly secrete them into the extracellular space. This approach allows for the delivered proteins to interact with cell surface receptors and/or other molecules, e.g., toxins or signaling molecules, in the extracellular space, such as the gut lumen. Additionally, as the engineered microbial cells described herein can be commensal, non-pathogenic cells, the cells can persist in a subject and provide continued delivery of proteins directly to the target area, such as in the gut, providing improved efficacy and reduced side effects compared to traditional methods of administering therapeutic molecules themselves. It is also known that some non-pathogenic bacteria or commensal bacteria, which do not naturally express T3SS, migrate from the gut or blood stream to tumors, and the inventors also surprisingly found that engineering such bacteria to express the modified T3SS system wherein the functionality to deliver payloads into cells has been removed allows targeted delivery of peptides into the extracellular space in and/or surrounding tumors thus reducing the side effects of any anti-cancer peptides and increases the effectiveness of any such anti-cancer therapy. The inventors also found that single domain antibodies, or nanobodies, can be delivered either with bacteria, such as commencal non-pathogenic bacterial cells, with the functional T3SS capable of injecting a payload into a cell or with the altered T3SS without the capability to inject the payload into the cell, depending on whether the target is inside the cell or outside the cell either on cell surface or in the extracellular space.

[0040] Accordingly, in one aspect of any of the embodiments, described herein is an engineered, non-pathogenic or commensal gram negative microbial cell, wherein the cell in its natural state does not comprise T3SS, the cell comprising: a) a first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS) and b) a second nucleic acid sequence encoding a T3SS-compatible payload polypeptide. In one aspect of any of the embodiments, described herein is an engineered, non-pathogenic, gram negative microbial cell comprising: a) a first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS) b) a second nucleic acid sequence encoding a T3SS-compatible payload

polypeptide; and c) a third nucleic acid sequence encoding a master T3SS regulator. As used herein, "T3SS-derived extracellular secretion system" or "TDESS" refers to a system of proteins obtained and/or derived from a T3SS that can form a structure capable of delivering polypeptides from the microbial cell into the extracellular space instead of into the intracellular space of a target cell. In some embodiments of any of the aspects, a TDESS cannot deliver polypeptides from the microbial cell into the intracellular space of a target cell. In some embodiments, a microbial cell which does not naturally comprise a T3SS (e.g., a non-pathogenic and/or commensal bacteria) is engineered to comprise a TDESS.

[0041] In some embodiments of any of the aspects, the TDESS comprises at least virB; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiD; mxiA; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40 or homologs thereof. In some embodiments of any of the aspects, the TDESS comprises at least virB; virF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiD; mxiA; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40 or homologs thereof. In some embodiments of any of the aspects, the TDESS comprises at least: virB; acp; ipaA; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiE; mxiD; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40 or homologs thereof. In some embodiments of any of the aspects, the TDESS comprises at least: virB; virF; acp; ipaA; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiE; mxiD; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40 or homologs thereof. Homologs of any of the foregoing from a given species are readily identified by one of skill in the art, e.g,, by querying a database of sequence information (e.g., using NCBI BLAST) with the gene name and/or sequence of one of the given genes and selecting the closest matching sequence found in the genome of the given species. In some embodiments of any of the aspects, the homolog has at least 80%, at least 85%, at least 90%, at least 95%, at least 98% sequence identity with the nucleic acid or polypeptide sequences described herein. T3SSs are known in a number of species and individual polypeptides of an engineered TDESS can be obtained from any T3SS. In some embodiments of any of the aspects, the TDESS polypeptides can be, or be derived from, T3SS polypeptides endogenous to a *Shigella* spp; *Salmonella* spp; enteropathogenic *E. coli*; or *Yersinia* spp. bacterium. In some embodiments of any of the aspects described herein, a homolog can be a polypeptide with the same function, functional characteristics, and/or activity as the reference polypeptide. By way of non-limiting example, a homolog with the same function as one of the T3SS polypeptides described herein can be identified by

engineering a bacteria to not express the given polypeptide and to instead express a putative functional homolog and then measuring the ability of the bacteria to secrete a payload polypeptide. If the bacteria retain at least 10% of the reference ability to secrete the payload polypeptide, the putative functional homolog is demonstrated to be a functional homolog. In some embodiments, a functional homolog has at least 10% of the activity of the reference polypeptide, e.g. 10% or more, 20% or more, 30% or more, 50% or more, 75% or more, 80% or more, 90% or more, 95% or more, or 100% or more of the activity of the reference polypeptide.

[0042] In some embodiments of any of the aspects, the TDESS comprises at least a needle monomer polypeptide (e.g. MxiH (e.g., NCBI Gene ID No: 1238256)); PrgI (e.g., NCBI Gene ID No: 1254396; YscF (e.g., NCBI Gene ID No: 1172700)); and/or EscF (e.g., NCBI Gene ID No: 8873370)), an inner rod polypeptide (e.g. MxiI (e.g., NCBI Gene ID No: 1238257)); PrgJ (e.g., NCBI Gene ID No: 1254395)); YscI (e.g., NCBI Gene ID No: 2767498)); and/or EscI (e.g., NCBI Gene ID No: 8219253)), ring polypeptides; a ruler polypeptide (e.g. Spa32 (e.g., NCBI Gene ID No: 876502); InvJ (e.g., NCBI Gene ID No: 1254415); YscP (e.g., NCBI Gene ID No: 5798302); and/or Orf16 (e.g., NCBI Gene ID No: 8219247)); and an ATPase (e.g. Spa47 (e.g., NCBI Gene ID No: 876429); InvC (e.g., NCBI Gene ID No: 1254417); YscN (e.g., NCBI Gene ID No: 10216379); and/or SepB (also known as EscN) (e.g., NCBI Gene ID No: 8873386)); and one or more of a switch polypeptide (e.g. Spa40 (e.g., NCBI Gene ID No: 876433); SpaS (e.g., NCBI Gene ID No: 1254410); YscU (e.g., NCBI Gene ID No: 2767517); and/or EscU (e.g., NCBI Gene ID No: 7062687)). In some embodiments, a TDESS can further comprise one or more translocators (e.g. IpaC (e.g., NCBI Gene ID No: 876448); SipB (e.g., NCBI Gene ID No: 1254408); SipC (e.g., NCBI Gene ID No: 1254407); YopB (e.g., NCBI Gene ID No: 1449456); YopD (e.g., NCBI Gene ID No: 1449455); EspD (e.g., NCBI Gene ID No: 885777); and/or EspB (e.g., NCBI Gene ID No: 8474872)) and/or a chaperone for the one or more translocators (e.g. IpgC (e.g., NCBI Gene ID No: 1238043); SicA (e.g., NCBI Gene ID No: 1254409); SycD (e.g., NCBI Gene ID No: 2767486); and/or CesD (e.g., NCBI Gene ID No: 7063867)). In some embodiments, a TDESS does not comprise one or more translocators (e.g. IpaC (e.g., NCBI Gene ID No: 876448); SipB (e.g., NCBI Gene ID No: 1254408); SipC (e.g., NCBI Gene ID No: 1254407); YopB (e.g., NCBI Gene ID No: 1449456); YopD (e.g., NCBI Gene ID No: 1449455); EspD (e.g., NCBI Gene ID No: 885777); and/or EspB (e.g., NCBI Gene ID No: 8474872)) and/or a chaperone for the one or more translocators (e.g. IpgC (e.g., NCBI

Gene ID No: 1238043); SicA (e.g., NCBI Gene ID No: 1254409); SycD (e.g., NCBI Gene ID No: 2767486); and/or CesD (e.g., NCBI Gene ID No: 7063867)).

[0043] A TDESS can be constructed from a T3SS by omitting and/or mutating key proteins that mediate protein delivery into a target cell. In some embodiments of any of the aspects, a microbial cell comprising a TDESS does not comprise or express at least one of IpaB (Invasion Plasmid Antigen B); IpaD Invasion Plasmid Antigen D); IpaC Invasion Plasmid Antigen C); and MxiC(Membrane eXpression of Invasion plasmid antigens C). In some embodiments of any of the aspects, a microbial cell comprising a TDESS does not comprise or express at least one of IpaB; IpaD; IpaC; and MxiC. In some embodiments of any of the aspects, a microbial cell comprising a TDESS does not comprise or express IpaB; IpaD; and MxiC. In some embodiments of any of the aspects, a microbial cell comprising a TDESS does not comprise or express IpaB; IpaD; IpaC; and MxiC. In some embodiments, a microbial cell comprising a TDESS does not comprise or express a gatekeeper polypeptide (e.g. MxiC (e.g., NCBI Gene ID No: 876426); InvE (e.g., NCBI Gene ID No: 1254420); YopN (e.g., NCBI Gene ID No: 2767534); and/or SepL (e.g., NCBI Gene ID No: 8873375)). In some embodiments, a microbial cell comprising a TDESS does not comprise or express a needle-tip polypeptide (e.g. IpaD (e.g., NCBI Gene ID No: 876444); SipD (e.g., NCBI Gene ID No: 1254406); LcrV (e.g., NCBI Gene ID No: 1172676); and/or EspA (e.g., NCBI Gene ID No: 960865)).

[0044] In some embodiments of any of the aspects, a microbial cell comprising a TDESS comprises or expresses a mutated form of MxiH that constitutively secretes T3SS effectors. Such mutations can be accmploished by mutating residues in the PSNP loop or residues P44 or Q51 and are described further in , e.g., Kenjale et al. J. Biol. Chem. 2005 280:42929-42937; which is incorporated by reference herein in its entirety. In some embodiments of any of the aspects, the mutated form of MxiH is a D73A mutation in MxiH.

[0045] In some embodiments of any of the aspects, the first nucleic acid sequence is located on a plasmid. In some embodiments of any of the aspects, the one or more genes encoding a TDESS polypeptide can be located on a plasmid. In some embodiments of any of the aspects, the first nucleic acid sequence is located on a chromosome (e.g. a naturally-ocurring chromosome, a modified endogenous chromosome, or a bacterial artificial chromosome (BAC)). In some embodiments of any of the aspects, the one or more genes encoding a TDESS polypeptide can be located on a chromosome. In some embodiments of any of the aspects, the first nucleic acid sequence can comprise one or more operons, e.g. one

operon, two operons, three operons, or more operons. In some embodiments, the first nucleic acid sequence can comprise one or more separate sequences and/or molecules (e.g. a portion of the genes are found on one plasmid and another portion of the genes are found on a second plasmid). In some embodiments, the first nucleic acid sequence can be integrated into the chromosome using, for example, landing pad technology, see, e.g. Kuhlman and Cox, 2010 Nucleic Acids Research 38:e92; which is incorporated by reference herein in its entirety.

[0046] A T3SS-compatible payload polypeptide refers to any polypeptide that can be transported out of the microbial cell by a T3SS and/or TDESS and which is exogenous to the microbial cell, i.e., not encoded by the microbial cell before introduction of said polypeptide-encoding nucleic acid into the microbial cell. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide can be exogenous to a target cell (i.e., not encoded by the target cell), target tissue or organ, and/or target organism or toxin. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide can be ectopic to a target cell, target tissue, and/or target organism. A payload polypeptide can be from any source, e.g. the polypeptide can have a prokaryotic origin, a eukaryotic origin, or a synthetic origin. A payload polypeptide can be a naturally-occuring polypeptide or a mutant and/or variant thereof. In a variant payload polypeptide, one or more residues can be altered, deleted, and/or added as compared to a naturally-occuring and/or wild-type polypeptide so long as the function remains substantially the same.

[0047] The term "exogenous" refers to a substance present in a cell other than its native source. The term "exogenous" when used herein can refer to a nucleic acid (e.g. a nucleic acid encoding a payload polypeptide) or a polypeptide (e.g., a payload polypeptide) that has been introduced by a process involving the hand of man into a biological system such as a cell or organism in which it is not normally found and one wishes to introduce the nucleic acid or polypeptide into such a cell or organism. Alternatively, "exogenous" can refer to a nucleic acid or a polypeptide that has been introduced by a process involving the hand of man into a biological system such as a cell or organism in which it is found in low amounts and one wishes to increase the amount of the nucleic acid or polypeptide in the cell or organism. In contrast, the term "endogenous" refers to a substance that is native to the biological system or cell (e.g. the microbial cell and/or target cell). As used herein, "ectopic" refers to a substance that is found in an unusual location and/or amount. An ectopic substance can be one that is normally found in a given cell, but at a much lower amount and/or at a different

time. Ectopic also includes substance, such as a polypeptide or nucleic acid, that is not naturally found or expressed in a given cell in its natural environment.

[0048] Naturally-occurring T3SS substrates comprise a secretion signal within the first 20 amino acids of the polypeptide. Certain naturally-occuring T3SS substrates comprise a chaperone-binding domain within the first 50 amino acids of the polypeptide. Accordingly, in some embodiments, the payload polypeptide can comprise an N-terminal type three secretion system (T3SS) signal, wherein the T3SS signal comprises a T3SS secretion signal. In some embodiments, the payload polypeptide can comprise an N-terminal type three secretion system (T3SS) signal, wherein the T3SS signal comprises the first 20 amino acids of a naturally occurring T3SS substrate. In some embodiments, the payload polypeptide can comprise an N-terminal type three secretion system (T3SS) signal, wherein the T3SS signal comprises a T3SS chaperone-binding domain. In some embodiments, the payload polypeptide can comprise T3SS chaperone-binding domain and an N-terminal type three secretion system (T3SS) signal, wherein the T3SS signal comprises from about the first 50 to about the first 70 amino acids of a naturally occurring T3SS substrate. In some embodiments, in the context of a T3SS signal polypeptide, the term "about" can refer to $\pm$ 3 amino acids. In some embodiments, in the context of a T3SS signal polypeptide, the term "about" can refer to $\pm$ 2 amino acids. In some embodiments, in the context of a T3SS signal polypeptide, the term "about" can refer to $\pm$ 1 amino acid.

[0049] Examples of T3SS secretion signals and chaperone-binding domains are known in the art, see, e.g. Costa et al. Mbio 2012 3:e00243-11 or Schmitz et al. Nat Methods 2009 6:500-2; which described the signals and domains of *Shigella* effectors and which is incorporated by reference herein in its entirety. Additional examples are known in the art, e.g. Sory et al. PNAS 1995 92:11998-20002; which is is incorporated by reference herein in its entirety.

[0050] In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a T3SS secretion sequence. In some embodiments of any of the aspects,

the T3SS-compatible payload polypeptide comprises an N-terminal T3SS secretion sequence. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a OspC3 T3SS secretion sequence. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a nanobody, VNA, or VHH and a OspC3 T3SS secretion sequence.

[0051] In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises an anti-inflammatory polypeptide, e.g., a human anti-inflammatory

polypeptide and/or an anti-inflammatory polypeptide that is ectopic to the target cell, tissue, or organism. An anti-inflammtory polypeptide can be a polypeptide that suppresses inflammatory signaling in the target cell, tissue, or organism, including but not limited to, polypeptides which are part of the endogenous signaling pathways in the target cell, tissue, or organism. Non-limiting examples of anti-inflammatory polypeptides can include, IL-10 (e.g., human IL-10, NCBI Gene ID: 3586) or IL-27 (e.g., human IL-27, NCBI Gene ID: 246778).

[0052] In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises an antibody reagent. As used herein an "antibody" refers to IgG, IgM, IgA, IgD or IgE molecules or antigen-specific antibody fragments thereof (including, but not limited to, a Fab, $F(ab')_2$, Fv, disulphide linked Fv, scFv, single domain antibody, closed conformation multispecific antibody, disulphide-linked scfv, diabody), whether derived from any species that naturally produces an antibody, or created by recombinant DNA technology; whether isolated from serum, B-cells, hybridomas, transfectomas, yeast or bacteria.

[0053] As described herein, an "antigen" is a molecule that is bound by a binding site on an antibody agent. Typically, antigens are bound by antibody ligands and are capable of raising an antibody response *in vivo*. An antigen can be a polypeptide, protein, nucleic acid or other molecule or portion thereof. The term "antigenic determinant" refers to an epitope on the antigen recognized by an antigen-binding molecule, and more particularly, by the antigen-binding site of said molecule.

[0054] As used herein, the term "antibody reagent" refers to a polypeptide that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence and which specifically binds a given antigen. Antibody reagents as used herein do not comprise a S-S bond or require a post-translational modification in order to specifically bind their target antigen. In some aspects, the antibody reagents have a molecular weight of 200kDa or lower. In some aspects, the antibody reagents have a molecular weight of 150kDa or lower. In some aspects, the antibody reagents have a molecular weight of 100kDa or lower. In some aspects, the antibody reagents have a molecular weight of 90kDa or lower. In some aspects, the antibody reagents have a molecular weight of between 50 and 100kDa or between 50 and 150 kDa

[0055] An antibody reagent can comprise an antibody or a polypeptide comprising an antigen-binding domain of an antibody. In some embodiments, an antibody reagent can comprise a monoclonal antibody or a polypeptide comprising an antigen-binding domain of a monoclonal antibody. For example, an antibody can include a heavy (H) chain variable

region (abbreviated herein as VH), and a light (L) chain variable region (abbreviated herein as VL). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term "antibody reagent" encompasses antigen-binding fragments of antibodies (e.g., single chain antibodies, Fab and sFab fragments, F(ab')2, Fd fragments, Fv fragments, scFv, and domain antibodies (dAb) fragments (see, e.g. de Wildt et al., Eur J. Immunol. 1996; 26(3):629-39; which is incorporated by reference herein in its entirety)) as well as complete antibodies. An antibody can have the structural features of IgA, IgG, IgE, IgD, IgM (as well as subtypes and combinations thereof). Antibodies can be from any source, including mouse, rabbit, pig, rat, and primate (human and non-human primate) and primatized antibodies. Antibodies also include midibodies, humanized antibodies, chimeric antibodies, and the like.

[0056] The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" ("FR"). The extent of the framework region and CDRs has been precisely defined (see, Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917; which are incorporated by reference herein in their entireties). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[0057] The terms "antigen-binding fragment" or "antigen-binding domain", which are used interchangeably herein are used to refer to one or more fragments of a full length antibody that retain the ability to specifically bind to a target of interest. Examples of binding fragments encompassed within the term "antigen-binding fragment" of a full length antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; (iii) an Fd fragment consisting of the VH and CH1 domains; (iv) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546; which is incorporated by reference herein in its entirety), which consists of a VH or VL domain; and (vi) an isolated complementarity determining region (CDR) that retains specific antigen-binding functionality.

[0058] In some embodiments, an antibody reagent can be a single domain antibody. A

single-domain antibody (sdAb, called Nanobody by Ablynx, the developer of these moleculer) is an antibody fragment consisting of a single monomeric variable antibody domain. Like a whole antibody, it is able to bind selectively to a specific antigen. With a molecular weight of only about 12–15 kDa, single-domain antibodies are much smaller than common antibodies (150–160 kDa) which are composed of two heavy protein chains and two light chains, and even smaller than Fab fragments (~50 kDa, one light chain and half a heavy chain) and single-chain variable fragments (~25 kDa, two variable domains, one from a light and one from a heavy chain.

[0059] As used herein, the term "specific binding" refers to a chemical interaction between two molecules, compounds, cells and/or particles wherein the first entity binds to the second, target entity with greater specificity and affinity than it binds to a third entity which is a non-target. In some embodiments, specific binding can refer to an affinity of the first entity for the second target entity which is at least 10 times, at least 50 times, at least 100 times, at least 500 times, at least 1000 times or greater than the affinity for the third nontarget entity. A reagent specific for a given target is one that exhibits specific binding for that target under the conditions of the assay being utilized.

[0060] Additionally, and as described herein, a recombinant humanized antibody, e.g., single domain antibody (VHH) can be further optimized to decrease potential immunogenicity, while maintaining functional activity, for therapy in humans. In this regard, functional activity means a polypeptide capable of displaying one or more known functional activities associated with a recombinant antibody or antibody reagent thereof as described herein. Such functional activities include, e.g. the ability to bind to a target.

[0061] In some embodiments of any of the aspects, the antibody reagent can be a single chain antibody reagent, e.g., one which, as a single polypeptide chain, can specifically bind the target antigen. In some embodiments of any of the aspects, the antibody reagent can be a VNA or a VHH. VHH's (camelid heavy-chain-only Ab V_H domain) are antibodies produced by camelids and which comprise only a heavy chain. VHH's can be naturally-occuring, naturally-produced, engineered, and/or humanized. A VNA (VHH (heavy-chain-only Ab V_H)-based neutralizing agent) is a polypeptide comprising at least two linked individual VHHs. VNAs can be heteromultimers, e.g., comprise at least two different individual VHHs. Nanobodies, VNA, and/or VHH are commercially available, see, e.g., Ablynx (Gent, Belgium).

[0062] In some embodiments of any of the aspects, the antibody reagent can be monomeric. In some embodiments of any of the aspects, the antibody reagent can be monospecific. In some embodiments of any of the aspects, the antibody reagent can be multimeric after secretion.

[0063] In some embodiments of any of the aspects, the cell comprises at least one further nucleic acid sequence encoding an additional T3SS-compatible payload polypeptide comprising an antibody reagent. In some embodiments of any of the aspects, the engineered cell can express multiple antibody reagents that are multimeric before or after secretion, e.g., they form a heteromeric complex. In some embodiments of any of the aspects, the engineered cell can express multiple antibody reagents that are multimeric after secretion, e.g., they form a heteromeric complex. In some embodiments of any of the aspects, the multimeric complex can be multispecific, e.g., each different antibody reagent can be specific for a different antigen. By way of non-limiting example, described below herein is $VNA^{TcdB/A}$, which comprises 4 VHH and specifically binds to both TcdB and TcdA. In some embodiments of any of the aspects, the microbial cell can comprise one or more payload polypeptides that collectively are specific for both TcdB and TcdA. In some embodiments of any of the aspects, the microbial cell can comprise one or more payload polypeptides that collectively are specific for both Stx1 and Stx2.

[0064] In some embodiments of any of the aspects, the antibody reagent specifically binds to an inflammatory cytokine receptor or an inflammatory cytokine. Non-limiting examples of inflammatory cytokine receptor or an inflammatory cytokine can include TNFα (e.g., human TNFα, NCBI Gene ID: 7124), IL-8 (e.g., human IL-8, NCBI Gene ID: 3576); IL-6 (e.g., human IL-6, NCBI Gene ID: 3569), IL-18 (e.g., human IL-18, NCBI Gene ID: 3606), IL-21 (e.g., human IL-12, NCBI Gene ID: 59067), IL-33 (e.g., human IL-33, NCBI Gene ID: 90865) and IL-13 (e.g., human IL-13, NCBI Gene ID: 3596).

[0065] In some embodiments of any of the aspects, the antibody reagent specifically binds to an extracellular cancer cell marker. Cancer cell markers are molecules expressed on the surface of the cancer cell and which are preferentially expressed on cancer cells as compared to healthy cells. Non-limiting examples of suitable cancer cell markers can include PD-L1 (e.g., human PD-L1, NCBI Gene ID: 29126), PD-1 (e.g., human PD-1, NCBI Gene ID: 5133), and CTLA-4 (e.g., human CTLA-4, NCBI Gene ID: 1493). In some embodiments of any of the aspects, the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 reagent. In some embodiments of any of the aspects, the antibody reagent is an anti-PD-L1;

anti-PD-1; or anti-CTLA-4 VNA or VHH. In some embodiments of any of the aspects, the antibody reagent specifically binds to a cancer checkpoint polypeptide, i.e. a polypeptide that serves as a checkpoint to inhibit activity of tumor immunite that blocks recognition and clearance by human immune response. Non-limiting examples of suitable cancer checkpoint polypeptides can include PD-L1 (e.g., human PD-L1, NCBI Gene ID: 29126), PD-1 (e.g., human PD-1, NCBI Gene ID: 5133), and CTLA-4 (e.g., human CTLA-4, NCBI Gene ID: 1493). In some embodiments of any of the aspects, the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 reagent.

[0066] In some embodiments of any of the aspects, the antibody reagent specifically binds to a bacterial toxin, e.g. a toxin released by a bacterium that is pathogenic to the target cell, tissue, and/or organism. In some embodiments of any of the aspects, the bacterial toxin is an *E. coli* or *C. difficile* toxin. Non-limiting examples of bacterial toxins can include shiga toxin (Stx, Stx1 and/or Stx2); *C. difficile* toxin A (TcdA); *C. difficile* toxin B (TcdB); cholera toxin; anthrax toxin; and botulinum toxin.

[0067] In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a toxin. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises an antigen. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a cytokine. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a pro-drug converting enzyme, e.g., cytosine deaminiase. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises an anti-inflammatory cytokine. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises a receptor agonist, e.g., to modulate immune responses. In some embodiments of any of the aspects, the T3SS-compatible payload polypeptide comprises an enzyme, e.g., a diagnostic factor or a lactose intolerance relevant enzyme.

[0068] In some embodiments of any of the aspects, the second nucleic acid sequence is located on a plasmid. In some embodiments of any of the aspects, the second nucleic acid sequence is located on a chromosome (e.g. a naturally-ocurring bacterial chromosome, a modified endogenous chromosome, or a bacterial artificial chromosome (BAC)). In some embodiments of any of the aspects, the second nucleic acid sequence can comprise one or more operons, e.g. one operon, two operons, three operons, or more operons. In some embodiments, the second nucleic acid sequence can be integrated into the chromosome using,

for example, landing pad technology, see, e.g. Kuhlman and Cox, 2010 Nucleic Acids Research 38:e92; which is incorporated by reference herein in its entirety.

[0069] In some embodiments of any of the aspects, expression of the TDESS-compatible payload polypeptide can controlled by an inducible promoter, e.g., to avoid constitutive expression of the polypeptide and/or control the timing and level of expression of the polypeptide. In some embodiments of any of the aspects, the second nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding an T3SS-compatible payload polypeptide.

[0070] As expression of the TDESS-compatible payload polypeptide is advantageous when the TDESS itself expressed, it can be desireable to have the TDESS-compatible payload polypeptide expression regulated by a T3SS regulator such that expression of the polypeptide occurs when a TDESS is expressed and available for delivery of the polypeptide. In some embodiments of any of the aspects, the inducible promoter sequence is regulated by a master T3SS regulator (i.e. master T3SS transcriptional regulator). In some embodiments of any of the aspects, the inducible promoter sequence comprises a (T3SS)-associated promoter or promoter element. In some embodiments of any of the aspects, the T3SS-associated promoter or promoter element is an MxiE recognition sequence. In some embodiments, a T3SS-associated promoter and/or promoter element is a promoter and/or promoter element which endogenously controls the expression of a structural T3SS component, and/or a T3SS chaperone, and/or a T3SS substrate. Non-limiting examples of T3SS-associated promoters and/or promoter elements include MxiE or VirB or VirF recognition sequences, which are described, e.g. in Mavris et al. J Bact 2002 184:4409-19 and Beloin et al. JBC 2002 277:15333-15344; which are incorporated by reference herein in their entirety.

[0071] In order to control the expression of the TDESS and/or the TDESS-compatible payload polypeptide, a master T3SS regulator (i.e. master T3SS transcriptional regulator) can be provided. In some embodiments of any of the aspects, the cell comprises a third nucleic acid sequence encoding a master T3SS regulator. In some embodiments of any of the aspects, the master T3SS regulator is VirB, VirF, or a homolog thereof. As the master regulator will control the expression of the TDESS and the TDESS-compatible payload polypeptide, the expression of the master regulator itself can be manipulated to ensure that the TDESS and its payload polypeptide are expressed at the desired time, location, and/or level. Accordingly, in some embodiments of any of the aspects, the third nucleic acid

sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding a master T3SS regulator.

[0072] In order for a polypeptide described herein to be expressed, the nucleic acid encoding the polypeptide can be operatively linked to a promoter. In some embodiments, the polypeptide can be constitutively expressed. In some embodiments, nucleic acids encoding the polypeptide can be operatively linked to a constitutive promoter. In some embodiments, the polypeptide can be inducibly expressed. In some embodiments, nucleic acids encoding the polypeptide can be operatively linked to an inducible promoter.

[0073] As described herein, an "inducible promoter" is one that is characterized by initiating or enhancing transcriptional activity when in the presence of, influenced by, or contacted by an inducer or inducing agent than when not in the presence of, under the influence of, or in contact with the inducer or inducing agent. An "inducer" or "inducing agent" may be endogenous, or a normally exogenous compound or protein that is administered in such a way as to be active in inducing transcriptional activity from the inducible promoter. In some embodiments, the inducer or inducing agent, e.g., a chemical, a compound or a protein, can itself be the result of transcription or expression of a nucleic acid sequence (e.g., an inducer can be a transcriptional repressor protein), which itself may be under the control or an inducible promoter. Non-limiting examples of inducible promoters include but are not limited to, the lac operon promoter, a nitrogen-sensitive promoter, an IPTG-inducible promoter, a salt-inducible promoter, and tetracycline, steroid-responsive promoters, rapamycin responsive promoters and the like. Inducible promoters for use in prokaryotic systems are well known in the art, see, e.g. the beta.-lactamase and lactose promoter systems (Chang et al., Nature, 275: 615 (1978, which is incorporated herein by reference); Goeddel et al., Nature, 281: 544 (1979), which is incorporated herein by reference), the arabinose promoter system, including the araBAD promoter (Guzman et al., J . Bacteriol., 174: 7716-7728 (1 992), which is incorporated herein by reference; Guzman et al., J. Bacteriol., 177: 4121-4130 (1995), which is incorporated herein by reference; Siegele and Hu, Proc. Natl. Acad. Sci. USA, 94: 8168-8172 (1997), which is incorporated herein by reference), the rhamnose promoter (Haldimann et al., J. Bacteriol., 180: 1277-1286 (1998), which is incorporated herein by reference), the alkaline phosphatase promoter, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8: 4057 (1980), which is incorporated herein by reference), the $P_{LtetO-1}$ and $P_{lac/are-1}$ promoters (Lutz and Bujard, Nucleic Acids Res., 25: 1203-1210 (1997), which is incorporated herein by reference), and hybrid promoters such

as the tac promoter. deBoer et al., Proc. Natl. Acad. Sci. USA, 80: 21-25 (1983), which is incorporated herein by reference.

[0074] An inducible promoter useful in the methods and systems as disclosed herein can be induced by one or more physiological conditions, such as changes in pH, temperature, radiation, osmotic pressure, saline gradients, cell surface binding, and the concentration of one or more extrinsic or intrinsic inducing agents. The extrinsic inducer or inducing agent may comprise amino acids and amino acid analogs, saccharides and polysaccharides, nucleic acids, protein transcriptional activators and repressors, cytokines, toxins, petroleum-based compounds, metal containing compounds, salts, ions, enzyme substrate analogs, hormones, and combinations thereof. In specific embodiments, the inducible promoter is activated or repressed in response to a change of an environmental condition, such as the change in concentration of a chemical, metal, temperature, radiation, nutrient or change in pH. Thus, an inducible promoter useful in the methods and systems as disclosed herein can be a phage inducible promoter, nutrient inducible promoter, temperature inducible promoter, radiation inducible promoter, metal inducible promoter, hormone inducible promoter, steroid inducible promoter, and/or hybrids and combinations thereof. Appropriate environmental inducers can include, but are not limited to, exposure to heat (i.e., thermal pulses or constant heat exposure), various steroidal compounds, divalent cations (including Cu2+ and Zn2+), galactose, tetracycline, IPTG (isopropyl-β-D thiogalactoside), as well as other naturally occurring and synthetic inducing agents and gratuitous inducers.

[0075] Inducible promoters useful in the methods and systems as disclosed herein also include those that are repressed by "transcriptional repressors" that are subject to inactivation by the action of environmental, external agents, or the product of another gene. Such inducible promoters may also be termed "repressible promoters" where it is required to distinguish between other types of promoters in a given module or component of the biological switch converters described herein. Preferred repressors for use in the present invention are sensitive to inactivation by physiologically benign agent. Thus, where a lac repressor protein is used to control the expression of a promoter sequence that has been engineered to contain a lacO operator sequence, treatment of the host cell with IPTG will cause the dissociation of the lac repressor from the engineered promoter containing a lacO operator sequence and allow transcription to occur. Similarly, where a tet repressor is used to control the expression of a promoter sequence that has been engineered to contain a tetO operator sequence, treatment of the host cell with tetracycline will cause the dissociation of

the tet repressor from the engineered promoter and allow transcription of the sequence downstream of the engineered promoter to occur. In some embodiments of any of the aspects, the inducible promoter is selected from the group consisting of: an arabinose-inducible promoter; pBAD arabinose-inducible promoter (e.g., VirFara); tumor-induced promoters; ansB promoter; pflE promoter; and napF promoter; and an inflammation-induced promoter.

[0076] As described below herein, expression of certain *Salmonella* genes is known to be induced when the bacterium is present in a tumor. The promoters of these genes and/or *E. coli* homologs of these genes can be utilized to induce TDESS and payload polypeptide expression only once a cell has reached a tumor, reducing off-target side effects. Non-limiting examples of suitable promoters can include the promoters of ansB; pflE; and napF of *Salmonella*, which are described further in, e.g, Arrach et al. Cancer Res, 2008. 68(12): p. 4827-32; which is incorporated by reference herein in its entirety.

[0077] In some embodiments of any of the aspects, the engineered microbial cell described herein can be created by engineering and/or modifying a microbial cell selected from the group consisting of: *E. coli* NISSLE 1917 (EcN); *E. coli* K12; MP; HS; human commensal bacteria, murine commensal bacteria, and derivative strains thereof. In some embodiments of any of the aspects described herein, strain which is derivative of *E. coli* K12 is selected from the group consisting of *E. coli* DH10β and *E. coli* DH5α. In some embodiments of any of the aspects, the engineered microbial cell described herein can be created by engineering and/or modifying *E. coli* NISSLE 1917 (EcN). In some embodiments of any of the aspects, the engineered microbial cell described herein can be created by engineering and/or modifying a commensal intestinal microbial cell. In some embodiments of any of the aspects, the commensal intestinal microbial cell is *E. coli* NISSLE 1917 (EcN).

[0078] In some embodiments of any of the aspects, the engineered microbial cell described herein can be created by engineering and/or modifying a pathogenic microbial cell, wherein the modifications include deletion or mutation of one or more T3SS components. In some embodiments of any of the aspects, the one or more T3SS components is selected from the group consisting of: a toxin; a T3SS effector; a structural T3SS polypeptide; and a master regulator of T3SS components. Such deletions and/or mutations have been described in the art, e.g. non-limiting examples include virulence-curing of *S. typhimurium* by removing a virulence plasmid (see, e.g. Gulig and Curtiss. Infect Immun 1987 55:2891-2901; which is incorporated by reference herein in its entirety) and virulence-curing of *S. typhimurium* by mutation and/or of master regulators, e.g. master regulators of genes encoding endogenous

T3SS substrates (see, e.g., Eichelberg and Galan. Infect immune 1999 67:4099-4105; which is incorporated by reference herein in its entirety). In some embodiments, the T3SS component is located on a plasmid. For example, *Yersinia* and *Shigella* encode type 3 secretion systems in plasmids. In some embodiments, a plasmid comprising the T3SS component is removed from the bacterial cell, such as *Shigella*. For example, one can introduce a T3SS encoding plasmid into virulence plasmid cured steains of *Shigella*. In some embodiments of any of the aspects, the pathogenic microbial cell is selected from the group consisting of: *Salmonella* spp.; *Shigella* Spp; and *Yersinia* spp. In some embodiments of any of the aspects, the pathogenic microbial cell is selected from the group consisting of: *Salmonella typhimurium* SPI1and *Shigella flexneri* mxi-spa.

[0079] In some embodiments of any of the aspects described herein, the engineered cell can be further engineered or modified to increase secretion of the payload polypeptide. Such modifications can include modifications to increase translation, transcription, T3SS system component production, and the like. In some embodiments of any of the aspects, the cell has been contacted with a mutagenic treatment; and selected for increased secretion. In some embodiments of any of the aspects, the cell has been contacted with a mutagenic treatment; and selected for increased secretion of the T3SS-compatible payload polypeptide.

[0080] In some embodiments of any of the aspects, delivery of the payload polypeptide to a desired location can be increased or improved by further engineering the microbial cell to permit binding and/or increased binding to a cell or molecule found at the desired location. Methods for modulating bacterial adherence to a given cell are known in the art. In some embodiments of any of the aspects, the cell further comprises a nucleic acid sequence encoding one or more polypeptides that increase adhesion to a target cell. In some embodiments of any of the aspects, the polypeptides that increase adhesion to the target cell comprise Tir and intimin, e.g. from enteropathogenic *E. coli*. Intimin is an outer membrane protein and Tir is a substrate of the T3SS which, upon delivery to a target cell, integrates into the plasma membrane and acts as a receptor for intimin. In some embodiments, an engineered microbial cell comprising a nucleic acid sequence encoding intimin and Tir can also comprise a nucleic acid sequence encoding the Tir chaperone CesT. In some embodiments of any of the aspects, the polypeptide that increases adhesion to the target cell is selected from a group consisting of: a bacterial adhesion; Afa1; AIDA; invasion; an antibody reagent specific for an extracellular epitope of a target cell polypeptide; and a single chain antibody specific for an extracellular epitope of a target cell polypeptide. Construction

of adhesins that are specific for a given target, e.g., different regions of the gut, is known to one of skill in the art and described further in, e.g., Pinero-Lambea et al. ACS Synthetic Biology 2015 4:463-473; which is incorporated by reference herein in its entirety.

[0081] The engineered microbial cells described herein can permit delivery of one or more payload polypeptides to a desired extracellular location. In one aspect of any of the embodiments, described herein is a method of introducing a polypeptide into a target tissue or organism, the method comprising contacting the target tissue or organism with an engineered microbial cell as described herein.

[0082] In one aspect of any of the embodiments, described herein is a method of reducing inflammation in a subject, the method comprising administering an engineered microbial cell or a composition comprising an engineered microbial cell as described herein to a subject in need thereof. In some embodiments of any of the aspects, the microbial cell can comprise a payload polypeptide that is an anti-inflammatory polypeptide or an antibody reagent that specifically binds to an inflammatory cytokine in exptracellular space or inflammatory cytokine receptor on a cell surface. In some embodiments of any of the aspects, the inflammation is inflammation of the gastrointestinal tract. In some embodiments of any of the aspects, the subject is in need of treatment for a condition selected from the group consisting of: asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis. In some embodiments of any of the aspects, the subject is a subject in need of treatment for inflammatory bowel disease. In some embodiments of any of the aspects, the microbial cell or composition comprising the microbial cell an a pharmaceutically acceptable carrier is administered orally. In some embodiments of any of the aspects, the microbial cell is engineered from an intestinal commensal bacterium.

[0083] In one aspect of any of the embodiments, described herein is a method of treating a proliferative disease in a subject, the method comprising administering an engineered microbial cell as described herein to a subject in need thereof. In some embodiments of any of the aspects, the proliferative disease is a cancer. In some embodiments of any of the aspects, the payload polypeptide can be a toxin or an antibody reagent specific for a cancer cell marker and/or regulator and/or checkpoint polypeptide. In some embodiments of any of the aspects, the microbial cell can be engineered from *E. coli* NISSLE 1917 (EcN), which demonstrates a surprising ability to localize to tumors without inducing any immune responses. In some embodiments of any of the aspects, the microbial cell is administered systemically or orally. In some embodiments of any of the aspects, the microbial cell is

administered intratumorally. In some embodiments of any of the aspects, the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally. In some embodiments of any of the aspects of the invention, the microbial cell is administered orally for targeting either targets in the gastrointestinal (GI) track or in tumors into which the microbial cell in question is known to migrate from the gut.

[0084] In one aspect of any of the embodiments, described herein is a method of treating an intestinal infection in a subject, the method comprising administering an engineered microbial cell as described herien to a subject in need thereof. In some embodiments of any of the aspects, the microbial cell can comprise a payload polypeptide that is an antibody reagent that specifically binds a bacterial toxin. In some embodiments of any of the aspects, the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin. In some embodiments of any of the aspects, the intestinal infection is a *C. difficile* infection and/or the subject has *C. difficile* colitis and the toxin is TcdA and/or TcdB. In some embodiments of any of the aspects, the intestinal infection is cholera and the toxin is cholera toxin. In some embodiments of any of the aspects, the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin. In some embodiments of any of the aspects, the intestinal infection is botulism and the toxin is botulinum toxin. In some embodiments of any of the aspects, the microbial cell is administered orally.

[0085] In some embodiments of any of the aspects, the method further comprises a step of inducing secretion of the T3SS-compatible payload polypeptide by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master regulator. In some embodiments of any of the aspects, expression of the T3SS-compatible payload polypeptide and/or the T3SS master regulator is controlled by an inducible promoter. In some embodiments of any of the aspects, the compound is arabinose and the inducible promoter is an arabinose-inducible promoter.

[0086] In one aspect of any of the embodiments, described herein is a method for delivering a polypeptide into a) the extracellular milieu of a subject's gastrointestinal tract or b) the extracellular milieu of a subject's tumor, the method comprising contacting administering a microbial cell as described herein to the subject. As used herein, "extracellular milieu" refers to the environment found in the extracellular space, e.g., the fluid environment in a subject which is not located within a cell. The extracellular milieu can include the interstitial fluid and/or the extracellular matrix.

[0087] In one aspect of any of the embodiments, described herein is a kit comprising an engineered microbial cell as described herein. A kit is any manufacture (e.g., a package or container) comprising at one engineered microbial cell in a container either in dry, or lyophilized form and usually with a pharmaceurically acceptable carrier as described herein, the manufacture being promoted, distributed, or sold as a unit for performing the methods described herein. When the kits, and methods described herein are used for diagnosis and/or treatment of a condition in patients, the reagents (e.g., detection probes) or systems can be selected such that a positive result is obtained in at least about 20%, at least about 40%, at least about 60%, at least about 80%, at least about 90%, at least about 95%, at least about 99% or in 100% of subjects having or developing a sensitivity to the therapeutics described herein.

[0088] A kit can further comprising reagents and/or components for the preservation, culture, and/or further engineering of a cell as described herein, e.g., a cryopreservation solution, media, an inducer, a vector for introduction of a second nucleic acid sequence, etc. Such ingredients are known to the person skilled in the art and may vary depending on the method carried out. Additionally, the kit may comprise an instruction leaflet and/or may provide information about the cell or further medication of the cell.

[0089] In some embodiments, the methods described herein relate to treating a subject, such as a mammalian subject, including human, farm animals or pets, such as swine, bowine, dog, or cat. In some embodimens the subject is a fowl. The compositions and methods as described herein can be used in human or veterinary treatment. Subjects having a condition described here (e.g. inflammation or cancer) can be identified by a physician/veterinarian using current methods of diagnosing such conditions. Symptoms and/or complications which characterize these conditions and aid in diagnosis.

[0090] The compositions and methods described herein can be administered to a subject in need of treatment, e.g. in need of treatment for inflammation or cancer. In some embodiments, the methods described herein comprise administering an effective amount of compositions described herein, e.g. engineered microbial cells to a subject in order to alleviate a symptom. As used herein, "alleviating a symptom" is ameliorating any condition or symptom associated with a given condition. As compared with an equivalent untreated control, such reduction is by at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, 99% or more as measured by any standard technique. A variety of means for administering the compositions described herein to subjects are known to those of skill in the art. Such methods

can include, but are not limited to oral, subcutaneous, transdermal, airway (aerosol), cutaneous, topical, injection, or intratumoral administration. Administration can be local or systemic.

[0091] The term "effective amount" as used herein refers to the amount of engineered microbial cells needed to alleviate at least one or more symptom of the disease or disorder, and relates to a sufficient amount of pharmacological composition to provide the desired effect. The term "therapeutically effective amount" therefore refers to an amount of engineered microbial cells that is sufficient to effect a particular effect when administered to a typical subject. An effective amount as used herein, in various contexts, would also include an amount sufficient to delay the development of a symptom of the disease, alter the course of a symptom disease (for example but not limited to, slowing the progression of a symptom of the disease), or reverse a symptom of the disease. Thus, it is not generally practicable to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

[0092] Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of an engineered microbial cell which achieves a half-maximal inhibition of symptoms) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay, e.g., assay for inflammation, among others. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

[0093] In some embodiments, the technology described herein relates to a pharmaceutical composition comprising an engineered microbial cell as described herein, and optionally a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers and diluents

include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. Some non-limiting examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C_2-C_{12} alcohols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" or the like are used interchangeably herein.

[0094] Pharmaceutical compositions comprising an engineered microbial cell can be formulated to be suitable for oral administration, for example as discrete dosage forms, such as, but not limited to, tablets (including without limitation scored or coated tablets), pills, caplets, capsules, chewable tablets, powder packets, cachets, troches, wafers, aerosol sprays, or liquids, such as but not limited to, syrups, elixirs, solutions or suspensions in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion, or a water-in-oil emulsion. Such compositions contain a predetermined amount of the pharmaceutically acceptable salt of the disclosed compounds, and may be prepared by methods of pharmacy well known to those skilled in the art. See generally, Remington: The Science and Practice of Pharmacy, 21st Ed., Lippincott, Williams, and Wilkins, Philadelphia PA. (2005).

[0095] The methods described herein can further comprise administering a second agent and/or treatment to the subject, e.g. as part of a combinatorial therapy.

[0096] The methods described herein can further comprise administering a second agent and/or treatment to the subject, e.g. as part of a combinatorial therapy. By way of non-limiting example, if a subject is to be treated for inflammation according to the methods described herein, the subject can also be administered a second agent and/or treatment known to be beneficial for subjects suffering from pain or inflammation. Examples of such agents and/or treatments include, but are not limited to, non-steroidal anti-inflammatory drugs (NSAIDs - such as aspirin, ibuprofen, or naproxen); corticosteroids, including glucocorticoids (e.g. cortisol, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, and beclometasone); methotrexate; sulfasalazine; leflunomide; anti-TNF medications; cyclophosphamide; pro-resolving drugs; mycophenolate; or opiates (e.g. endorphins, enkephalins, and dynorphin), steroids, analgesics, barbiturates, oxycodone, morphine, lidocaine, and the like.

[0097] Non-limiting examples of a second agent and/or treatment for a subject in need of treatment for cancer can include radiation therapy, surgery, gemcitabine, cisplastin, paclitaxel, carboplatin, bortezomib, AMG479, vorinostat, rituximab, temozolomide, rapamycin, ABT-737, PI-103; alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin,

authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE® Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Ill.), and TAXOTERE® doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE.RTM. vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids

such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (Tykerb.RTM.); inhibitors of PKC-alpha, Raf, H-Ras, EGFR (*e.g.*, erlotinib (Tarceva®)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[0098] In certain embodiments, an effective dose of a composition comprising engineered microbial cells as described herein can be administered to a patient once. In certain embodiments, an effective dose of a composition comprising engineered microbial cells can be administered to a patient repeatedly. In some embodiments, the dose can be a daily administration,for example oral administration, of, e.g., a capsule comprising bacterial cells as described herein. In some embodiments, the dose can be, e.g. an injection of bacterial cells into the desired area, e.g. a tumor. In some embodiments, the dose can be administered systemically, e.g. by intravenous injection. In some embodiments, a dose can comprise from 10^6 to 10^{12} cells. In some embodiments, a dose can comprise from about 10^8 to 10^{10} cells. A composition comprising engineered microbial cells can be administered over a period of time, such as over a 5 minute, 10 minute, 15 minute, 20 minute, or 25 minute period. The administration can be repeated, for example, on a regular basis, such as hourly for 3 hours, 6 hours, 12 hours, daily (i.e. one a day) or longer or such as once a week, or biweekly (i.e., every two weeks) for one month, two months, three months, four months or longer.

[0099] In some embodiments, after an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after treatment biweekly for three months, treatment can be repeated once per month, for six months or a year or longer. Treatment according to the methods described herein can reduce levels of a marker or symptom of a condition, e.g. by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80 % or at least 90% or more.

[00100] The dosage of a composition as described herein can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. With respect to duration and frequency of treatment, it is typical for skilled clinicians to monitor subjects in order to determine when the treatment is providing therapeutic benefit, and to determine whether to increase or decrease dosage, increase or decrease administration frequency, discontinue treatment, resume treatment, or make other alterations to the treatment regimen. The dosing schedule can vary from once a week to daily depending on a number of clinical factors, such as the subject's sensitivity to engineered microbial cells. The desired dose or amount of activation can be administered at one time or divided into subdoses, e.g., 2-4 subdoses and

administered over a period of time, e.g., at appropriate intervals through the day or other appropriate schedule. In some embodiments, administration can be chronic, e.g., one or more doses and/or treatments daily over a period of weeks or months. Examples of dosing and/or treatment schedules are administration daily, twice daily, three times daily or four or more times daily over a period of 1 week, 2 weeks, 3 weeks, 4 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, or 6 months, or more.

[00101] The dosage ranges for the administration of engineered microbial cells, according to the methods described herein depend upon, for example, the form of the cells, their potency, and the extent to which symptoms, markers, or indicators of a condition described herein are desired to be reduced, for example the percentage reduction desired. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, and sex of the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

[00102] The efficacy of engineered microbial cells in, e.g. the treatment of a condition described herein, or to induce a response as described herein can be determined by the skilled clinician. However, a treatment is considered "effective treatment," as the term is used herein, if any one or all of the signs or symptoms of a condition described herein are altered in a beneficial manner, other clinically accepted symptoms are improved, or even ameliorated, or a desired response is induced e.g., by at least 10% following treatment according to the methods described herein. Efficacy can be assessed, for example, by measuring a marker, indicator, symptom, and/or the incidence of a condition treated according to the methods described herein or any other measurable parameter appropriate. Efficacy can also be measured by a failure of an individual to worsen as assessed by hospitalization, or need for medical interventions (i.e., progression of the disease is halted). Methods of measuring these indicators are known to those of skill in the art and/or are described herein. Treatment includes any treatment of a disease in an individual or an animal (some non-limiting examples include a human or an animal) and includes: (1) inhibiting the disease, e.g., preventing a worsening of symptoms (e.g. pain or inflammation); or (2) relieving the disease, e.g., causing regression of symptoms. An effective amount for the treatment of a disease means that amount which, when administered to a subject in need thereof, is sufficient to result in effective treatment as that term is defined herein, for that disease. Efficacy of an agent can be determined by assessing physical indicators of a condition or desired response. It is well within the ability of one skilled in the art to monitor efficacy of administration

and/or treatment by measuring any one of such parameters, or any combination of parameters. Efficacy can be assessed in animal models of a condition described herein. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change in a marker is observed.

[00103] In some aspects of any of the embodiments, provided herein are methods and compositions relating to the delivery or translocation of antibody reagents, e.g., single chain antibodies or VHH, to the intracellular space of a target eukaryotic cell by engineering non-pathogenic bacterial cells which express a functional T3SS to create a biological delivery system. Also described herein are compositions and methods that relate to non-pathogenic microbial cells that have been engineered to express both 1) a functional type three secretion system (T3SS) that is capable of injecting a payload into another cell and at least one antibody reagent that is compatible with the T3SS.

[00104] In one aspect of any of the embodiments, described herein is an engineered non-pathogenic microbial cell comprising (1) a first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS); (2) a second nucleic acid sequence encoding an T3SS-compatible antibody reagent; wherein the engineered microbial cell is non-pathogenic with respect to the target cell or target organism. In one aspect of any of the embodiments, described herein is an engineered non-pathogenic microbial cell comprising (1) a first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS); (2) a second nucleic acid sequence encoding an T3SS-compatible VHH; wherein the engineered microbial cell is non-pathogenic with respect to the target cell or target organism. For example, a plasmid comprising two operons encoding a functional *Shigella* T3SS (covering 31 kb) can be utilized.

[00105] As used herein, a "target cell" is a cell which can receive a polypeptide delivered by a bacterial T3SS. In some embodiments, a target cell is a eukaryotic cell. In some embodiments, a target cell is a cell comprised by, or originating from, a vertebrate. In some embodiments, a target cell is a cell comprised by, or originating from, a mammal. A "target organism" is an organism comprising at least one "target cell." A target cell can be located *in vitro* or *in vivo*. In some embodiments, a target cell is an isolated target cell. In some embodiments, a target cell is not an isolated target cell. In some embodiments, the target cell is part of the target organism.

[00106] A T3SS is a multi-protein structure found in gram negative bacteria. It moves polypeptides from the cytoplasm of the bacterial cell through the interior of the T3SS "needle" into the cytoplasm of a target cell. T3SS's are found in pathogenic strains and have been observed in pathogenic isolates of, e.g., *Shigella, Salmonella, E. coli, Burkholderia, Yersinia, Chlamydia, Pseudomonas, Erwinia, Ralstonia, Rhizobium, Vibrio*, and *Xanthamonas*. Further discussion of T3SS's can be found, e.g. in Izore et al. Structure 2011 19:603-612; Korotkov et al. Nature Reviews

Microbiology 2012 10:336-351; Wooldridge, K. (ed) Bacterial Secreted Proteins. Caster Academic Press 2009; Snyder and Champness (eds.) Molecular Genetics of Bacteria. 3rd Ed. ASM Press: 2007; each of which is incorporated by reference herein in its entirety.

[00107] The suite of T3SS-related proteins in a given wild-type cell is typically divided into structural proteins (those proteins which form the needle itself), substrate proteins (those proteins which are transported through the needle to the host), and chaperones (those proteins that bind effectors in the cytoplasm to protect, process, and/or shuttle the effectors to the needle). As used herein, a "functional T3SS" refers, minimally, to the set of structural proteins which are required in order to transfer at least one polypeptide to a target cell. In some embodiments, a functional T3SS system can comprise one or more chaperone proteins. In some embodiments, a functional T3SS can comprise one or more, for example, two, three, or four, substrates which are not virulence factor (e.g. certain translocators). In some embodiments, a functional T3SS does not comprise a virulence factor which is delivered to the target cell.

[00108] As used herein, a "virulence factor" refers to those substrates which affect and/or manipulate a target cell in a manner which is beneficial to infection and deleterious to the target cell, i.e. they perturb the normal function of the target cell. Examples of actions of virulence factors include, but are not limited to, modulation of actin polymerization, induction of apoptosis, modulation of the cell cycle, modulation of gene transcription. Not all substrates are necessarily virulence factors. By way of non-limiting example, a T3SS (and a functional T3SS) can comprise proteins referred to as transcloators. These substrates are secreted by the T3SS as it nears a complete form and create a pore in the target cell membrane, allowing further substrates to be delievered into the cytoplasm of the target cell, i.e. translocators are substrates in that they travel through the needle to the target cell and are also structural proteins in that they form part of the structure through which other substrates are delivered into the target cell. In some embodiments, a single polypeptide can be both a translocator and a virulence factor (e.g. IpaB of *Shigella*).

[00109] In some embodiments, a functional T3SS can comprise one or more translocators.

[00110] In some embodiments, a functional T3SS does not comprise a translocator that also has virulence factor activity.

[00111] The minimal set of proteins required for a functional T3SS can vary depending upon, e.g. the identity of the polypeptide which is to be transferred, the origin of the T3SS, the identity of the non-pathogenic bacterial cell, and/or the identity of the host cell.

[00112] In some embodiments, a functional T3SS can comprise one or more of a needle monomer polypeptide, an inner rod polypeptide, ring polypeptides, one or more translocators, a needle-tip polypeptide, a ruler polypeptide, and/or an ATPase.

[00113] In some embodiments, a functional T3SS can comprise a needle monomer polypeptide (e.g. MxiH (e.g., NCBI Gene ID No: 1238256 (DNA sequence disclosed as SEQ ID NO: 19; PRT

sequence disclosed as SEQ ID NO: 20)); PrgI (e.g., NCBI Gene ID No: 1254396 (DNA sequence disclosed as SEQ ID NO: 21; PRT sequence disclosed as SEQ ID NO: 22)); YscF (e.g., NCBI Gene ID No: 1172700 (DNA sequence disclosed as SEQ ID NO: 23; PRT sequence disclosed as SEQ ID NO: 24)); and/or EscF (e.g., NCBI Gene ID No: 8873370 (DNA sequence disclosed as SEQ ID NO: 25; PRT sequence disclosed as SEQ ID NO: 26) or NC_013941.1 (4477882..4478103) (SEQ ID NO: 1) or WP_001053840 (SEQ ID NO: 2))), an inner rod polypeptide (e.g. MxiI (e.g., NCBI Gene ID No: 1238257 (DNA sequence disclosed as SEQ ID NO: 27; PRT sequence disclosed as SEQ ID NO: 28)); PrgJ (e.g., NCBI Gene ID No: 1254395 (DNA sequence disclosed as SEQ ID NO: 29; PRT sequence disclosed as SEQ ID NO: 30)); YscI (e.g., NCBI Gene ID No: 2767498 (DNA sequence disclosed as SEQ ID NO: 31; PRT sequence disclosed as SEQ ID NO: 32) or NC_005813.1 (18395..18733) (SEQ ID NO: 3) or WP_032465675.1 (SEQ ID NO: 4)); and/or EscI (e.g., NCBI Gene ID No: 8219253 (DNA sequence disclosed as SEQ ID NO: 33; PRT sequence disclosed as SEQ ID NO: 34) or NC_013008.1 (4669108..4669485) (SEQ ID NO: 5) or WP_001302733.1 (SEQ ID NO: 6))), ring polypeptides, one or more translocators (e.g. IpaC (e.g., NCBI Gene ID No: 876448 (DNA sequence disclosed as SEQ ID NO: 35; PRT sequence disclosed as SEQ ID NO: 36)); SipB (e.g., NCBI Gene ID No: 1254408 (DNA sequence disclosed as SEQ ID NO: 37; PRT sequence disclosed as SEQ ID NO: 38)); SipC (e.g., NCBI Gene ID No: 1254407 (DNA sequence disclosed as SEQ ID NO: 39; PRT sequence disclosed as SEQ ID NO: 40)); YopB (e.g., NCBI Gene ID No: 1449456 (DNA sequence disclosed as SEQ ID NO: 41; PRT sequence disclosed as SEQ ID NO: 42)); YopD (e.g., NCBI Gene ID No: 1449455 (DNA sequence disclosed as SEQ ID NO: 43; PRT sequence disclosed as SEQ ID NO: 44)); EspD (e.g., NCBI Gene ID No: 885777 (DNA sequence disclosed as SEQ ID NO: 45; PRT sequence disclosed as SEQ ID NO: 46)); and/or EspB (e.g., NCBI Gene ID No: 8474872 (DNA sequence disclosed as SEQ ID NO: 47; PRT sequence disclosed as SEQ ID NO: 48))); a needle-tip polypeptide (e.g. IpaD (e.g., NCBI Gene ID No: 876444 (DNA sequence disclosed as SEQ ID NO: 49; PRT sequence disclosed as SEQ ID NO: 50)); SipD (e.g., NCBI Gene ID No: 1254406 (DNA sequence disclosed as SEQ ID NO: 51; PRT sequence disclosed as SEQ ID NO: 52)); LcrV (e.g., NCBI Gene ID No: 1172676 (DNA sequence disclosed as SEQ ID NO: 53; PRT sequence disclosed as SEQ ID NO: 54)); and/or EspA (e.g., NCBI Gene ID No: 960865 (DNA sequence disclosed as SEQ ID NO: 55; PRT sequence disclosed as SEQ ID NO: 56))); a ruler polypeptide (e.g. Spa32 (e.g., NCBI Gene ID No: 876502 (DNA sequence disclosed as SEQ ID NO: 57; PRT sequence disclosed as SEQ ID NO: 58)); InvJ (e.g., NCBI Gene ID No: 1254415 (DNA sequence disclosed as SEQ ID NO: 59; PRT sequence disclosed as SEQ ID NO: 60)); YscP (e.g., NCBI Gene ID No: 5798302 (DNA sequence disclosed as SEQ ID NO: 61; PRT sequence disclosed as SEQ ID NO: 62) or NC_010157.1 (27606..28973) (SEQ ID NO: 7) or WP_002212950.1 (SEQ ID NO: 8)); and/or Orf16 (e.g., NCBI Gene ID No: 8219247 (DNA sequence disclosed as SEQ ID NO: 63; PRT sequence disclosed as SEQ ID NO: 64) or NC_013008.1 (4664050..4664466) (SEQ ID NO:

9) or WP_001303723.1 (SEQ ID NO: 10))), and an ATPase (e.g. Spa47 (e.g., NCBI Gene ID No: 876429 (DNA sequence disclosed as SEQ ID NO: 65; PRT sequence disclosed as SEQ ID NO: 66)); InvC (e.g., NCBI Gene ID No: 1254417 (DNA sequence disclosed as SEQ ID NO: 67; PRT sequence disclosed as SEQ ID NO: 68)); YscN (e.g., NCBI Gene ID No: 10216379 (DNA sequence disclosed as SEQ ID NO: 69; PRT sequence disclosed as SEQ ID NO: 70)); and/or SepB (also known as EscN) (e.g., NCBI Gene ID No: 8873386 (DNA sequence disclosed as SEQ ID NO: 71; PRT sequence disclosed as SEQ ID NO: 72) or NC_013941.1 (4493198..4494538) (SEQ ID NO: 11) or WP_000622545.1 (SEQ ID NO: 12))). In some embodiments, a functional T3SS can further comprise a chaperone for the one or more translocators (e.g. IpgC (e.g., NCBI Gene ID No: 1238043 (DNA sequence disclosed as SEQ ID NO: 73; PRT sequence disclosed as SEQ ID NO: 74)); SicA (e.g., NCBI Gene ID No: 1254409 (DNA sequence disclosed as SEQ ID NO: 75; PRT sequence disclosed as SEQ ID NO: 76)); SycD (e.g., NCBI Gene ID No: 2767486 (DNA sequence disclosed as SEQ ID NO: 77; PRT sequence disclosed as SEQ ID NO: 78) or NC_005813.1 (38937..39443) (SEQ ID NO: 13) or WP_002222758.1 (SEQ ID NO: 14)); and/or CesD (e.g., NCBI Gene ID No: 7063867 (DNA sequence disclosed as SEQ ID NO: 79; PRT sequence disclosed as SEQ ID NO: 80))). In some embodiments, a functional T3SS can further comprise one or more of a switch polypeptide (e.g. Spa40 (e.g., NCBI Gene ID No: 876433 (DNA sequence disclosed as SEQ ID NO: 81; PRT sequence disclosed as SEQ ID NO: 82)); SpaS (e.g., NCBI Gene ID No: 1254410 (DNA sequence disclosed as SEQ ID NO: 83; PRT sequence disclosed as SEQ ID NO: 84)); YscU (e.g., NCBI Gene ID No: 2767517 (DNA sequence disclosed as SEQ ID NO: 85; PRT sequence disclosed as SEQ ID NO: 86)); and/or EscU (e.g., NCBI Gene ID No: 7062687 (DNA sequence disclosed as SEQ ID NO: 87; PRT sequence disclosed as SEQ ID NO: 88))) and a gatekeeper polypeptide (e.g. MxiC (e.g., NCBI Gene ID No: 876426 (DNA sequence disclosed as SEQ ID NO: 89; PRT sequence disclosed as SEQ ID NO: 90)); InvE (e.g., NCBI Gene ID No: 1254420 (DNA sequence disclosed as SEQ ID NO: 91; PRT sequence disclosed as SEQ ID NO: 92)); YopN (e.g., NCBI Gene ID No: 2767534 (DNA sequence disclosed as SEQ ID NO: 93; PRT sequence disclosed as SEQ ID NO: 94) or NC_005813.1 (32887..33768) (SEQ ID NO: 15) or WP_011171994.1 (SEQ ID NO: 16)); and/or SepL (e.g., NCBI Gene ID No: 8873375 (DNA sequence disclosed as SEQ ID NO: 95; PRT sequence disclosed as SEQ ID NO: 96) or NC_013941.1 (4481286..4482341) (SEQ ID NO: 17) or WP_001273445.1 (SEQ ID NO: 18))).

[00114] In some embodiments, the functional type three secretion system (T3SS) can comprise polypeptides endogenous to a bacterium selected from the group consisting of: *Shigella* spp; *Salmonella* spp; enteropathogenic *E. coli*; and *Yersinia* spp. In some embodiments, the genes encoding a functional type three secretion system (T3SS) comprise: virB; acp; ipaA; ipaB; ipaC; ipaD; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL;

mxiM; mxiE; mxiD; mxiC; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40 and/or homologs thereof.

[00115] In some embodiments, the first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS) can comprise one contiguous sequence. In some embodiments, the first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS) is located on a plasmid. In some embodiments, the first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS) is located on a chromosome (e.g. a naturally-ocurring chromosome, a modified endogenous chromosome, or a bacterial artificial chromosome (BAC)). In some embodiments, the first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS) can comprise one or more operons, e.g. one operon, two operons, three operons, or more operons. In some embodiments, the first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS) can comprise one or more separate sequences and/or molecules (e.g. a portion of the genes are found on one plasmid and another portion of the genes are found on a second plasmid). In some embodiments, the first nucleic acid sequence can be integrating into the chromosome using, for example, landing pad technology, see, e.g. Kuhlman and Cox, 2010 Nucleic Acids Research 38:e92; which is incorporated by reference herein in its entirety.

[00116] In some embodiments, a functional T3SS system can be introduced into a non-pathogenic bacterial cell. In alternative embodiments, a pathogenic bacterial cell comprising a functional T3SS can be engineered to be non-pathogenic, e.g. by deleting or mutation one or more T3SS components. Non-limiting examples of T3SS components that can be deleted or mutated to engineer a non-pathogenic bacterial cell include: a toxin; a T3SS substrate; a structural T3SS polypeptide; a master regulator of T3SS components; and any combination thereof. Such deletions and/or mutations have been described in the art, e.g. non-limiting examples include virulence-curing of *S. typhimurium* by removing a virulence plasmid (see, e.g. Gulig and Curtiss. Infect Immun 1987 55:2891-2901; which is incorporated by reference herein in its entirety) and virulence-curing of *S. typhimurium* by mutation and/or of master regulators, e.g. master regulators of genes encoding endogenous T3SS substrates (see, e.g., Eichelberg and Galan. Infect immune 1999 67:4099-4105; which is incorporated by reference herein in its entirety). In some embodiments, the T3SS component is located on a plasmid. For example, *Yersinia* and *Shigella* encode type 3 secretion systems in plasmids. In some embodiments, a plasmid comprising the T3SS component is removed from the bacterial cell, such as *Shigella*. In some embodiments, the pathogenic microbial cell is selected from the group consisting of: *Salmonella* spp.; *Shigella* Spp; and *Yersinia* spp. In some embodiments, the pathogenic microbial cell is selected from the group consisting of: *Salmonella typhimurium* SPI1 and *Shigella felxneri* mxi-spa. For example, one can introduce the T3SS encoding plasmid into virulence plasmid cured steains of *Shigella*.

[00117] In some embodiments, the first nucleic acid sequence is no greater than 4 kb in size. In some embodiments, the first nucleic acid sequence is no greater than 3 kb in size, e.g., no greater than 2.5 kb, 2 kb, 1/5 kb, or 1 kb. In some embodiments, the first nucleic acid sequence and third nucleic acid sequence are, cumulatively, no greater than 4 kb in size. In some embodiments, the first nucleic acid sequence and third nucleic acid sequence are, cumulatively, no greater than 3 kb in size, e.g., no greater than 2.5 kb, 2 kb, 1/5 kb, or 1 kb.

[00118] In some embodiments, the first nucleic acid sequence and optionally, the third nucleic acid sequence, are found in or introduced to the cells by means of a plasmid or vector which is no greater than 6 kb in size, e.g., 6 kb or less, 5 kb or less, 4 kib or less, 3 kb or less, 2.5 kb or less, or 2 kb or less. In some embodiments, the first nucleic acid sequence and optionally, the third nucleic acid sequence, are found in or introduced to the cells by means of a plasmid or vector which is no greater than 3 kb in size. The delivery of an antibody reagent, e.g., a VHH, via a T3SS requires close proximity of the microbial cell and the target cell. Accordingly, in some embodiments, delivery of an antibody reagent can be increased or enhanced by causing and/or increasing adhesion of the microbial cell to the target cell. In some embodiments, the engineered microbial cell further comprises a nucleic acid sequence encoding one or more polypeptides that increase adhesion to the target cell. A number of polypeptides can increase adhesion.

[00119] In some embodiments, the polypeptides that increase adhesion to the target cell comprise Tir and intimin, e.g. from enteropathogenic *E. coli*. Intimin is an outer membrane protein and Tir is a substrate of the T3SS which, upon delivery to a target cell, integrates into the plasma membrane and acts as a receptor for intimin. In some embodiments, an engineered microbial cell comprising a nucleic acid sequence encoding intimin and Tir can also comprise a nucleic acid sequence encoding the Tir chaperone CesT.

[00120] In some embodiments, the polypeptide that increases adhesion to the target cell can be selected from the group consisting of a bacterial adhesin; Afal; AIDA; invasin; or a single chain antibody specific for an extracellular epitope of a target cell polypeptide. In some embodiments, the extracellular epitope of the target cell can be specific for a certain type of target cell, e.g. a cancer-cell specific epitope and/or a tissue-specific epitope in order to target delivery of polypeptides to a particular cell type.

[00121] As described herein, four endogenous *Shigella* polypeptides, when secreted by a functional type 3 secretion system, are sufficient to enable a bacterial cell to mediate its uptake into a target cell. Accordingly, engineered bacterial cells as described herein that comprise these four polypeptides (or homologs thereof) can be internalized by a target cell and can deliver their T3SS-compatible antibody reagent(s) before, during, and/or after internalization into the target cell. Their uptake by the target cell can also trigger, e.g. innate immune responses leading to cell death. In some embodiments, uptake can trigger innate immune responses potentially leading to cell death. Such

engineered bacterial cells can be suitable, for example, for use in methods where it is desired to kill the target cell, e.g. in treating a solid tumor. In some embodiments, a commensal cell is engineered to comprise the four *Shigella* polypeptides and/or homologs thereof. In some embodiments, a pathogenic cell engineered to be avirulent is engineered such that it retains the four *Shigella* polypeptides and/or homologs thereof. In some embodiments, any one of the four *Shigella* polypeptides is sufficient to induce the uptake of the bacterial cell. In some embodiments, an engineered cell is engineered such that the engineered cell introduces less than four *Shigella* polypeptides and/or homologs thereof, e.g. only one of the polypeptides, only two of the polypeptides, or only three of the polypeptides.

[00122] Conversely, cells lacking all four of these endogenous *Shigella* polypeptides cannot mediate uptake by the target cell and remain in the extracellular environment. Such engineered bacterial cells can be suitable for use in methods where is undesirable to activate innate immune responses, e.g. when delivering anti-inflammatory antibody reagents to the target cell. In some embodiments, a commensal cell is engineered such that it does not any of the four *Shigella* polypeptides and/or homologs thereof, e.g. it retains none of the polypeptides. In some embodiments, a pathogenic cell engineered to be avirulent is engineered such that it does not any of the four *Shigella* polypeptides and/or homologs thereof, e.g. it retains none of the polypeptides.

[00123] The four endogenous Shigella polypeptides referred to above are IpgB1 (e.g., NCBI Ref Seq: NP_858263 (SEQ ID NO: 97)); IpgD (e.g., NCBI Ref Seq: NP_085296 (SEQ ID NO: 98)); IpaA (e.g., NCBI Ref Seq: NP_858264 (SEQ ID NO: 99)) and IcsB (e.g., NCBI Ref Seq: NP_085294 (SEQ ID NO: 100)). Homologs of the foregoing Shigella polypeptides are also contemplated for use in the compositions and methods described herein. By way of non-limiting example, SopB (e.g., NCBI Ref Seq: NP_460064 (SEQ ID NO: 101)) is a homolog of IpgD and SipA (e.g., NCBI Ref Seq: NP_461803 (SEQ ID NO: 102)) is a homolog of IpaA. Numerous species have a Rho GTP exchange factor that is a homolog of IpgB1.

[00124] Homologs of any given polypeptide or nucleic acid sequence can be found using, e.g., BLAST programs (freely available on the world wide web at http:// blast.ncbi.nlm.nih.gov/), e.g. by searching freely available databases of sequence for homologous sequences, or by querying those databases for annotations indicating a homolog (e.g. search strings that comprise a gene name or describe the activity of a gene). The homologous amino acid or DNA sequence can be at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more, identical to a reference sequence. The degree of homology (percent identity) between a reference and a second sequence can be determined, for example, by comparing the two sequences using freely available computer programs commonly employed for this purpose on the world wide web.

[00125] In some embodiments of any of the aspect described herein, a homolog can be a polypeptide with the same function, functional characteristics, and/or activity as the reference

polypeptide. By way of non-limiting example, a homolog with the same function as one of the four endogenous *Shigella* polypeptides mentioned above (i.e. IpgB1; IpgD; IpaA; and IcsB) can be identified by engineering a bacteria to not express one of IpgB1; IpgD; IpaA; and IcsB and to instead express a putative functional homolog and then measuring the ability of the bacteria to invade a target cell. If the bacteria retains at least 10% of the reference ability to invade a target cell, the putative functional homolog is demonstrated to be a functional homolog. In some embodiments, a functional homolog has at least 10% of the activity of the reference polypeptide, e.g. 10% or more, 20% or more, 30% or more, 50% or more, 75% or more, 80% or more, 90% or more, 95% or more, or 100% or more of the activity of the reference polypeptide.

[00126] In addition to a first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS), the engineered non-pathogenic microbial cells described herein further comprise a second nucleic acid sequence encoding a T3SS-compatible antibody reagent. As used herein the term "T3SS-compatible antibody reagent" refers to any antibody reagent expressed in the microbial cell that, in the presence of a functional T3SS, can be delivered to the cytoplasm of a target cell. A T3SS-compatible antibody reagent can be from any source, e.g. the reagent can have a eukaryotic origin, or a synthetic origin. A T3SS-compatible antibody reagent can be a naturally-occuring antibody reagent or a mutant and/or variant thereof. In a variant T3SS-compatible antibody reagent, one or more residues can be altered, deleted, and/or added as compared to a naturally-occuring and/or wild-type antibody reagent so long as the function remains substantially the same. Antibody reagents, including VHH, are discussed in further detail elsewhere herein.

[00127] In some embodiments, the T3SS-compatible antibody reagent is exogenous to the microbial cell. In some embodiments, the T3SS-compatible antibody reagent is ectopic with respect to the target cell. In some embodiments, the T3SS-compatible antibody reagent is exogenous with respect to the target cell. In some embodiments, the T3SS-compatible polypeptide is antibody reagent with respect to the target cell.

[00128] In some embodiments, the T3SS-compatible antibody reagent can comprise an N-terminal type three secretion system (T3SS) signal. Naturally-occurring T3SS substrates comprise a secretion signal within the first 20 amino acids of the polypeptide. Certain naturally-occuring T3SS substrates comprise a chaperone-binding domain within the first 50 amino acids of the polypeptide.

[00129] In order for the T3SS-compatible antibody reagent to be expressed, the nucleic acid encoding the T3SS-compatible antibody reagent can be operatively linked to a promoter. In some embodiments, the T3SS-compatible antibody reagent can be constitutively expressed. In some embodiments, nucleic acids encoding the T3SS-compatible antibody reagent can be operatively linked to a constitutive promoter. In some embodiments, the T3SS-compatible antibody reagent can be inducibly expressed. In some embodiments, nucleic acids encoding the T3SS-compatible antibody reagent can be operatively linked to an inducible promoter. In some embodiments, the T3SS-

compatible antibody reagent to can be operatively linked to a type three secretion system (T3SS)-associated promoter or promoter element.

[00130] In some embodiments, a nucleic acid encoding a T3SS-compatible antibody reagent is present within the prokaryotic genome, e.g. the nucleic acids can be incorporated into the genome. Typically, in bacteria, one uses homologous recombination to target genes to specific sites on bacterial chromosomes. In some embodiments, a nucleic acid encoding a T3SS-compatible antibody reagent is present within a vector. In some embodiments, a nucleic acid encoding a T3SS-compatible antibody reagent is present within a portion of a plasmid. In some embodiments, a nucleic acid encoding a T3SS-compatible single domain antibody reagent is present within a vector. In some embodiments, a nucleic acid encoding a T3SS-compatible single-domain antibody reagent is present within a portion of a plasmid.

[00131] In addition to a first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS) and a second nucleic acid sequence encoding a T3SS-compatible antibody reagent, in some embodiments, the engineered non-pathogenic microbial cells described herein further comprise a third nucleic acid sequence encoding a master T3SS regulator.

[00132] In some embodiments, an engineered microbial cell can comprise nucleic acid sequence(s) encoding multiple T3SS-compatible antibody reagents. The multiple T3SS-compatible antibody reagents can be encoded as part of the same operon and/or as part of separate operons.

[00133] In one aspect, described herein is a kit comprising an engineered microbial cell comprising a first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS); and a second nucleic acid sequence encoding an T3SS-compatible antibody reagent; wherein the engineered microbial cell is non-pathogenic with respect to a target cell.

[00134] The antibody reagent comprised by an engineered microbial cell comprising a first nucleic acid sequence comprising genes encoding a functional type three secretion system (T3SS), can bind specifically to, e.g., pro-inflammatory factors (e.g., pro-inflammatory cytokines or signaling molecules) or cancer cell markers and/or factors that promote the growth, metastasis, and/or survivial of cancer cells.

[00135] In one aspect, the technology described herein relates to a method of introducing a polypeptide into a target cell, the method comprising contacting the target cell with an engineered microbial cell comprising a T3SS and a T3SS-compatible antibody reagent as described herein. In some embodiments, the methods described herein relate to treating a subject. Subjects having a condition described here (e.g. inflammation or cancer) can be identified by a physician using current methods of diagnosing such conditions. For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular

embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. If there is an apparent discrepancy between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[00136] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[00137] The terms "decrease", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease by a statistically significant amount. In some embodiments of any of the aspects, "reduce," "reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, "reduction" or "inhibition" does not encompass a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition as compared to a reference level. A decrease can be preferably down to a level accepted as within the range of normal for an individual without a given disorder.

[00138] The terms "increased", "increase", "enhance", or "activate" are all used herein to mean an increase by a statically significant amount. In some embodiments of any of the aspects, the terms "increased", "increase", "enhance", or "activate" can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, an "increase" is a statistically significant increase in such level.

[00139] A T3SS is a multi-protein structure found in gram negative bacteria. It moves polypeptides from the cytoplasm of the bacterial cell through the interior of the T3SS "needle" into the cytoplasm of a target cell. T3SS's are found in pathogenic strains and have been observed in pathogenic isolates of, e.g., *Shigella, Salmonella, E. coli, Burkholderia, Yersinia, Chlamydia, Pseudomonas, Erwinia, Ralstonia, Rhizobium, Vibrio,* and *Xanthamonas.* Further discussion of T3SS's can be found, e.g. in Izore et al. Structure 2011 19:603-612; Korotkov et al. Nature Reviews Microbiology 2012 10:336-351; Wooldridge, K. (ed) Bacterial Secreted Proteins. Caster Academic Press 2009; Snyder and Champness (eds.) Molecular Genetics of Bacteria. 3rd Ed. ASM Press: 2007; each of which is incorporated by reference herein in its entirety. The suite of T3SS-related proteins in a given wild-type cell is typically divided into structural proteins (those proteins which form the needle itself), substrate proteins (those proteins which are transported through the needle to the host), and chaperones (those proteins that bind effectors in the cytoplasm to protect, process, and/or shuttle the effectors to the needle).

[00140] In some embodiments, a nucleic acid encoding a polypeptide is present within the prokaryotic genome, e.g. the nucleic acids can be incorporated into the genome. Typically, in bacteria, one uses homologous recombination to target genes to specific sites on bacterial chromosomes. In some embodiments, a nucleic acid encoding a polypeptide is present within a vector. The term "vector", as used herein, refers to a nucleic acid construct designed for delivery to cell or transfer between different cells. Many vectors useful for transferring exogenous genes into target cells are available, e.g. the vectors may be episomal, e.g., plasmids, virus derived vectors or may be integrated into the target cell genome, through homologous recombination or random integration. In some embodiments, a vector can be an expression vector. As used herein, the term "expression vector" refers to a vector that has the ability to incorporate and express heterologous nucleic acid fragments in a cell. An expression vector may comprise additional elements. The nucleic acid incorporated into the vector can be operatively linked to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence.

[00141] In some embodiments, a nucleic acid encoding a polypeptide is present within a portion of a plasmid. Plasmid vectors can include, but are not limited to, pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993)

from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," *Gene Expression Technology*, vol. 185 (1990), which is hereby incorporated by reference in its entirety). In some embodiments, the plasmid is a low-copy number plasmid, e.g., pBR, pACYC, and/or SC101.

[00142] As used herein, a "subject" means a human or non-human animal. Usually the non-human animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, "individual," "patient" and "subject" are used interchangeably herein.

[00143] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of a given condition. A subject can be male or female and an adult or a child, including infants.

[00144] A subject can be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment or one or more complications related to such a condition, and optionally, have already undergone treatment. Alternatively, a subject can also be one who has not been previously diagnosed as having a condition or one or more complications related to the condition as described herein.

[00145] A "subject in need" of treatment for a particular condition can be a subject having that condition, diagnosed as having that condition as desrcibed herein, such as inflammation or cancer.

[00146] As used herein, the terms "protein" and "polypeptide" are used interchangeably herein to designate a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g., phosphorylated, glycated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but

usage of these terms in the art overlaps. The terms "protein" and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[00147] In the various embodiments described herein, it is further contemplated that variants (naturally occurring or otherwise), alleles, homologs, conservatively modified variants, and/or conservative substitution variants of any of the specific polypeptides described are encompassed. As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid and retain the desired activity of the polypeptide. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles consistent with the disclosure.

[00148] A given amino acid can be replaced by a residue having similar physiochemical characteristics, e.g., substituting one aliphatic residue for another (such as Ile, Val, Leu, or Ala for one another), or substitution of one polar residue for another (such as between Lys and Arg; Glu and Asp; or Gln and Asn). Other such conservative substitutions, e.g., substitutions of entire regions having similar hydrophobicity characteristics, are well known. Polypeptides comprising conservative amino acid substitutions can be tested in any one of the assays described herein to confirm that a desired activity, e.g. antigen-binding activity and specificity of a native or reference polypeptide is retained.

[00149] Amino acids can be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q); (3) acidic: Asp (D), Glu (E); (4) basic: Lys (K), Arg (R), His (H). Alternatively, naturally occurring residues can be divided into groups based on common side-chain properties: (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; (6) aromatic: Trp, Tyr, Phe. Non-conservative substitutions will

entail exchanging a member of one of these classes for another class. Particular conservative substitutions include, for example; Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

[00150] In some embodiments, the polypeptide described herein can be a variant of a sequence described herein. In some embodiments, the variant is a conservatively modified variant. Conservative substitution variants can be obtained by mutations of native nucleotide sequences, for example. A "variant," as referred to herein, is a polypeptide substantially homologous to a native or reference polypeptide, but which has an amino acid sequence different from that of the native or reference polypeptide because of one or a plurality of deletions, insertions or substitutions. Variant polypeptide-encoding DNA sequences encompass sequences that comprise one or more additions, deletions, or substitutions of nucleotides when compared to a native or reference DNA sequence, but that encode a variant protein or fragment thereof that retains activity. A wide variety of PCR-based site-specific mutagenesis approaches are also known in the art and can be applied by the ordinarily skilled artisan.

[00151] A variant amino acid or DNA sequence can be at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more, identical to a native or reference sequence. The degree of homology (percent identity) between a native and a mutant sequence can be determined, for example, by comparing the two sequences using freely available computer programs commonly employed for this purpose on the world wide web (e.g. BLASTp or BLASTn with default settings).

[00152] Alterations of the native amino acid sequence can be accomplished by any of a number of techniques known to one of skill in the art. Mutations can be introduced, for example, at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion. Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered nucleotide sequence having particular codons altered according to the substitution, deletion, or insertion required. Techniques for making such alterations are very well established and include, for example,

those disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985, 12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462, which are herein incorporated by reference in their entireties. Any cysteine residue not involved in maintaining the proper conformation of the polypeptide also can be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) can be added to the polypeptide to improve its stability or facilitate oligomerization.

[00153] As used herein, the term "nucleic acid" or "nucleic acid sequence" refers to any molecule, preferably a polymeric molecule, incorporating units of ribonucleic acid, deoxyribonucleic acid or an analog thereof. The nucleic acid can be either single-stranded or double-stranded. A single-stranded nucleic acid can be one nucleic acid strand of a denatured double- stranded DNA. Alternatively, it can be a single-stranded nucleic acid not derived from any double-stranded DNA. In one aspect, the nucleic acid can be DNA. In another aspect, the nucleic acid can be RNA. Suitable nucleic acid molecules are DNA, including genomic DNA or cDNA. Other suitable nucleic acid molecules are RNA, including mRNA.

[00154] As used herein, "engineered" refers to the aspect of having been manipulated by the hand of man. For example, a cell is considered to be "engineered" when at least one aspect of the cell has been manipulated by the hand of man to differ from the aspect as it exists in nature. As is common practice and is understood by those in the art, progeny an engineered cell are typically still referred to as "engineered" even though the actual manipulation was performed on a prior entity.

[00155] As used herein, "microbe" or "microorganism" refers to an organism which is microscopic. A microbe can be a single-celled organism. In some embodiments of any of the aspects, a microbe can be a bacterium.

[00156] The term "expression" refers to the cellular processes involved in producing RNA and proteins and as appropriate, secreting proteins, including where applicable, but not limited to, for example, transcription, transcript processing, translation and protein folding, modification and processing. "Expression products" include RNA transcribed from a gene, and polypeptides obtained by translation of mRNA transcribed from a gene. The term "gene" means the nucleic acid sequence which is transcribed (DNA) to RNA *in vitro* or *in vivo* when operatively linked to appropriate regulatory sequences. A gene may or may not include

regions preceding and following the coding region, e.g. 5' untranslated (5'UTR) or "leader" sequences and 3' UTR or "trailer" sequences.

[00157] The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed, and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence, and, optionally, production of the desired polypeptide encoded by the polynucleotide sequence. In some examples, transcription of a nucleic acid is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the nucleic acid in a cell-type in which expression is intended. It will also be understood that the nucleic acid can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of a protein.

[00158] The term "isolated" or "partially purified" as used herein refers, in the case of a nucleic acid or polypeptide, to a nucleic acid or polypeptide separated from at least one other component (*e.g.*, nucleic acid or polypeptide) that is present with the nucleic acid or polypeptide as found in its natural source and/or that would be present with the nucleic acid or polypeptide when expressed by a cell, or secreted in the case of secreted polypeptides. A chemically synthesized nucleic acid or polypeptide or one synthesized using *in vitro* transcription/translation is considered "isolated."

[00159] As used herein, a "target cell" is a cell close to which an engineered microbial cell as described herein delivers a payload polypeptide, e.g., the payload polypeptide is delivered to the extracellular space surrounding the target cell. In some embodiments, a target cell is a eukaryotic cell. In some embodiments, a target cell is a cell comprised by, or originating from, a vertebrate. In some embodiments, a target cell is a cell comprised by, or originating from, a mammal. A "target organism" is an organism comprising at least one "target cell." A target cell can be located *in vitro* or *in vivo*. In some embodiments, a target cell is an isolated target cell. In some embodiments, a target cell is not an isolated target cell. In some embodiments, the target cell is part of the target organism.

[00160] As used herein, the term "non-pathogenic" refers to a microbial cell which does not have a deleterious effect upon a target cell, i.e. in the presence of the non-pathogenic microbial cell, a target cell will not have a statistically significantly increased rate of cell death, nor a statistically significantly decreased metabolic rate or altered rate of growth and/or division. It is recognized that, therefore, whether a cell is non-pathogenic with respect

to a target cell may vary depending upon, e.g. the environment in which the target cell is located and the concentration of the microbial cells. In some embodiments, a non-pathogenic microbial cell is non-pathogenic if it does not have a deleterious effect upon a target cell *in vitro* when the microbial cell is present at a concentration of less than 50x relative to the target cell. In some embodiments, a non-pathogenic microbial cell can be one that does not express a toxin having a deleterious effect upon the target cell. In some embodiments, a non-pathogenic microbial cell can be one that does not replicate within the target cell. In some embodiments, a non-pathogenic microbial cell can be one that is not found in the cytoplasm of the target cell. In some embodiments a non-pathogenic microbial cell can be one that is not found in the cytoplasm of the target cell but is found in the phagosome of the target cell. In some embodiments, a non-pathogenic microbial cell can be a commensal microbial cell. In some embodiments, a non-pathogenic microbial cell can be a non-immunogenic microbial cell, i.e. a cell that does not cause a target cell to secrete increased levels of, e.g. IL-8 when the microbial cell is present.

[00161] Non-limiting examples of non-pathogenic microbial cells with respect to human target cells can include, but are not limited to: *E. coli* K12; *E. coli* DH5α, *E. coli* HB101, *E. coli* BL21, *E. coli* DH10beta, *E. coli* JM110, *E. coli* MinT3, and virulence-cured *Shigella* strains (e.g. those missing the virulence plasmid encoding the T3SS and >20T3SS-compatible effectors). Non-limiting examples of commensal microbial cells with respect to human subjects include, but are not limited to: *E. coli* NISSLE 1917 (EcN); *E. coli* 83972; *E. coli* M17. In some embodiments of any of the aspects, *E. coli* NISSLE 1917 are used in some applications. In some embodiments of any of the aspects, *E. coli* K12 and/or DH5α are used in some applications.

[00162] As used herein, "commensal" refers to one of two organisms living in permanent close association, the referred to organism being one which gains a benefit from the association without causing serious disadvantage to the second organism under normal conditions. Commensal bacteria can include, e.g., non-pathogenic bacteria which form part of the normal flora of a healthy human alimentary tract.

[00163] A "cancer cell" is a cancerous, pre-cancerous, or transformed cell, either *in vivo*, *ex vivo*, or in tissue culture, that has spontaneous or induced phenotypic changes that do not necessarily involve the uptake of new genetic material. Although transformation can arise from infection with a transforming virus and incorporation of new genomic nucleic acid, or uptake of exogenous nucleic acid, it can also arise spontaneously or following exposure to a

carcinogen, thereby mutating an endogenous gene. Transformation/cancer is associated with, e.g., morphological changes, immortalization of cells, aberrant growth control, foci formation, anchorage independence, malignancy, loss of contact inhibition and density limitation of growth, growth factor or serum independence, tumor specific markers, invasiveness or metastasis, and tumor growth in suitable animal hosts such as nude mice. *See, e.g.,* Freshney, CULTURE ANIMAL CELLS: MANUAL BASIC TECH. (3rd ed., 1994). As used herein, the term "cancer" refers to an uncontrolled growth of cells that interferes with the normal functioning of the bodily organs and systems. A subject who has a cancer or a tumor is a subject having objectively measurable cancer cells present in the subject's body. Included in this definition are benign and malignant cancers, as well as dormant tumors or micrometastases. Cancers that migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs.

[00164] A "tumor" as used herein refers to an uncontrolled growth of cells tumor interferes with the normal functioning of the bodily organs and systems. The terms "cancer" and "malignancy" refer to a tumor that is metastatic, i.e. that is it has become invasive, seeding tumor growth in tissues remote from the original tumor site. A subject that has a cancer or a tumor is a subject having objectively measurable cancer cells present in the subject's body. Included in this definition are benign tumors and malignant cancers, as well as potentially dormant tumors or micrometastatses. Cancers that migrate from their original location and seed other vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. Hematopoietic cancers, such as leukemia, are able to out-compete the normal hematopoietic compartments in a subject, thereby leading to hematopoietic failure (in the form of anemia, thrombocytopenia and neutropenia) ultimately causing death.

[00165] Examples of cancer include but are not limited to, carcinoma, blastoma, sarcoma, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and CNS cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma (GBM); hepatic carcinoma; hepatoma; intra-epithelial neoplasm.; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and

squamous carcinoma of the lung); lymphoma including Hodgkin's and non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (e.g., lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; as well as other carcinomas and sarcomas.

[00166] As used herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with a disease or disorderThe term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a condition. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality, whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00167] As used herein, the term "pharmaceutical composition" refers to the active agent in combination with a pharmaceutically acceptable carrier e.g. a carrier commonly used in the pharmaceutical industry. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00168] As used herein, the term "administering," refers to the placement of a compound as disclosed herein into a subject by a method or route which results in at least partial delivery of the agent at a desired site. Pharmaceutical compositions comprising the

compounds disclosed herein can be administered by any appropriate route which results in an effective treatment in the subject.

[00169] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[00170] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[00171] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[00172] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00173] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[00174] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[00175] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3);

Robert S. Porter *et al.* (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), Taylor & Francis Limited, 2014 (ISBN 0815345305, 9780815345305); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737), the contents of which are all incorporated by reference herein in their entireties.

[00176] One of skill in the art can readily identify a chemotherapeutic agent of use (*e.g.* see Physicians' Cancer Chemotherapy Drug Manual 2014, Edward Chu, Vincent T. DeVita Jr., Jones & Bartlett Learning; Principles of Cancer Therapy, Chapter 85 in Harrison's Principles of Internal Medicine, 18th edition; Therapeutic Targeting of Cancer Cells: Era of Molecularly Targeted Agents and Cancer Pharmacology, Chs. 28-29 in Abeloff's Clinical Oncology, 2013 Elsevier; and Fischer D S (ed): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 2003).

[00177] Other terms are defined herein within the description of the various aspects of the invention.

[00178] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely

for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00179] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. Moreover, due to biological functional equivalency considerations, some changes can be made in protein structure without affecting the biological or chemical action in kind or amount. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00180] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[00181] The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

[00182] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. An engineered, non-pathogenic, gram negative microbial cell comprising:

a) a first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS); wherein the TDESS comprises at least virB; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiD; mxiA; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40; and

b) a second nucleic acid sequence encoding an T3SS-compatible payload polypeptide.

2. The microbial cell of paragraph 1, wherein the cell does not comprise or express at least one of:

a. IpaB;

b. IpaD; and

c. MxiC.

3. The microbial cell of paragraph 2, wherein the cell does not comprise or express at least one of:

a. IpaB and IpaD; and

b. MxiC.

4. The microbial cell of paragraph 3, wherein the cell does not comprise or express IpaB; IpaD; and MxiC.

5. The microbial cell of paragraph 3, wherein the cell does not comprise or express IpaB; IpaD; IpaC; and MxiC.

6. The microbial cell of any of paragraphs 1-5, wherein the cell has a mutated MxiH.

7. The microbial cell of paragraph 6, wherein the cell has a D73A mutation in MxiH.

8. The microbial cell of any of paragraphs 1-7, wherein the second nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding an T3SS-compatible payload polypeptide.

9. The microbial cell of paragraph 8, wherein the inducible promoter sequence is regulated by a master T3SS regulator.

10. The microbial cell of any of paragraphs 8-9, wherein the inducible promoter sequence comprises a (T3SS)-associated promoter or promoter element.

11. The microbial cell of paragraph 10, wherein the T3SS-associated promoter or promoter element is a MxiE recognition sequence.

12. The microbial cell of any of paragraphs 1-11, wherein the cell comprises a third nucleic acid sequence encoding a master T3SS regulator.

13. The microbial cell of paragraph 12, wherein the master T3SS regulator is selected from the group consisting of:

VirB and VirF.

14. The microbial cell of any of paragraphs 12-13, wherein the third nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding a master T3SS regulator.

15. The microbial cell of paragraph 14, wherein the inducible promoter is selected from the group consisting of:
an arabinose-inducible promoter; pBAD arabinose-inducible promoter; tumor-induced promoters; ansB promoter; pflE promoter; napF promoter; and an inflammation-induced promoter.

16. The microbial cell of any of paragraphs 1-15, wherein the TDESS comprises at least: virB; acp; ipaA; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiE; mxiD; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40.

17. The microbial cell of any of paragraphs 1-16, wherein the TDESS comprises polypeptides endogenous to a bacterium selected from the group consisting of:
Shigella spp; Salmonella spp; enteropathogenic E. coli; and Yersinia spp.

18. The microbial cell of any of paragraphs 1-17, wherein the first nucleic acid sequence is located on a plasmid.

19. The engineered microbial cell of any of paragraphs 1-17, wherein the first nucleic acid sequence is located on a chromosome.

20. The microbial cell of any of paragraphs 1-19, wherein the second nucleic acid sequence is located on a plasmid.

21. The engineered microbial cell of any of paragraphs 1-19, wherein the second nucleic acid sequence is located on a chromosome.

22. The microbial cell of any of paragraphs 1-21, wherein the T3SS-compatible payload polypeptide comprises a T3SS secretion sequence.

23. The microbial cell of any of paragraphs 1-22, wherein the T3SS-compatible payload polypeptide comprises an N-terminal T3SS secretion sequence.

24. The microbial cell of any of paragraphs 1-23, wherein the T3SS-compatible payload polypeptide comprises a OspC3 T3SS secretion sequence.

25. The microbial cell of any of paragraphs 1-24, wherein the T3SS-compatible payload polypeptide comprises an anti-inflammatory polypeptide.

26. The microbial cell of paragraph 25, wherein the anti-inflammatory polypeptide is IL-10 or IL-27.

27. The microbial cell of any of paragraphs 1-24, wherein the T3SS-compatible payload polypeptide comprises an antibody reagent.

28. The microbial cell of paragraph 27, wherein the antibody reagent is selected from the group consisting of:
a nanobody; a VNA; and a VHH.

29. The microbial cell of any of paragraphs 27-28, wherein the cell comprises at least one further nucleic acid sequence encoding an additional T3SS-compatible payload polypeptide comprising an antibody reagent.

30. The microbial cell of any of paragraphs 27-28, wherein the one or more antibody reagents form a multimeric complex.

31. The microbial cell of paragraph 30, wherein the multimeric complex is multispecific.

32. The microbial cell of any of paragraphs 27-31, wherein the antibody reagent specifically binds to a cancer cell marker.

33. The microbial cell of any of paragraphs 27-31, wherein the antibody reagent specifically binds to a cancer checkpoint polypeptide.

34. The microbial cell of any of paragraphs 27-33, wherein the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 reagent.

35. The microbial cell of any of paragraphs 27-34, wherein the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 VNA or VHH.

36. The microbial cell of any of paragraphs 27-31, wherein the antibody reagent specifically binds to an inflammatory cytokine receptor or an inflammatory cytokine.

37. The microbial cell of paragraph 36, wherein the antibody reagent binds to a molecule selected from the group consisting of:
TNFα, IL-8; IL-6, IL-18, IL-21, Il-33 and IL-13.

38. The microbial cell of any of paragraphs 27-31, wherein the antibody reagent specifically binds to a bacterial toxin.

39. The microbial cell of paragraph 38, wherein the bacterial toxin is an E. coli or C. difficile toxin.

40. The microbial cell of any of paragraphs 38-39, wherein the bacterial toxin is selected from the group consisting of:
shiga toxin; C. difficile toxin A (TcdA); C. difficile toxin B (TcdB); cholera toxin; anthrax toxin; and botulinum toxin.

41. The microbial cell of any of paragraphs 1-24, wherein the T3SS-compatible payload polypeptide comprises a toxin.
42. The microbial cell of any of paragraphs 1-24, wherein the T3SS-compatible payload polypeptide comprises an antigen.

43. The microbial cell of any of paragraphs 1-42, wherein the microbial cell is engineered from a microbial cell selected from the group consisting of:
E. coli NISSLE 1917 (EcN); E. coli K12; MP; HS; and derivative strains thereof.
44. The microbial cell of paragraph 43, wherein the strain which is derivative of E. coli K12 is selected from the group consisting of:
E. coli DH10β and E. coli DH5α.
45. The microbial cell of any of paragraphs 1-43, wherein the microbial cell is engineered from E. coli NISSLE 1917 (EcN).
46. The microbial cell of any of paragraphs 1-45, wherein the microbial cell is engineered from a commensal intestinal microbial cell.
47. The microbial cell of paragraph 46, wherein the commensal intestinal microbial cell is E. coli NISSLE 1917 (EcN).
48. The microbial cell of any of paragraphs 1-42, wherein the non-pathogenic microbial cell is engineered from a pathogenic microbial cell organism by deletion or mutation of one or more T3SS components.
49. The microbial cell of paragraph 48, wherein the one or more T3SS components is selected from the group consisting of:
a toxin; a T3SS effector; a structural T3SS polypeptide; and a master regulator of T3SS components.
50. The microbial cell of any of paragraphs 48-49, wherein the pathogenic microbial cell is selected from the group consisting of:
Salmonella spp.; Shigella Spp; andYersinia spp.

51. The microbial cell of paragraph 48-50, wherein the pathogenic microbial cell is selected from the group consisting of:
Salmonella typhimurium SPI1and Shigella felxneri mxi-spa.

52. The microbial cell of any of paragraphs 1-51, wherein cell has been:
a. contacted with a mutagenic treatment; and
b. selected for increased secretion.
53. The microbial cell of any of paragraphs 1-52, wherein cell has been:
a. contacted with a mutagenic treatment; and
b. selected for increased secretion of the T3SS-compatible payload polypeptide.

54. The microbial cell of any of paragraphs 1-53, wherein the cell further comprises a nucleic acid sequence encoding one or more polypeptides that increase adhesion to a target cell.
55. The microbial cell of paragraph 54, wherein the polypeptides that increase adhesion to the target cell comprise Tir and intimin.
56. The microbial cell of paragraph 55, wherein the polypeptide that increases adhesion to the target cell is selected from a group consisting of:
a bacterial adhesion; Afa1; AIDA; invasion; an antibody reagent specific for an extracellular epitope of a target cell polypeptide; and a single chain antibody specific for an extracellular epitope of a target cell polypeptide.

57. A method of introducing a polypeptide into a target tissue or organism, the method comprising contacting the target tissue or organism with a microbial cell of any of paragraphs 1-56.
58. A method of reducing inflammation in a subject, the method comprising administering an microbial cell of any of paragraphs 25-26 or 36-37 to a subject in need thereof.
59. The method of paragraph 58, wherein the inflammation is inflammation of the gastrointestinal tract.
60. The method of any of paragraphs 58-59, wherein the subject is in need of treatment for a condition selected from the group consisting of:
asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis.

61. The method of paragraph 60, wherein the subject is a subject in need of treatment for inflammatory bowel disease.

62. The method of any of paragraphs 58-61, wherein the microbial cell is administered orally.

63. A method of treating cancer in a subject, the method comprising administering an microbial cell of any of paragraphs 26-35 to a subject in need thereof.

64. The method of paragraph 63, wherein the microbial cell is administered systemically.

65. The method of paragraph 63, wherein the microbial cell is administered intratumorally.

66. The method of paragraph 63, wherein the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally.

67. The method of any of paragraphs 63-66, wheiren the microbial cell is engineered from E. coli NISSLE 1917 (EcN).

68. A method of treating an intestinal infection in a subject, the method comprising administering a microbial cell of any of paragraphs 38-39 to a subject in need thereof.

69. The method of paragraph 68, wherein the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin.

70. The method of paragraph 68, wherein the intestinal infection is a C. difficile infection and/or the subject has C. difficile colitis and the toxin is TcdA and/or TcdB.

71. The method of paragraph 68, wherein the intestinal infection is cholera and the toxin is cholera toxin.

72. The method of paragraph 68, wherein the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin.

73. The method of paragraph 68, wherein the intestinal infection is botulism and the toxin is botulinum toxin.

74. The method of any of paragraphs 68-73, wherein the microbial cell is administered orally.

75. The method of any of paragraphs 57-74, wherein secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master regulator.

76. The method of paragraph 75, wherein the compound is arabinose.

77. A method for delivering a polypeptide into a) the extracellular milieu of a subject's gastrointestinal tract or b) the extracellular milieu of a subject's tumor , the method comprising contacting administering a microbial cell of any of paragraphs 1-56 to the subject.

78. A kit comprising the microbial cell of any of paragraphs 1-56.

79. The use of a microbial cell of any of paragraphs 25-26 or 36-37 to reduce inflammation in a subject in need thereof.

80. The use of paragraph 79, wherein the inflammation is inflammation of the gastrointestinal tract.

81. The use of any of paragraphs 79-80, wherein the subject is in need of treatment for a condition selected from the group consisting of:
asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis.

82. The use of paragraph 81, wherein the subject is a subject in need of treatment for inflammatory bowel disease.

83. The use of any of paragraphs 79-82, wherein the microbial cell is administered orally.

84. The use of an microbial cell of any of paragraphs 26-35 treat cancer in a subject in need thereof.

85. The use of paragraph 84, wherein the microbial cell is administered systemically.

86. The use of paragraph 84, wherein the microbial cell is administered intratumorally.

87. The use of paragraph 84, wherein the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally.

88. The use of any of paragraphs 84-87, wheiren the microbial cell is engineered from E. coli NISSLE 1917 (EcN).

89. The use of a microbial cell of any of paragraphs 38-39 to treat an intestinal infection in a subject in need thereof.

90. The use of paragraph 89, wherein the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin.

91. The use of paragraph 89, wherein the intestinal infection is a C. difficile infection and/or the subject has C. difficile colitis and the toxin is TcdA and/or TcdB.

92. The use of paragraph 89, wherein the intestinal infection is cholera and the toxin is cholera toxin.

93. The use of paragraph 89, wherein the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin.

94. The use of paragraph 89, wherein the intestinal infection is botulism and the toxin is botulinum toxin.

95. The use of any of paragraphs 89-94, wherein the microbial cell is administered orally.

96. The use of any of paragraphs 79-95, wherein secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master regulator.

97. The use of paragraph 96, wherein the compound is arabinose.

[00183] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. An engineered, non-pathogenic, gram negative microbial cell comprising:
    a) a first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS); wherein the TDESS comprises at least virB; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiD; mxiA; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40; and
    b) a second nucleic acid sequence encoding an T3SS-compatible payload polypeptide.
2. The microbial cell of paragraph 1, wherein the cell does not comprise or express at least one of:
    a. IpaB;
    b. IpaD; and
    c. MxiC.
3. The microbial cell of paragraph 2, wherein the cell does not comprise or express at least one of:
    a. IpaB and IpaD; and
    b. MxiC.
4. The microbial cell of paragraph 3, wherein the cell does not comprise or express IpaB; IpaD; and MxiC.
5. The microbial cell of paragraph 3, wherein the cell does not comprise or express IpaB; IpaD; IpaC; and MxiC.

6. The microbial cell of any of paragraphs 1-5, wherein the cell has a mutated MxiH.
7. The microbial cell of paragraph 6, wherein the cell has a D73A mutation in MxiH.
8. The microbial cell of any of paragraphs 1-7, wherein the second nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding an T3SS-compatible payload polypeptide.
9. The microbial cell of paragraph 8, wherein the inducible promoter sequence is regulated by a master T3SS transcriptional regulator.
10. The microbial cell of any of paragraphs 8-9, wherein the inducible promoter sequence comprises a (T3SS)-associated promoter or promoter element.
11. The microbial cell of paragraph 10, wherein the T3SS-associated promoter or promoter element is a MxiE recognition sequence.
12. The microbial cell of any of paragraphs 1-11, wherein the cell comprises a third nucleic acid sequence encoding a master T3SS transcriptional regulator.
13. The microbial cell of paragraph 12, wherein the master T3SS transcriptional regulator is selected from the group consisting of:

    VirB and VirF.
14. The microbial cell of any of paragraphs 12-13, wherein the third nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding a master T3SS transcriptional regulator.
15. The microbial cell of paragraph 14, wherein the inducible promoter is selected from the group consisting of:

    an arabinose-inducible promoter; pBAD arabinose-inducible promoter; an IPTG-inducible promoter; tumor-induced promoters; ansB promoter; pflE promoter; napF promoter; and an inflammation-induced promoter.
16. The microbial cell of any of paragraphs 1-15, wherein the TDESS comprises at least: virB; acp; ipaA; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiE; mxiD; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40.
17. The microbial cell of any of paragraphs 1-16, wherein the TDESS comprises polypeptides endogenous to a bacterium selected from the group consisting of:

    Shigella spp; *Salmonella* spp; enteropathogenic *E. coli*; and *Yersinia* spp.
18. The microbial cell of any of paragraphs 1-17, wherein the first nucleic acid sequence is located on a plasmid.

19. The engineered microbial cell of any of paragraphs 1-17, wherein the first nucleic acid sequence is located on a chromosome.
20. The microbial cell of any of paragraphs 1-19, wherein the second nucleic acid sequence is located on a plasmid.
21. The engineered microbial cell of any of paragraphs 1-19, wherein the second nucleic acid sequence is located on a chromosome.
22. The engineered microbial cell of any of paragraphs 1-21, wherein the first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS) and/or the genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS) are exogenous to the microbial cell.
23. The engineered microbial cell of any of paragraphs 1-22, wherein the first nucleic acid sequence is no greater than 3kb in size.
24. The engineered microbial cell of any of paragraphs 1-22, wherein the first nucleic acid sequence and third nucleic acid sequence are cumulatively no greater than 3 kb in size.
25. The engineered microbial cell of any of paragraphs 1-24, wherein the cell did not comprise a T3SS prior to being engineered to comprise the first and second nucleic acid sequences.

26. The microbial cell of any of paragraphs 1-25, wherein the T3SS-compatible payload polypeptide comprises a T3SS secretion sequence.
27. The microbial cell of any of paragraphs 1-26, wherein the T3SS-compatible payload polypeptide comprises an N-terminal T3SS secretion sequence.
28. The microbial cell of any of paragraphs 1-27, wherein the T3SS-compatible payload polypeptide comprises a OspC3 T3SS secretion sequence.
29. The microbial cell of any of paragraphs 1-28, wherein the T3SS-compatible payload polypeptide comprises an anti-inflammatory polypeptide.
30. The microbial cell of paragraph 29, wherein the anti-inflammatory polypeptide is IL-10 or IL-27.
31. The microbial cell of any of paragraphs 1-30, wherein the T3SS-compatible payload polypeptide comprises an antibody reagent.

32. The microbial cell of paragraph 31, wherein the antibody reagent is selected from the group consisting of:

a nanobody; a VNA; and a VHH.

33. The microbial cell of any of paragraphs 31-32, wherein the cell comprises at least one further nucleic acid sequence encoding an additional T3SS-compatible payload polypeptide comprising an antibody reagent, VHH, or VNA.
34. The microbial cell of any of paragraphs 31-32, wherein the one or more antibody reagents form a multimeric complex.
35. The microbial cell of paragraph 34, wherein the multimeric complex is multispecific.
36. The microbial cell of any of paragraphs 31-35, wherein the antibody reagent specifically binds to a cancer cell marker.
37. The microbial cell of any of paragraphs 31-35, wherein the antibody reagent specifically binds to a cancer checkpoint polypeptide.
38. The microbial cell of any of paragraphs 31-37, wherein the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 reagent.
39. The microbial cell of any of paragraphs 31-38, wherein the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 VNA or VHH.
40. The microbial cell of any of paragraphs 31-39, wherein the antibody reagent specifically binds to an inflammatory cytokine receptor or an inflammatory cytokine.
41. The microbial cell of paragraph 40, wherein the antibody reagent binds to a molecule selected from the group consisting of:

TNFα, IL-8; IL-6, IL-18, IL-21, Il-33 and IL-13.

42. The microbial cell of any of paragraphs 31-35, wherein the antibody reagent specifically binds to a bacterial toxin.
43. The microbial cell of paragraph 42, wherein the bacterial toxin is an *E. coli* or *C. difficile* toxin.
44. The microbial cell of any of paragraphs 42-43, wherein the bacterial toxin is selected from the group consisting of:

shiga toxin; *C. difficile* toxin A (TcdA); *C. difficile* toxin B (TcdB); cholera toxin; anthrax toxin; and botulinum toxin.

45. The microbial cell of any of paragraphs 1-28, wherein the T3SS-compatible payload polypeptide comprises a toxin.
46. The microbial cell of any of paragraphs 1-28, wherein the T3SS-compatible payload polypeptide comprises an antigen.

47. The microbial cell of any of paragraphs 1-46, wherein the microbial cell is engineered from a microbial cell selected from the group consisting of:

    E. coli NISSLE 1917 (EcN); *E. coli* K12; MP; HS; and derivative strains thereof.

48. The microbial cell of paragraph 47, wherein the strain which is derivative of *E. coli* K12 is selected from the group consisting of:

    E. coli DH10β and *E. coli* DH5α.

49. The microbial cell of any of paragraphs 1-47, wherein the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).
50. The microbial cell of any of paragraphs 1-49, wherein the microbial cell is engineered from a commensal intestinal microbial cell.
51. The microbial cell of paragraph 50, wherein the commensal intestinal microbial cell is *E. coli* NISSLE 1917 (EcN).
52. The microbial cell of any of paragraphs 1-51, wherein the non-pathogenic microbial cell is engineered from a pathogenic microbial cell organism by deletion or mutation of one or more T3SS components.
53. The microbial cell of paragraph52, wherein the one or more T3SS components is selected from the group consisting of:

    a toxin; a T3SS effector; a structural T3SS polypeptide; and a master transcriptional regulator of T3SS components.

54. The microbial cell of any of paragraphs 52-53, wherein the pathogenic microbial cell is selected from the group consisting of:

    Salmonella spp.; *Shigella* Spp; and*Yersinia* spp.

55. The microbial cell of paragraph 52-54, wherein the pathogenic microbial cell is selected from the group consisting of:

    Salmonella typhimurium SPI1and *Shigella felxneri* mxi-spa.

56. The microbial cell of any of paragraphs 1-55, wherein cell has been:

a. contacted with a mutagenic treatment; and
b. selected for increased secretion.

57. The microbial cell of any of paragraphs 1-56, wherein cell has been:
    a. contacted with a mutagenic treatment; and
    b. selected for increased secretion of the T3SS-compatible payload polypeptide.

58. The microbial cell of any of paragraphs 1-57, wherein the cell further comprises a nucleic acid sequence encoding one or more polypeptides that increase adhesion to a target cell.
59. The microbial cell of paragraph 58, wherein the polypeptides that increase adhesion to the target cell comprise Tir and intimin.
60. The microbial cell of paragraph 59, wherein the polypeptide that increases adhesion to the target cell is selected from a group consisting of:
    a bacterial adhesion; Afa1; AIDA; invasion; an antibody reagent specific for an extracellular epitope of a target cell polypeptide; and a single chain antibody specific for an extracellular epitope of a target cell polypeptide.

61. A method of introducing a polypeptide into a target tissue or organism, the method comprising contacting the target tissue or organism with a microbial cell of any of paragraphs 1-60.
62. A method of reducing inflammation in a subject, the method comprising administering an microbial cell of any of paragraphs 29-30 or 40-41 to a subject in need thereof.
63. The method of paragraph 62, wherein the inflammation is inflammation of the gastrointestinal tract.
64. The method of any of paragraphs 62-63, wherein the subject is in need of treatment for a condition selected from the group consisting of:
    asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis.
65. The method of paragraph 64, wherein the subject is a subject in need of treatment for inflammatory bowel disease.
66. The method of any of paragraphs 62-65, wherein the microbial cell is administered orally.

67. A method of treating cancer in a subject, the method comprising administering an microbial cell of any of paragraphs 30-39 to a subject in need thereof.
68. The method of paragraph 67, wherein the microbial cell is administered systemically.
69. The method of paragraph 67, wherein the microbial cell is administered intratumorally.
70. The method of paragraph 67, wherein the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally.
71. The method of any of paragraphs 67-70, wheiren the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).
72. A method of treating an intestinal infection in a subject, the method comprising administering a microbial cell of any of paragraphs 42-43 to a subject in need thereof.
73. The method of paragraph 72, wherein the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin.
74. The method of paragraph 72, wherein the intestinal infection is a *C. difficile* infection and/or the subject has *C. difficile* colitis and the toxin is TcdA and/or TcdB.
75. The method of paragraph 72, wherein the intestinal infection is cholera and the toxin is cholera toxin.
76. The method of paragraph 72, wherein the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin.
77. The method of paragraph 72, wherein the intestinal infection is botulism and the toxin is botulinum toxin.
78. The method of any of paragraphs 72-77, wherein the microbial cell is administered orally.
79. The method of any of paragraphs 61-78, wherein secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master transcriptional regulator.
80. The method of paragraph 79, wherein the compound is arabinose.

81. A method for delivering a polypeptide into a) the extracellular milieu of a subject's gastrointestinal tract, b) the lumen of a tumor, or c) the extracellular milieu of a subject's tumor , the method comprising contacting administering a microbial cell of any of paragraphs 1-60 to the subject.

82. A kit comprising the microbial cell of any of paragraphs 1-60.

83. The use of a microbial cell of any of paragraphs 29-30 or 40-41 to reduce inflammation in a subject in need thereof.
84. The use of paragraph 83, wherein the inflammation is inflammation of the gastrointestinal tract.
85. The use of any of paragraphs 83-84, wherein the subject is in need of treatment for a condition selected from the group consisting of:

asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis.

86. The use of paragraph 85, wherein the subject is a subject in need of treatment for inflammatory bowel disease.
87. The use of any of paragraphs 83-86, wherein the microbial cell is administered orally.
88. The use of an microbial cell of any of paragraphs 30-39 treat cancer in a subject in need thereof.
89. The use of paragraph 88, wherein the microbial cell is administered systemically.
90. The use of paragraph 88, wherein the microbial cell is administered intratumorally.
91. The use of paragraph 88, wherein the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally.
92. The use of any of paragraphs 88-91, wheiren the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).
93. The use of a microbial cell of any of paragraphs 42-4339 to treat an intestinal infection in a subject in need thereof.
94. The use of paragraph 93, wherein the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin.
95. The use of paragraph 93, wherein the intestinal infection is a *C. difficile* infection and/or the subject has *C. difficile* colitis and the toxin is TcdA and/or TcdB.
96. The use of paragraph 93, wherein the intestinal infection is cholera and the toxin is cholera toxin.
97. The use of paragraph 93, wherein the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin.

98. The use of paragraph 93, wherein the intestinal infection is botulism and the toxin is botulinum toxin.

99. The use of any of paragraphs 93-97, wherein the microbial cell is administered orally.

100. The use of any of paragraphs 83-99, wherein secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master transcriptional regulator.

101. The use of paragraph100, wherein the compound is arabinose.

EXAMPLES

[00184] EXAMPLE 1: Engineering Novel Probiotics For The Treatment Of Inflammatory Bowel Disease

[00185] Inflammatory bowel disease (IBD) which includes ulcerative colitis and Crohn's disease is a chronic intestinal disorder that affects over 3 million individuals in the Westernized world [1]. The inflammation associated with these disorders often results from a dysregulated immune response to the commensal microbiota that causes abdominal pain, diarrhea, and rectal bleeding, which in severe cases can require surgical interventions [1, 2]. The mainstay of current treatments is the use of anti-inflammatory drugs, including systemic immunosuppressants like parenteral antibodies that block TNFα activity. However, these treatment options, which are not always sufficient to relieve symptoms, are associated with serious side effects. For example, agents that cause systemic immunosuppression greatly increase the susceptibility of patients for developing serious infections including reactivation of latent tuberculosis, the development of brain abscesses, and disseminated fungal infections [1]. Described herein is a novel treatment for IBD that circumvents these issues by reengineering specialized bacterial secretion systems to target the delivery of the anti-inflammatory cytokine IL-10 to the intestines, thus limiting systemic side effects by directly targeting immunosuppression to the site of disease.

[00186] Role of IL-10 in Inflammatory Bowel Disease (IBD). IL-10 is one of the most important anti-inflammatory cyokines of the intestinal immune system. Its immunosuppressive activities include downregulating synthesis of pro-inflammatory cytokines by regulatory T cells and macrophages, increasing production of anti-inflammatory mediators, and inhibiting antigen presentation in macrophages and dendritic cells [3]. There is extensive data that IL-10 plays a role in suppressing the development of IBD. For example,

genome-wide association studies (GWAS) demonstrate that a subset of patients with IBD have IL-10 promoter polymorphisms associated with reduced IL-10 serum levels [4-6] and IL-10 deficient mice develop colitis similar to that observed in IBD patients [7]. These observations indicate that the administration of recombinant IL-10 could be a good therapeutic candidate, at least for those patients with IBD that exhibit lower circulating levels of IL-10 [7]. However, limited clinical trials investigating parenteral IL-10 therapy in Crohn's disease patients observed no differences between the treatment and placebo groups, likely due to the short half-life and resultant low levels of recombinant IL-10 that reach the intestines [8-11].

[00187] Localized delivery of IL-10 by probiotics as IBD treatment. Treatment options that locally deliver recombinant IL-10 have been investigated as a therapeutic strategy. For example, the oral administration of IL-10 to the intestines of IBD patients by genetically-modified probiotic bacteria was pursued. For these studies, Lactococcus lactis, a Gram-positive lactic acid producing bacteria found in fermented diary products including cheese and yogurt, was genetically modified to express and secrete recombinant IL-10 [12, 13]. Ingestion of this strain results in a 50% reduction in gut inflammation in dextran sulfate sodium (DSS) and IL-10-/- mouse models of IBD [13]. However, when administered in human clinical trails, only minimal improvement in symptoms was observed when comparing treatment and placebo groups [13, 14]. It is contemplated here in that this outcome may be due to the inabilty of L. lactis to colonize the gut, such that recombinant IL-10 is only transiently delivered to the patient during passage of the bacteria through the intestines [15]. Described herein are genetically engineered bacteria that continuously deliver IL-10 to the intestines to provide more effective IBD treatment.

[00188] Described herein are methods and compositions which permit functional IL-10 to be secreted through the Shigella flexneri type 3 secretion system. Many Gram-negative enteric pathogens, including Shigella, Salmonella and Yersinia species, directly deliver proteins into host intestinal cells through specialized type 3 secretion systems (T3SSs) [16]. These protein delivery systems are complex nanomachines that form a syringe-like structure that spans the inner and outer membranes of Gram-negative bacteria to form a conduit for the direct delivery of bacterial proteins into the cytoplasm of target cells [17]. Proteins are recognized as secreted substrates by the type 3 machinery through the presence of an N-terminal secretion sequence. Notably, the addition of a type 3 secretion sequence to heterologus proteins is sufficient to target their secretion through the T3SS [18, 19].

[00189] Interestingly, IL-10 is functional when fused to a type 3 secretion sequence. Mice infected with wild type Shigella flexneri strains that express and secrete IL-10 exhibit a 2-fold reduction of inflammation in response to infection with Shigella [18]. Notably, IL-10 exerts its activity by binding to receptors on the outer cell surfaces [3]. However, the Shigella T3SS primarily delivers proteins into the cytosol of targeted cells. Therefore, the presumably small amounts of extracellular IL-10 released by the secretion system are sufficient to partially block Shigella induced inflammation. It is contemplated herein that increased localized secretion of IL-10 into the intestinal lumen by the T3SS can more effectively limit inflammation, including in the context of IBD.

[00190] Engineering type 3 secretion systems as therapeutic vectors. Repurposing the T3SSs of pathogens to deliver therapeutic proteins as opposed to virulence factors is currently being pursued as a therapeutic strategy. This strategy has shown some promise delivering antigenic molecules for vaccine development and transcription factors to alter gene expression in mammalian cells [20-24]. However, to date, a major limitation of this approach has been the use of virulence-attenuated pathogenic bacteria for protein delivery. These attenuated strains still encode known, and likely unknown, virulence determinants, limiting their use in patients, particularly those that are immunocompromised. Described herein is the development of a system that takes advantage of the protein delivery capabilites of T3SSs that circumvents the issues associated with attenuated pathogens. Using recombineering, non-pathogenic laboratory strains of E. coli have been engineered to express the T3SS from Shigella flexneri [Reeves et al,]. These laboratory strains secrete and deliver a variety of heterologous proteins into mammalian cells at levels similar to pathogenic Shigella strains. Based on the success of this approach, the Shigella T3SS was introduced into the probiotic bacteria, E. coli Nissle 1917 (EcN) to develop therapeutic commensal bacterial strains. Wild-type EcN is already given as a treatment to patients with IBD in Europe and Canada where it has been observed to be as efficacious at preventing IBD flares as the oral agent, mesalazine [20-22]. Additionally, EcN is capable of colonizing of the gut of mice and humans, a trait that can provide a means for prolonged delivery of therapeutic proteins to patients [15]. Contemplated herein is the directed targeting of recombinant IL-10 to the intestines via a type 3 secretion competent strain of EcN could prove to be an effective innovative treatment for IBD (schematic in Fig 1).

[00191] Described herein is the engineering of type 3 secretion competent strains of the probiotic E. coli Nissle 1917 (EcN) to secrete high levels of functional IL-10 into the

intestinal lumen. This approach enhances the ability of this probiotic to target anti-inflammatory effects to the site of disease, thereby limiting the systemic immunosuppression observed with current therapeutic modalities like TNFα blockers. The ability of EcN to colonize the intestines means this strategy can provide a long-term cost-effective treatment for IBD patients. Importantly, although this proposal focuses specifically on delivery of IL-10, the paradigm described within can be applied to secreted alleles of additional cytokines/proteins of therapeutic value thus providing a highly adaptable and efficient platform for developing new treatment strategies that act to promote the localized delivery of therapeutic agents.

[00192] Engineer type 3 secretion competant strains of commensal E. coli Nissle 1917 (EcN) that secrete functional IL-10. These strains can be permit identification of conditions that maximize the expression of functional IL-10 alleles that are recognized and secreted into the extracellular millieu via genetically engineered EcN strains.

[00193] Development of non-pathogenic type 3 secretion competent E. coli. Described herein are non-pathogenic laboratory strains of E. coli that encode a functional T3SS, which enables them to secrete as well as deliver defined proteins directly into mammalian cells. This was accomplished by using yeast and bacterial homologous recombeering approaches to capture a 31 kB region that encodes all of the 25 proteins needed to form a functional Shigella flexneri T3SS onto an autonomously replicating plasmid. This plasmid was designed such that the large region of Shigella-derived DNA it contains can be maintained on the plasmid or integrated into a non-essential locus of the chromosome of DH10β E. coli, a strain referred to here as T3-E.coli. Similar to wild type Shigella, these bacteria can utilize their T3SS to deliver proteins into >80% of mammalian cells they come encounter (data not shown). After developing this cloning strategy in DH10β, it was used to introduce the type 3 secretion operons into the chromosome of E. coli Nissle 1917 (T3-EcN), which also expresses the T3SS (data not shown). Both strains are particularly well suited for use as in vivo therapeutic protein delivery systems for several reasons: (1) isolating the type 3 secretion components with this recombinational cloning strategy allows for the generation of delivery strains devoid of virulence factors, as opposed to attenuated pathogens which likely retain virulence determinants and (2) the introduction of the type 3 secretion operons into the chromosome alleviates the need for antibiotic selection and prevents horizontal transfer of these genes into other bacterial species.

[00194] Development of a screening platform to identify optimal type 3 secretion sequence-target protein combinations. All type 3 secreted proteins (effectors) are defined by an N-terminal secretion sequence within their first 20-100 amino acid residues [17, 23]. Previous studies have found that the addition of these seqeunces to heterologous proteins generates alleles that are recognized as secreted proteins [18, 24]. To rapidly identify the regions of Shigella effectors that are sufficient to generate a secreted allele when fused to heterologous proteins, a secretion sequence screening platform was developed. A collection of 14 plasmids, each of which carries the first 30 or 50 residues of a Shigella effector plus an upstream consensus Shine-Dalgarno sequence in a Gateway recombination-based entry plasmid was constructed. Using this plasmid collection, along with a Gateway-compatible destination vector for a target heterologous protein, it is possible to rapidly generate and test the secretion of a variety of N-terminal secretion sequence-target fusion proteins.

[00195] The secretion sequence screening platform was used to identify sequences that promote the recognition of mammalian MyoD protein as a type 3 secreted substrate. Fusion of MyoD to 50 but not 30 residues of all effectors tested, resulted in alleles recognized as secreted substrates by T3-E. coli (50 amino acid fusions shown, Fig 2A and 2B). However, only a subset of the secreted alleles were detected within extracts of mammalian cells indicating that the individual secretion sequences differ in how efficiently they deliver heterologous proteins into mammalian cells (Fig 2C). The MyoD fusion proteins were also tested for how the secretion sequence affects protein stabilty and localization (data not shown). Taken together, these data were used to successfully identify several secretion sequences that affectively transformed MyoD to be recognized as a type 3 secretion substrate without preturbing the normal properties of the wild type protein. Applying this strategy to each of four induced pluripotent stem (iPS) cell reprogramming factors, Oct4, Sox2, Klf4 and c-Myc [25], as well as two cardiac reprogramming factors (Mef2c and Tbx5) [26], and a TALE (transcription activator-like effector) protein [27], also resulted in alleles that are recognized as secreted substrates by T3-E. coli (Fig 3). Secretion of IL-10 can be optimized using a similar strategy.

[00196] Engineer type 3 secretion competant strains of E. coli Nissle 1917 that secrete functional IL-10.

[00197] Rationale: A previous study demonstrated that Shigella expressing IL-10 limits inflammation in a mouse model of infection [18]. However, in addition to IL-10, Shigella delivers >20 virulence factors into cells, including several that promote inflammation in

direct competition with the anti-inflammatory functions of IL-10. It is contemplated herein that IL-10 delivery from a non-pathogenic bacteria is even more efficacious.

[00198] The T3SS from Shigella has been introduced into the probiotic EcN using the genetic methodology described above. Though T3SSs normally deliver proteins into the cytosol of targeted cells; however, in order to confer anti-inflammatory activity, IL-10 can be secreted directly into the intestinal lumen and bind to extracellullar IL-10 receptors. Thus, described herein are modifications to the T3SS in T3-EcN strains such that IL-10 is exclusively secreted, rather than delivered into mammalian cells. Secretion and function of IL-10 can be optimized by screening a library of secretion sequences fused to IL-10.

[00199] Generate functional IL-10 alleles that are recognized as type 3 secreted substrates.

[00200] The screening platform decribed above (Fig. 7) can permit identification of type 3 secretion sequences that when fused to IL-10 promote its recognition as a type 3 secreted substrate, but do not perturb its activity. Each allele can be cloned into a vector that places expression of the IL-10 fusion protein under control of the strong constitutive promoter, BBa_J23100, from the Registry of Standard Biological Parts. This promoter is optimized for binding to σ70, the main RNA polymerase sigma factor expressed in E. coli [28]. Placing IL-10 expression under σ70 control will yield constitutive expression of IL-10, thereby preventing the need for the addition of an inducer for expression. Lambda red recombination can be used to introduce the IL-10 expression construct into the non-essential lacZ locus of the T3-EcN chromosome. Secretion of the IL-10 fusion proteins can be evaluated in T3-EcN using standard secretion assay conditions [29]. The amount of IL-10 secreted can be monitored by quantitative western blot analyses of cell lysates and supernants as well as via a standard IL-10 ELISA kit. Commerically available IL-10 cam be used as controls. Each supernatant sample can be probed for the presence of the cytoplasmic protein, DnaK, to check for bacterial cell lysis. The activity of the secretion sequence-IL-10 fusion proteins can be assayed by measuring proliferation of MC/9 mast cells following 3 days of incubation with T3-EcN supernatant containing the IL-10 fusion proteins and compared to purified commercial human IL-10 as reference [30]. IL-10 alleles that are efficiently secreted and maintain wild type immunomodulatory activity can be further pursued.

[00201] If the IL-10 fusion proteins do not express well from the BBa_J23100 promoter, additional variants available in the Standard Registry of Biological Parts can be screened. If the activity of IL-10 fusion proteins is disrupted, a flexible linker between IL-10 and the type 3 secretion sequence can be incorporated.

[00202] Engineer T3-EcN strains able to secrete IL-10 into the extracellular mileu.

[00203] Normally, in the absence of a signal from a host cell the type 3 secretion apparatus is inactive and plugged by the tip complex proteins, IpaB and IpaD (Fig 4A) [31, 32]. Traffic through the secretion apparatus is also regulated by MxiC, which is present within in the machine physically blocking the path of type 3 substrates until secretion is activated [32]. IT is contemplated herein that deletion of either of the tip complex proteins or MxiC from Shigella can result in unregulated, constitutive secretion of type 3 effectors [31, 32] (Fig. 4B). further contemplated herein that cumulative loss of all three proteins might lead to even greater levels of secreted effectors proteins. Thus, to generate a strain of T3-EcN that constitutively secretes maximal levels of IL-10, a T3SS that lacks IpaB, IpaD and/or MxiC can be generated using a lambda red recombination based approach. After generating knockout strains in T3-EcN, ecretion of the IL-10 fusion protein can be tested in the individual as well as the triple knockout strains. A time course can be performed in which supernatant of the strains will be collected every 2 hours for 12 hours to determine the kinetics of secretion in each genetic background. These experiments can identify whether any of these strains can consistently secrete IL-10 over an extended time, a useful trait for a anti-inflammatory probiotic. Genetic backgrounds that secrete the most IL-10 for the most prolonged amount of time will be further tested.

[00204] If robust IL-10 secretion from T3-EcN strains is not demonstrated, the approach described herein can be applied to the T3-E. coli (DH10β) strain instead. Strains that lack IpaB, IpaD and/or MxiC can be generated and secretion of the IL-10 fusion proteins in each genetic background assessed. As an additional consideration, particularly before such strains are considered for administration to patients, auxotrophic derivatives of these therapeutic strains can be generated to prevent the growth of any bacteria that are inadvertently shed into the environment [33].

[00205] Evaluate anti-inflammatory properties of type 3 secretion competant E. coli Nissle 1917 IL-10 strains in mouse models of IBD.

[00206] The efficacy of the IL-10-secreting commensal bacteria can be tested, for example, in three complementary mouse models of IBD: (i) the administration of dextran sulfate sodium (DSS) which models acute colitis in wild type hosts, (ii) IL-10-/- mice to model chronic enterocolitis in the setting of intact adaptive and innate immune responses, and (iii) a translational humanized mouse model of colitis using newly developed immunodeficient NSGAb0DR1 mice treated with 2,4,6-trinitrobenzene sulfonic acid (TNBS)

to more closely mimic the effect of T3-EcN IL-10 on a human immune system. By using mouse models with different underlying pathophysiologies, the divergent host and microbial heterogeneity observed with IBD patients can be modeled.

[00207] Over a 14-day period, individual mice (n=5 per strain) will be evaluated by both daily fecal sampling to assess the presence/shedding of T3-EcN and by tissue examination upon sacrifice (1 mouse/strain genotype on d3, d6, d9, d12, d14) to assess inflammation. For these preliminary studies, the mice will receive a daily oral inoculation 1-2 $x10^7$ CFU of T3-EcN IL-10 by providing bacteria in the drinking water in order to facilitate the bacteria taking up residence in the gut microbiota. For each condition, all mice can be administered the inocula when mild inflammation or injury has been observed in the specific pathogen free (SPF) mouse facility in each designated model: DSS treated wild type mice (day 3 of DSS exposure of 6 week old mice), IL-10-/- mice (6 weeks of age), and NSGAb0DR1 humanized mice (14 weeks of age). For each model, control groups can be included that receive either no bacteria, wild type EcN, or T3-EcN without IL-10. The latter control can be included to ensure that any differences in inflammation are due to IL-10 and not the persence of the type 3 secretion system.

[00208] To determine the overall effects on inflammation, the intestinal tissues can be fixed and paraffin embedded for histology-based assessment of intestinal inflammation. Sections can be scored in a blinded fashion with respect to intervention. For humanized and IL-10-/- mice, four parameters [mononuclear cell infiltration, polymorphonuclear cell infiltration, epithelial cell hyperplasia, and epithelial cell injury] can be used and scored as absent (0), mild (1), moderate (2), or severe (3) and summed for a final histologic severity score [34]. For DSS colitis, parameters can be scored on a scale of 0-4: percentage of colon involved by inflammation, percentage of crypt loss, presence of lymphoid follicles, edema, erosions, and density of inflammatory cells and the individual parameters are summed to give a total severity score. Statistical analysis can be performed to determine whether differences between groups are significant using a Student's T-test or analysis of variance (ANOVA) using STATA software.

[00209] To monitor for specific changes in the cytokine present in the inflammatory milieu of the treated mice, organ explant cultures [35] can be used. The distal colon (0.5 g dry weight) of humanized mice and DSS-treated mice and transverse colon (0.5 g dry weight) of IL-10-/- mice can be isolated [36] and explant supernatants will be analyzed using multiplex cytokine analysis for IL-1a, IL-1b IL-2, IL-4, IL-6, KC, TNF-a, IFNγ, IL-10 (not applicable

for IL-10-/-), IL-12p40, IL-12p70, IL-13, IL-17A, IL-21, and IL-23 using the Luminex platform. In addition, for the IL-10-/- mice, their peripheral blood can be sampled on a weekly basis for granulocytes, as peripheral blood granulocyte number increases and correlates with enterocolitis lesion development [37].

[00210] It is contemplated herei that the combination of EcN's natural anti-inflammatory properties combined with localized delivery of IL-10 to mouse intestines reduce inflammation more than administration of EcN alone. It is possible that expression of the T3SS could induce inflammation as purified needle components have been shown to induce TLR2 signalling [38], though given that type 3 secretion systems do not secrete needle subunits once the apparatus is assembled, this is not expected to be an issue. If no improvement in inflammatory symptoms is observed with T3-EcN IL-10, the amount of IL-10 delivered to the mouse intestines can be increased by generating T3-EcN strains that can colonize their host more efficiently. The introduction of an EnvZ P41L point mutation into the T3-EcN chromosome alters the outer membrane profile of EcN leading to enhanced colonization of mouse intestines and can lead to enhanced or prolonged delivery of IL-10 [39]. Alternatively, if minimal inhibition of inflammation is observed over the 14 day period, increasing the duration of T3-EcN IL-10 treatment to 4 or 6 weeks can be performed.

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[00212] EXAMPLE 2

[00213] Standard cancer therapy includes surgical resection, chemotherapy and radiation; approaches that often are not sufficient to lead to cure or stable disease and generally associated with numerous side effects. While targeted therapies have made significant inroads in survival, the war on cancer wages on. Current and evolving knowledge of immunotherapy and synthetic engineering of bacteria hold the potential to revolutionize cancer care and treatment. The genesis of these fields leads back to the first immune-based bacteriotherapy therapy called Coley's toxin and this application aims to effectively leverage this history along with decades of subsequent science and technology to develop a new cancer therapeutics platform (for review see [1, 2]).

[00214] A variety of bacterial species are inherently well suited to function as potential anti-cancer agents, as some exhibit a profound propensity to home to and colonize the hypoxic and often nutrient rich environments of tumors and neoplastic deposits. Upon reaching tumor tissue, flagellated bacteria can effectively penetrate areas remote from vasculature. In this way, bacteria offer a means to introduce 'anti-neoplastic payloads' in a highly targeted fashion by specifically accessing tumor tissue. Select bacterial pathogens, including Salmonella and Clostridial species [3, 4], can promote tumor clearance by direct immune-stimulatory effects in tumor environments which can be thought of as immune privileged, exhausted, or suppressed. Synthetic biology based approaches have recently capitalized on this characteristic by modifying bacteria to deliver therapeutic payloads, i.e., pro-inflammatory cytokines, siRNAs and cytotoxins directly to tumors to promote tumor killing and rouse anti-tumor immunity [5]. Such modifications have generally been developed using attenuated bacterial pathogens, given concerns of administrating live pathogens to patients, particularly late stage cancer patients who are systemically immunosuppressed from chemotherapy and radiation treatments. While these strains work well in mouse tumor models, to date they have demonstrated limited success in human clinical trials, presumably

because the attenuated strains are cleared from the systemic circulation too rapidly to reach and establish residence in tumors [6, 7].

[00215] Nissle 1917 Escherichia coli (EcN), a probiotic commonly used in Europe and Canada for the treatment of inflammatory bowel disease [8], also shows a strong propensity for homing to tumors where it replicates to titers as high as 10^{10} colony forming units /gm [9-12]. However, unlike Salmonella which only reach titers of ~10^{5} [11], the presence of EcN within tumors does not induce any immune responses or inhibit tumor growth. It is contemplated herein that modifications to EcN can engineer the strain to promote tumor cell death. For example, tumor colonization with EcN engineered to secrete azurin, a Pseudomonas redox protein that promotes tumor cell apoptosis, inhibits tumor growth but does not promote eradication [12]. While this result is encouraging, it does indicate that additional modifications to EcN are required to actualize EcN as a cancer therapeutic. To address these issues, described herein are synthetic biology based approaches to develop EcN strains capable of secreting proteins either directly into cancerous cells or into the tumor milieu. As a first step regarding the potential clinical utility of such strains, they are engineered to deliver single domain antibodies (nanobodies) that inhibit the activity of tumor cell immune checkpoints (PD-1 and CTLA-4) as the targeted deposition of such nanobodies into the tumor milieu promotes the recognition and clearance of neoplastic lesions resistant to anti-tumor immunity.

[00216] Both non-pathogenic and commensal Gram-negative bacteria, including EcN, rarely, if at all, secrete proteins directly into the extracellular environment. Rather they utilize type I and type II secretion systems to deliver proteins to the periplasmic space of the cell envelope. In contrast, many Gram-negative bacterial pathogens utilize complex protein delivery machines to efficiently transfer proteins directly from bacteria into the cytosol of mammalian cells. Type 3 secretion systems (T3SSs) are one, if not the best characterized, of these protein delivery nanomachines [13]. These complex machines have been reengineered to secrete therapeutic payloads, as opposed to virulence proteins, directly into or within the vicinity of mammalian cells. While most efforts have focused on engineering the T3SS systems of attenuated pathogens to deliver therapeutic payloads [14, 15], described herein is, e.g., the introduction and expression of a regulatable and functional type 3 secretion systems in non-pathogenic *E. coli*. In some embodiments, described herein are regulatable and functional T3SS-derived extracellular secretion systems in non-pathogenic bacteria. Furthermore, described herein is a platform to rapidly identify sequences that efficiently

promote the recognition of a variety of heterologous proteins as type 3 secreted substrates. These approaches can be applied to the development of type 3 secretion competent strains of EcN that deliver nanobodies that activate anti-tumor immunity within the tumor microenvironment.

[00217] VHH are small (~12-15kDa) single domain antibodies composed of a single variable immunoglobulin domain and are commonly found in camelids and cartilaginous fish [16]. These proteins are more stable than their traditional antibody counterparts and less dependent on disulfide bond formation for proper folding. These molecules have tremendous therapeutic potential, particularly those that mimic the activity of monoclonal immunomodulatory antibodies. Nanobodies that bind to and inhibit the activity of PD-L1 (programmed death ligand 1), PD-1 (programmed cell death inhibitor) and CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) have been described. Furthermore, the administration of the PD-L1 nanobody is effective as that of a commercially available PD-L1 antibody in promoting the regression of tumors in the B16 mouse model of melanoma. Nanobodies are readily produced by bacteria and are functional when engineered to be recognized as enteropathogenic E. coli type 3 secreted proteins [17].

[00218] Immune system checkpoints essentially serve as "brakes" that act to prevent over-activation of the immune system in response to pathogens as well as to maintain tolerance of self-antigens, thus protecting healthy tissues from damage. However, many malignant tumors block recognition by the host immune responses through the dysregulation of proteins that promote checkpoint activation. Three of the best-studied checkpoints in this scenario are PD-1, PD-L1 and CTLA-4, proteins that dampen T cell responses and are critical effectors in blocking anti-tumor T cell immunity [18-20]. Monoclonal antibodies that bind to and block PD-1, PD-L1 and CTLA-4 have shown extraordinary promise in clinical trials, particularly in the treatment of melanoma, renal cell cancer, and lung cancer. Therapies that combine the two agents show the most success, although they are often associated with marked side effects, including numerous 'autoimmune' sequelae and adverse immune related events including: life threatening colitis, hepatitis, pancreatitis, hypophysitis, and thyroiditis, due to the systemic effects of disinhibiting immune checkpoints [21]. It is contemplated herein that therapies that target the delivery of these checkpoint inhibitors to tumors and neoplastic deposits improve their efficacy while decreasing their off-target complications.

[00219] Stable integration of the Shigella operons needed to form a type 3 secretion system onto the *E. coli* chromosome. Described herein is a means to efficiently transfer the

secretion apparatus from the highly related human pathogen, *Shigella flexneri*, into non-pathogenic strains of *E. coli* (Fig. 5). Using a combination of yeast and bacterial homologous recombination-based technologies, a 31 kB region that contains the operons that encode all of the ~25 proteins needed to form a functional T3SS [22] were transferred from the *Shigella* virulence plasmid onto an autonomously replicating plasmid. The backbone of this plasmid contains an oriT sequence which enables its transfer from strain to strain via conjugation. In addition, the T3SS operons are flanked on each sides by a synthetic unique landing pad (LP) sequences which enables the efficient insertion of this DNA fragment at defined sites on the *E. coli* chromosome engineered to have the corresponding "landing pad" sequence (Fig. 5) [23].

[00220] Introduction of a functional, regulatable type 3 secretion system into *E. coli*. The introduction of the *Shigella* T3SS operons into *E. coli* is not sufficient to generate a functional protein delivery strain as this region of DNA does not include the master *Shigella* T3SS transcriptional regulator, VirF. However, as shown in Fig. 6, secretion can be activated in a regulated manner, with introduction of a second plasmid that expresses under control of its endogenous or a regulatable promoter. This data demonstrates that by controlling VirF expression, via exogenously added agents like arabinose or EcN promoters active specifically within tumors, it is possible to restrict the expression of the T3SS.

[00221] Development of a platform for rapid identification of signal sequences that promote secretion of heterologous proteins. To complement the development of type 3 secretion competent non-pathogenic *E. coli*, a recombination based platform to rapidly identify secretion sequences that promote the recognition of heterologous proteins as type 3 secreted substrates was constructed. While all type 3 secreted proteins are defined by a N-terminal secretion sequence of ~20 residues, a region essentially only defined by its unstructured nature [24], many also encode and require a downstream chaperone-binding domain. As shown in Fig. 7, it is observed that the fusion of the first 50, but not 30, amino acids of native *Shigella* type 3 secreted proteins is sufficient to generate alleles of heterologous proteins that are recognized as a secreted substrate. As shown for MyoD, the levels of secreted proteins can vary. Similarly, fusion to some signals does effect MyoD function, particularly once the proteins are delivered into host cells, indicating that the secretion sequence-heterologous combination for each protein can be optimized (data not shown and [25]).

[00222] Develop type 3 secretion competent strains of EcN that secrete immunomodulatory nanobodies. Described herein is the development of type 3 secretion-competent strains of Nissle 1917 E. coli (T3EcN) that recognize and secrete functional nanobodies. In some embodiments, the secretion can be into the extracellular environment. In some embodiments, the secretion can be into the intracellular space of a target cell. Versions of T3EcN capable of either directly injecting nanobodies into the cytosol of cancerous cells or into the extracellular milieu of tumors can be developed. Described herein are strains of EcN that secrete proteins into the media rather than host cells, the identification of modifications to single domain antibodies that promote their efficient recognition as type secreted proteins but do not perturb their activity, and versions of EcN that induce the expression of the T3SS in tumors by administration of a small molecule inducer, such as arabinose, or via endogenous EcN promoters specifically activated within tumors.

[00223] Introduce the operons needed to form a functional type 3 secretion system into EcN. The *Shigella* T3SS operons can be introduced onto the chromosome of EcN. The operons can be integrated onto the chromosome to ensure that this large ~31kB fragment of DNA is stably maintained when the bacteria are introduced into mice. A "landing pad" site in the EcN chromosome can be utilized. After the T3SS operons are introduced into EcN, the functionality of the strain, T3EcN, can be confirmed using conventional assays to monitor the ability of the strain to secrete as well as deliver type 3 effectors into host cells, (Figs. 5,6 [26, 27]). The expression of the T3SS can be controlled by the introduction of a plasmid that conditionally expresses VirF and study epitope-tagged effectors expressed from an IPTG-inducible promoter on a second plasmid. Using this approach, a functional type 3 secretion has been introduced into multiple strains of *E. coli* including DH10b, DH5a, HB101 and BL21.

[00224] Develop strains of EcN strains that efficiently secrete proteins into the extracellular milieu. Once T3EcN is confirmed as functional, a screen can be performed for modifications to the T3SS components that act to promote the efficient secretion proteins into the surrounding media as opposed to the direct injection of proteins into mammalian cells. Strains that lack combinations of each of the following 3 proteins can be generated: (1) IpaD, the outer most protein of the secretion apparatus which forms a "cap" that holds the secretion apparatus in an "off" position prior to contact with host cells [28], (2) IpaB, one of two proteins at that tip of the needle (the conduit through which the secreted substrates travel) that, upon contact with host cells, serves to form the pore in the plasma membrane through

which proteins are translocated [28] and (3) MxiC, the "gatekeeper" protein, which prevents the internal loading of proteins into the needle prior to secretion apparatus [29]. A T3EcN strain that lacks the ipaBCD operon can be constructed first and then deletion of mxiC can be tested for increases the levels of constitutively secreted proteins. It can also be confirmed that these strains can no longer deliver proteins into host cells using the conventional TEM-1 β-lactamase translocation assay [26, 30].

[00225] Generate functional nanobodies of PD-1, PD-L1 and CTLA-4 that are recognized as type 3 secreted substrates. Alpaca-derived anti-mouse nanobodies that block the activity of three checkpoint proteins, PD-1, PD-L1 and CTLA-4 are available. Each VHH can be modified to be recognized as type 3 secreted substrates using the platform described herein (Fig.6). Basically, Gibson cloning can be employed to generate "Gateway" destination vectors whereby alleles of the PD-1, PD-L1 and CTLA-4 nanobodies fused to 11 different secretion signal sequences that are expressed under the control of the virF promoter can be quickly generated. The vectors can also be designed to introduce a flexible linker between the type secretion signal sequence and the nanobody and an epitope-tag at the carboxy termini of the nanobody. The levels of secreted nanobodies fused to each secretion signal sequence can be confirmed and whether the modified type 3 secreted nanobodies retain function confirmed by quantifying their ability to recognize their cognate binding proteins. Notably, for the later, the binding activities of the type 3 secreted nanobodies can be compared to their unmodified purified cognate nanobodies. The genes encoding each of the three modified nanobody proteins under the control of the virF promoter can be introduced onto the chromosome of T3EcN strain using CRIM, Landing Pad, and/or lambda InCh methodologies [23, 32, 33].

[00226] Introduction of regulatable versions of the transcriptional regulator VirF. As demonstrated in Fig.5, expression of the *Shigella* T3SS operons in *E. coli*, requires the addition of a plasmid that carries the transcriptional regulator, VirF. Notably, by placing VirF under the control of a regulatable promoter it should prove possible to control the expression of the T3SS in T3EcN in space and time, i.e., only once the bacteria colonize the tumors. Described herein are two complementary strategies to control VirF expression. First, virF can be placed under the control of the pBAD arabinose-inducible promoter (VirFara). This promoter, in EcN that carry a luciferase gene driven by the pBAD promoter, caused cells that have colonized tumors to exhibit GFP fluorescence when the mice are administered an L-arabinose solution orally or via a tail vein injection [11, 34]. A light signal is detected from tumors as early as 15-30 minutes post-arabinose administration, reaches a maximal intensity

at 1-3 hours and is undetectable after 8 hours. Repeat administration of L-arabinose on consecutive days results in the "re-induction" of light emission [11]. Second, virF can be placed under the control of endogenous EcN promoters predicted to be markedly up-regulated once the bacteria colonize tumors. For example, multiple *Salmonella* promoters that are specifically induced when the bacteria invade solid tumors as compared to organs like the spleen and organs have been identified[35]. Based on these studies, we will generate alleles of virF under the control of the homologous EcN ansB (VirFansB) and pflE (VirFpflE) promoters that are induced in hypoxic conditions and napF (VirFnapF), a promoter that regulates a gene involved in flagella biosynthesis [35]. Using λ-InCh technology [32] fragments of DNA that carries the virF gene under the control of each of the 4 promoters (pBAD, ansB, pflE and napF) can be stably integrated at a specific site on the EcN chromosome. The ability of each to drive expression of a virF driven allele of phiLOV, a fluorescent protein that in contrast to GFP folds even under hypoxic conditions [36, 37], when EcN are grown in the presence of arabinose or under hypoxic conditions can be tested.

[00227] Investigating the efficacy of T3EcN nanobody secreting strains in melanomas

[00228] Described herein is bacteria-mediated direct delivery into the tumor microenvironment of immunostimulatory nanobodies that block PD-1 and CTLA-4 activity to promote tumor regression. Further described herein is the establishment of conditions under which the genetically modified T3EcN strains selectively express VirF, the master T3SS transcriptional regulator, within tumors identification of inoculation conditions that ensure that the modified T3EcN_VirF strains home to and replicate within tumors. The ability of the nanobody-secreting T3EcN strains to promote mouse tumor clearance can be tested. It is contemplated herein that the directed delivery of immunostimulatory nanobodies that inhibit the activity of tumor checkpoints can treat a variety of tumor types.

[00229] Identify conditions under which VirF, the master type 3 secretion transcriptional regulator, is functional within tumors. Conditions under which VirF, the master T3SS transcription regulator, is activated only after the T3EcN strains colonize tumors can be identified. Two strategies are described herien: one strain, T3EcN_VirFara, where VirF activity is controlled by the administration of a small molecule, like arabinose and a second strain, T3EcN_VirFend, where VirF expression is activated by one of the 3 endogenous promoters discussed above herein (VirFansB, VirFPlfE or VirFnapF) that is activated by cues unique to the tumor microenvironment. To initially characterize the transcriptional activity of VirF under the control of each of the different promoters, T3EcN strains that carry an eGFP

VirF-driven transcriptional reporter can be examined. BALB/c mice that have implanted syngeneic ~ 0.2 cm^3 B16 tumors via the earlier injection of 1 x 10^5 B16 cells on the mid-right side of their flank [8] can be treated with the strain. Each mouse can receive a single tail vein inoculation of 2 x 10^7 CFU for each of 4 experimental strains plus strains that express no GFP, constitutive GFP or arabinose-inducible GFP, as controls. A non-bacteria/media alone injection can be performed as well. When administered at this dose, EcN has been observed to transiently colonize the spleen and liver at day 1 after which point it is rapidly cleared from the circulation. The pBAD-virF strain recipients can receive 200 ul of 25% arabinose daily via a gentle oral instillation, a condition previously demonstrated to promote activation of the pBAD promoter present within strains of EcN within solid tumors [44]. One (n=4 per group) and 3 (n = 4 per group) days post-inoculation, the patterns of bacterial colonization within tumors and solid organs as well as their eGFP expression levels will be examined upon sacrifice. The organs and tumors of each can be formalin-fixed and paraffin embedded, sectioned and stained with commercially available antibodies that recognize both *E. coli* as well as eGFP. By visualizing GFP expression via indirect immunofluorescence, GFP expression can be detected within microaerophilic regions of the tumor, a condition that can inhibit the correct folding of GFP.

[00230] Expression of the arabinose-driven genes carried by T3EcN can be induced within tumors and the endogenous promoters with the best performance for particularl conditions can be identified. In addition, by examining the distribution of GFP+ bacteria within the tumors and solid organs (liver and spleen), an understanding regarding the relative activity of the different VirF alleles within different regions of the tumor can be developed, as well as confirming that EcN is cleared from the spleens and livers of mice within three days [12].

[00231] Identify conditions that promote the colonization of syngeneic B16 derived tumors by T3EcN. Delivery conditions that ensure homing to and residence of T3EcN_VIrFara and T3EcN_VirFend strains within tumors can be identified. Several published studies have characterized the ability of unmodified EcN to colonize tumors [9-12]. However, given that the strains described herein have been modified to express a functional T3SS, the behavior of these strains in mice can be characterized, as it is theoretically possible that the expression of the T3SS alone could perturb EcN growth and/or induce host responses that promote the clearance of these bacteria. The published literature suggests that post-inoculation of 2 x10^6 CFU or 2 x10^7 CFU via tail vein injection into BALB/c mice, EcN will reach titers of 10^8-10^9CFU vs.10^{10} CFU within tumors, respectively, by 24 hours. The bacteria will then persist

at high titers for at least 14 days. While the group that inoculated the mice with 2 x10^7 CFU observed low-levels of bacteria within the liver (~10^4) and spleen (~10^5), the group that utilized a 10x lower inoculum observed none. Based on these results, the levels of EcN, T3EcN_VirFara and T3EcN_VirFend that localize to the tumor, spleen and liver when administered at inoculums of 2 x 10^6CFU or 2 x 10^7CFU via tail vein injection can be compared. For the T3EcN_VirFara strain, starting one day post-inoculation, a time whereby the majority of EcN have presumably been cleared from the liver and spleen, the mice wcanll receive a daily oral instillation of 200ul of 25% arabinose solution. A cohort of mice infected with unmodified EcN will also receive arabinose to control for any effects of the sugar inoculum on colonization. On days 1, 2, 3, 7 and 14, 4 mice receiving each inoculum can be sacrificed and each of these time courses can be repeated two additional times. Tumors, spleens, and livers can be dissected out, weighed and homogenized and plated to determine colony counts.

[00232] Given that the T3EcN we are studying should not invade mammalian cells, it is not expected that any host cell innate immune signaling responses will be triggered. Nevertheless, if it is observed that the T3EcN are altered in their ability to colonize tumors, the inoculum frequency and/or dose can be altered to modulate tumor colonization. Lastly, if the strains do not reach tumors after introduced via tail vein injections, the bacteria can be directly introduced into the neoplastic lesions via intratumoral injections, as once the bacteria reach the tumor they will be an immune-privileged environment protected from host innate immune response and thus able to establish residence and act to inject the immunostimulatory nanobodies into the tumors.

[00233] T3EcN immunostimulatory nanobody secreting strains can promote the regression of melanoma. T3EcN strains that secrete the anti-PD-1, anti-PD-L1 and anti-CTLA-4 immunostimulatory nanobodies promote B16 tumor regression. In clinical trials, combinations therapies that combine two of these three antibodies have shown great promise but patients experience a high rate of severe related adverse effects [21]. It is contemplated herien that the targeted delivery of nanobodies to tumors via T3EcN_VirF prevents or markedly alleviates the development of systemic off-target effects. T3EcN_VirF strains that secrete all three immunostimulatory nanobodies can be utilized. Using the inocula established above to promote T3EcN_VirF tumor residence, B16+ mice that have ~0.2cm^3 flank tumors can be inoculated with each of the following 5 strains: EcN, T3EcN_VirFara, T3EcN_VirFend. T3EcN_VirFara(PD-1/PD-L1/CTLA-4) and T3EcN_VirFend(PD-1/PD-

L1/CTLA-4) plus a media-only control. The mice can be injected weekly with each strain. Mice that receive the arabinose-driven system can receive daily oral doses of 200ul of 25% arabinose. The size of the tumors, located on their flanks, can be assessed every two days using calipers. The mice can be monitored daily for up to 4 weeks, a time frame which permit assessment of the health status of the mice more fully, as based on the published literature, untreated mice (those receiving unmodified EcN) are expected to become moribound at ~ 14 days, at which point they will be sacrificed for humane endpoint considerations. The experiment can be repeated thee times.

[00234] If the mice do not tolerate the targeted delivery of all 3 immunostimulatory nanobodies, strains that only deliver combinations of 2 nanobodies, i.e., PD-1/CTLA-4 vs. PD-L1/CTLA-4 can be utilized. In addition, minimal tumor regression is observed under a given condtion, tumors can be isolated and flow cytometry utilized to interrogate whether the T3EcN immunostimulatory nanobody secreting strains act to alter intratumoral T-cell populations, specifically the balance of effector T cells (both activated and exhausted) and regulatory T cells, as well as investigate the distribution of secreted nanobodies present in the tumors.

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[00236] EXAMPLE 3: Designer probiotics for the prevention/treatment of intestinal infection and inflammation

[00237] New drug delivery platforms are vitally needed for the targeted delivery of high-specificity therapeutics to sites of disease to maximize efficacy and limit off-target side

effects. To address this challenge, described herein is a synthetic biology approach to equip a safely administered probiotic, *Escherichia coli* Nissle 1917, with a programmable type 3 secretion system, a nanomachine used by bacteria to inject proteins into host cells. These nanomachines can be genetically reengineered to directly secrete therapeutic payloads into the gut milieu, providing a novel *in situ* platform for delivery to the intestinal mucosa. This targeted delivery of cargo can be capitalized by engineering these designer probiotics to recognize and secrete single domain antibodies (VHHs), a new class of therapeutic biomolecules with exquisite neutralizing specificity. VHH-based neutralizing agents (VNAs) that target essential bacterial toxins and pro-inflammatory cytokines can be used with the compositions and methods described herein, e.g., for treatment of intestinal infections and inflammation disorders, including *Clostridium difficile* colitis, hemolytic uremic syndrome (HUS) and inflammatory bowel disease (IBD).

[00238] The majority of efforts currently underway for the development of targeted drug delivery systems are focused on the development of synthetic nanoparticles, materials which are costly to produce, store, and distribute. Described herein is are cost-effective, self-replicating and flexible, programmable designer probiotics for the targeted delivery of therapeutics directly to sites of disease. Such an approach can overcome many issues associated with the wide-spread usages of antibiotics and systemic immunosuppressive agents. In some embodiments of any of the aspects, the methods and compositions described herein can further comprise means for biocontainment of "escaped" strains including kill switches that can engineered into the strains proposed herein before moving forward into human clinical trials.

[00239] Described herein are designer strains of the probiotic *E. coli* Nissle 1917 for the prevention and treatment of intestinal infection and inflammation. VNA can serve as novel therapeutics for the treatment of *Clostridium difficile* infections, HUS, botulism and anthrax. Described herein are *E. coli* Nissle 1917 that can recognize modified VHH as secreted substrates and secrete VNAs.

[00240] Designer probiotics are described herein for the treatment of *Clostridium difficile* infections, Shiga-toxin driven HUS, and IBD, and these designer probiotics can be modified to deliver a variety of protein-based therapeutic payloads, including cytokines, such as IL-10, that suppress intestinal inflammation or VNAs designed to target essential exposed virulence proteins of enteric bacterial pathogens, e.g., adhesins or essential components of virulence factor delivery systems. Furthermore, given the predilection of *E. coli* Nissle 1917 to colonize

solid tumors when administered via a parenteral route, these stains can potentially be engineered for the targeted delivery of cancer therapeutics including VHH that act as immune checkpoint inhibitors.

[00241] **Urgent need for new therapies for diarrheal illnesses and inflammatory bowel disease.** Gastrointestinal diseases of inflammatory or infectious origin are major sources of morbidity and mortality worldwide. Diarrheal diseases are responsible for the deaths of an estimated 2.2 million people globally each year (WHO), mostly children in the developing world. Indeed, one in nine child deaths are due to diarrheal illness. Inflammatory bowel disease (IBD) is more prevalent in developed countries, with 1.6 million cases annually in the U.S., and an annual direct cost estimated as high as $28 billion (Crohn's and Colitis Foundation of America). The high burden of these diseases reflects the unfortunate limitations of treatments to combat them. Both conventional antibiotics and anti-inflammatories are eventually distributed throughout the body, and with (often) limited specificity, promote detrimental off-target effects. For example, anti-inflammatory treatments are associated with systemic immunosuppression, and antibiotics alter the normal microbial flora, leading to overgrowth of pathogens such as *C. difficile* or enhanced virulence factor production such as Shiga toxin from enterohemorrhagic *E. coli*. Finally, the general efficacy of antibiotics is being diminished due to the inexorable emergence of drug resistance—indeed, widespread antibiotic use undoubtedly promotes the spread of resistance, already a growing medical crisis.

[00242] In response to the limitations of conventional antibiotics and immunomodulatory treatments, two therapeutic strategies have recently received considerable attention. First, probiotics have shown considerable therapeutic promise and have been used for the treatment of diarrheal illnesses and IBD. Unfortunately, most current probiotic strategies are not based on a mechanistic understanding of pathogenesis of these illnesses, resulting in empiric treatment and limited application. Second, monoclonal antibody-based "magic bullet" therapeutics with high target molecule specificity have revolutionized treatments of some cancers and chronic inflammatory diseases. However, these antibody-based therapies still suffer from frequent off-target effects due to their systemic administration and from high cost due to the specialized methodologies needed to manufacture and purify these complex proteins.

[00243] It is clear that new drug delivery platforms are vitally needed to enable the directed delivery of novel high-specificity therapeutics to sites of disease in order to both

maximize efficacy and limit off-target side effects. As described below, described herein is the utilization of synthetic biology to generate genetically engineered probiotics that locally deliver to the site of disease a new class of well-documented therapeutic biomolecules of exquisite neutralizing specificity and at a fraction of the cost of conventional biological drugs.

[00244] **VHH (nanobodies), small versatile antibody-based high affinity therapeutic agents.** A new exciting avenue of antibody-mediated therapies is currently emerging that circumvents many of the obstacles of conventional monoclonal antibody-based therapies. In the 1990s, studies of the immunoglobulin repertoire of Camelidae (such as camels, llamas and alpacas) revealed that some of their heavy chain immunoglobulins are naturally devoid of light chains [1]. These "heavy chain only antibodies" (HcAbs) bind antigens via a single variable-domain heavy-chain region, a VHH. VHH, small ~15 kDa protein domains, bind substrates with K_d's in the nM to pM range and exhibit a predilection for binding protein active sites [2]. Furthermore they can be used as modular building blocks to generate multimeric constructs that exhibit enhanced binding potential, both in terms of binding affinity and breath of epitope recognition. Their remarkable solubility, stability and small size overcome many of the barriers that currently limit the production of monoclonal antibodies, resulting in significant decreases in production costs. Several VHH- (or nanobody-) based therapies are now in clinical trials. For example, caplaczumab, an anti-vWF (van Willebrand factor) VHH produced by Abynx, is now in phase 3 trials for the treatment of acquired thrombotic thrombocytopenia purpura (TTP).

[00245] Although the small size of VHH and their concomitant utility in the construction of multimeric proteins of exceedingly high target specificity and affinity provides new therapeutic opportunities, potential off-target effects associated with their systemic administration remain a major concern. Described herein is the direct delivery of therapeutic payloads such as VHH into the intestinal lumen for the prevention and treatment of infection and inflammation. Specifically, as outlined in detail below, the well established and widely administered human probiotic, *E. coli Nissle 1917*, is engineered with a flexible programmable protein delivery machine that can be tuned to deliver therapeutic protein payloads, including VHH, into the intestinal lumen.

[00246] ***E. coli* Nissle 1917, a human commensal exhibits probiotic activity in intestinal infection and inflammation.** *E. coli* Nissle 1917, referred to hereafter as EcN, was originally isolated from the feces of a WWI soldier who was unique in not developing

enterocolitis during a shigellosis outbreak. Analyses of its genome sequence suggest that although the strain does not produce known virulence factors, it encodes multiple adhesins [3]. Thus, unlike some other probiotic strains, such as *Lactococcus lactis*, which are being evaluated for the delivery of therapeutics, EcN can colonize both human and murine intestines, where it resides predominantly in the cecum and colon [4, 5]. Moreover, EcN is flagellated and able to penetrate the mucus to reside in close proximity to intestinal cells. Although it is not yet known why the strain was protective against *Shigella,* it was recently shown that, due its strong affinity for binding iron [6], EcN outcompeted intestinal *Salmonella typhimurium*, reducing *S. typhimurium* colonization, in murine acute colitis and chronic persistent infection models. Today, due to its earlier observed general anti-inflammatory properties, EcN is widely administered as a probiotic in Canada and Europe, where it has been observed to be as effective as an oral agent, mesalazine, in preventing flares in patients with ulcerative colitis [7]. The genetic tractability, impressive safety record and intestinal colonization properties, make EcN a highly attractive substrate for the development of designer probiotics.

[00247] **Bacterial type 3 secretion systems, nanomachines for protein translocation into mammalian cells.** Bacteria use a variety of secretion systems to deliver proteins into their periplasmic space, a major structural component of their outer cell envelopes, but generally few, if any, secrete proteins into their extracellular environment. Notably, however, many Gram-negative pathogens utilize complex nanomachines to directly deliver or translocate tens or even hundreds of virulence proteins and/or toxins into the cytosol of targeted mammalian host cells. Type 3 secretion systems (T3SSs) are currently the best characterized of these protein delivery machines [8]. They are composed of 20-25 proteins that form a conduit to deliver proteins directly from the bacterial cytosol, through its inner and outer membranes as well as the host cell plasma membrane, into the cytosol of the target cell (Fig. 8).

[00248] **Engineering non-pathogenic laboratory strains of *E. coli* into a protein delivery system.** Most of the work focused on reengineering bacterial T3SSs for therapeutic purposes has focused on the generation of virulence-attenuated versions of pathogenic bacteria that still encode a functional machine. However, the introduction of such strains into patients, particularly immunocompromised patients, will likely be limited. Instead, described herein is a synthetic biology based approach to transfer the T3SS from a pathogen, *Shigella flexneri,* into its close phylogenetic relative, *E. coli* [12]. As outlined in Fig. 8, described

herein is a tunable protein delivery system composed of three discrete components: (1) the delivery apparatus which encodes the genes required to form a fully assembled and functionally T3SS, (2) an activator of the *Shigella* T3SS operons whose production can be controlled via constitutive or regulated promoters and (3) a system to produce alleles of heterologous proteins of therapeutic valve that are recognized as type 3 secreted substrates. Notably, as described in detail below, by introducing additional well defined modifications, this system can be converted from one that delivers proteins into mammalian cells to one that secretes defined proteins into the extracellular environment, i.e., the intestinal lumen.

[00249] **Recognition of heterologous proteins as type 3 secreted substrates.** While all type 3 secreted proteins encode an N-terminal secretion sequence, a stretch of 15-20 residues characterized only by its unstructured nature [13], many also encode and require a downstream chaperone-binding domain. Little is known regarding what determines the relative levels of effectors that are delivered into cells, particularly in the context of an infection. It appears that all type 3 secretion sequences are not equivalent and, curiously, that the type 3 secretion sequence optimal for the secretion of one protein may not promote the optimal secretion of another [14]. To extend the development of the of type 3 secretion competent non-pathogenic *E. coli* as a general platform for the recognition and secretion of heterologous proteins, including those of therapeutic value, a recombination-based platform is described herein, to rapidly identify secretion sequences that promote the recognition of heterologous proteins as type 3 secreted substrates [12]. This platform permitted the modification of multiple proteins, including many mammalian reprogramming factors, to be recognized as type 3-secreted factors [12]. An N-terminal type 3 secretion sequence does not perturb the function of the transcription factor MyoD within mammalian cells [12]. Thus, fusion of type 3 secretion sequences to VHH and VNA, described below, is very unlikely to interfere with their function. This is particularly true in light of the fact that VHH exhibit full function when assembled into heteromers as well as when flanked by epitope-tags at both their amino and carboxy termini [15, 16].

[00250] **Conversion of a human probiotic into an intestine-specific protein delivery machine capable of recognizing VHH as secreted substrates.** Given the well established safety record of EcN (*E. coli* Nissle 1917) as a human probiotic, its genetic tractability and its close phylogenetic relationship to laboratory K12 strains of *E. coli*, it was investigated whether the *Shigella* T3SS could also function when present in EcN. Given the observations that, in the absence of antibiotic selection, laboratory strains of *E. coli* do not maintain the

large (>40kB) plasmid that carries the operons encoding the components needed to form the T3SS, landing pad" technology [17] was used to stably introduce this region of DNA into the EcN chromosome, to generate T3EcN. The introduction of a plasmid encoding the *Shigella* master transcriptional regulator VirF provides a means to regulate expression of these operons. Indeed, these T3EcN strains that express this regulator, can express and secrete type 3 secreted proteins at levels similar to that of *Shigella flexneri*. Thus, described herein is the first probiotic strain engineered to express a transkingdom protein delivery system, a system capable of directly transferring proteins from bacteria into the cytosol of mammalian cells. As a first test of the possibility of developing a T3EcN VHH delivery system, the fate of two distinct VHH when fused to four different N-terminal type 3 secretion sequences was examined. Interestingly, only one of the four secretion sequences promoted T3SS recognition of both VHH sequences (Fig 10). Notably, this secretion signal also promoted the recognition of 3 additional VHH tested as type 3 secreted substrates, each of which was efficiently secreted (Fig 10).

[00251] VNAs, VHH-based neutralizing agents with vastly improved activity. Described herein is the use of VHH-based neutralizing agents or VNAs. VNAs exploit the modularity of VHH subunits. When fused together, multiple VHH act synergistically (rather than simply additively) in binding and inactivating bacterial toxins. This is especially true with combinations of VHH that bind non-overlapping epitopes. Using this strategy VNAs that neutralize numerous bacterial toxins. including those from *Clostridium difficile* (TcdA and TcdB) [19], *Clostridium botulinim* (BoNT/A) [15], enterohemorrhagic *E. coli 0157* (Stx1 and Stx2) [20] and *Bacillus anthracis* (PA) [21], have been generated. A similar phenomenon occurs with dimeric VHH that recognize TNFα [22].

[00252] VNAs are can be recognized as type 3 secreted substrates and maintain activity when secreted into the intestinal lumen. Described herein are EcN strains that recognize and secrete these more complex proteins as their therapeutic payloads. The increased size of VNAs as compared to VHH, is highly unlikely to pose a problem, as the addition of an N-terminal T3SS secretion sequence is sufficient to generate variants of heterologous proteins as large as 98.6 kDA that are recognized as secreted substrates [12]. Furthermore, VHH do not require disulfide bonds for proper folding and are markedly stable, e.g. bivalent functional VHH have been found within the intestinal lumen after secretion in an unfolded state by the Sec system of *Lactococcus* and *Lactobacillus* species [23, 24]. Hence,

T3EcN can be successfully engineered to secrete functional, highly stable VNAs into the intestinal milieu.

[00253] Described herein are T3EcN that secrete VNAs into the intestinal lumen, permitting their use as therapeutics for intestinal infection and inflammation. For example, described herein are variants of T3EcN for the treatment of disease cause by EHEC and *C. difficile*, two toxin-driven enteric infections, and inflammatory bowel disease, a disease well established to be responsive to agents that neutralize the activity of TNF. T3EcN can also be engineered for the treatment of a variety of diseases by engineering them to deliver a variety of protein-based therapeutic payloads, including cytokines, like IL-10, that suppress intestinal inflammation, or VNAs that neutralize essential exposed virulence proteins of enteric bacterial pathogens, i.e., adhesins or outer-bacterial components of virulence factor delivery systems. In addition, given the predilection of *E. coli* Nissle 1917 to colonize solid tumors when administered via a parenteral route [25-28], these stains can be engineered for the targeted delivery of cancer therapeutics including VHH that act as immune checkpoint inhibitors.

[00254] **Develop T3EcN^{VNA} that maximally secrete functional VNA into the intestinal lumen**

[00255] Development of T3EcN that secrete high levels of functional VNAs into the intestinal lumen. Described herein is the development of strains of EcN that secrete proteins into the media rather than into host cells, identification of promoters that promote expression of high levels of the EcN T3SS, and screening for modifications to VNA that promote maximal secretion without perturbing function. The ability of the T3EcN^{VNA} strains to colonize and deliver VNA into the intestinal lumen can be evaluated as described below herein.

[00256] **Develop strains of EcN that efficiently secrete proteins into the media rather than host cells.** Under physiologic conditions, prior to contact with host cells, T3SSs including that present in T3EcN, are fully assembled and held in an "off" but primed condition such that upon contact with host cells type 3 secreted substrates are rapidly injected into host cells. This "off" conformation is maintained by the presence of proteins at the exposed and inner surfaces of the type 3 secretion conduit. In the case of the *Shigella* T3SS, the system produced by T3EcN, the outer proteins are IpaB and IpaD, and the inner "gatekeeper" protein is MxiC (see schematic, Fig. 4A). Strains that lack any of these three proteins no longer deliver proteins into host cells in a regulated manner, but rather

constitutively secrete proteins into the media when grown at 37°C, a condition sufficient to activate expression of the T3SS, when present in either *Shigella* or *E. coli* [29, 30]. A similar phenotype is observed in a few strains that carry mutations in MxiH, the subunit protein that forms the channel that extends from the bacteria to host cells. Such mutations, i.e., MxiH D73A, are hypothesized to result in conformational changes that mimic those relayed from the needle to the secretion apparatus upon contact with host cells [31, 32].

[00257] The T3SS present in T3EcN can be converted into one that secretes proteins into the extracellular milieu rather than host cells by removing the operon that encodes IpaB, IpaC, IpaD and their cognate chaperone as well as MxiC. The wild type MxiH gene can be replaced with one that encodes for MxiH D73A variant, the mutant previously observed to result in the highest observed levels of constitutive secretion. The resulting secretor strain will be used for the initial (phase 1) T3EcN^{VNA} studies in our animal model experiments.

[00258] Genetic screens can identify either mutations in MxiH or T3EcN that result in increased VNA secretion. Described herein is a quantitative plate-based type 3 secretion assay to facilitate these studies (Fig. 10). In this screen, bacteria, like T3EcN, are grown on solid media under conditions that induce type 3 secretion and then overlaid with nitrocellulose filters. The secreted proteins are transferred to the filters, which are then probed with an antibody that recognizes the secreted protein. In this assay, only under conditions that induce activity of the nanomachines are secreted proteins seen, i.e., bacterial lysis is not an issue. Selected mutations can be tested to determine if they alter the ability of the EcN strains to colonize the murine intestinal tract.

[00259] **Development of T3EcN that exhibit maximal type 3 secretion activity.** As illustrated in Fig. 8, the expression of the operons that carry the genes needed to form a functional T3SS are controlled in trans via a plasmid encoded transcription factor. Interestingly, the levels of expression and activity of the T3SS in *E. coli* depends on the level of expression of the master regulator. The modularity of the system permits control of the levels and the timing of delivery of VNA into the intestinal lumen, i.e., in response to the development of intestinal inflammation or a bacterial pathogen. Described herein is the development of strains of EcN that express high levels of the *Shigella* T3SS, as this will correlate with increased levels of secreted VNAs. Thus, alleles of the master *Shigella* T3SS transcriptional factor, VirF, that are under control of strong constitutive promoters, i.e., BBa_J23100, a synthetic sequence optimized for binding by σ70, the main RNA polymerase sigma factor expressed in *E. coli* [33] are generated. A promoter identified as permitting high

levels of secreted VNAs can be further examined to confirm that high level constitutive activity of the T3SS does not perturb T3EcN growth, and the the transcription factor expression DNA cassettes can be introduced onto the chromosome of T3EcN using λ inch [35] or "landing pad" technology [17], ensuring that this DNA is stably maintained in the absence of antibiotic selection.

[00260] **Generate type 3 secreted VNA variants that maximally neutralize TcdA/TcdB, Stx2 and TNFα.** The following strategy will be used develop type 3 secreted active variants of VNAs that neutralize TcdA/TcdB, Stx2, the causative agents of CDI and HUS, respectively, and TNFα, a proinflammatory cytokine linked to IBD. The maximal number of fused VHH, up to six, that are recognized as secreted substrates when linked to an OspC3 type 3 secretion sequence can be determined. Whether fusion to any of the as of yet untested ~15 secretion signal sequences in this context, results in increased levels of secreted VNAs can be determined. Combinations of VHH that, when combined to form VNAs and secreted via T3EcN exhibit maximal toxin neutralization, can be tested using cell culture intoxication assays [19, 20, 36]. For each target VHH demonstrated to exhibit strong TcdA, TcdB and Stx2 neutralization and binding activities can be combined [19, 20]. A VHH library generated from lymphocytes isolated from two alpacas immunized with purified murine TNFα using approaches that were previously used to identify and characterize VHH that recognize ovine TNFα [18] can be generated. Genes for VNAs for each of the targets that exhibit the desired activity can be placed under the control of the same promoter that is chosen to drive the expression of the type 3 secretion system master regulator, VirF, such that expression of the T3SS and the secreted VNA are coordinately regulated. A DNA fragment that carries this expression cassette can be introduced onto the EcN chromosome at a defined loci using λ inch [35] or "landing pad" technology [17].

[00261] **Characterization of colonization and secretion patterns of T3EcN^{VNA} within the intestines of mice.** The biogeography, persistence and colonization dynamics of T3EcN^{VNA} when administered orally to mice can be investigated to optimize these parameters to ensure ideal efficacy. Although EcN is not a 'normal' constituent of the mouse intestinal microbiota, it can stably colonize the intestines of mice at high titers after a single inoculum [5, 37]. The biogeography, persistence and colonization dynamics of EcN, T3EcN and T3EcN^{VNA} after administration of 10^9 -10^{10} bacteria [5, 37] to mice by gavage can be characterized. T3EcN colonization can be monitored initially by quantifying (using conventional culture and confirmatory qPCR) bacterial load in shed feces as well as in

homogenates of various intestinal segments. To facilitate visualization of the EcN strains, versions of T3EcN^{VNA} that stably express eGFP can be used, a modification previously established to have no effect on EcN murine colonization [38]. To characterize VNA localization, particularly to the mucosa, the distribution of epitope-tagged type 3 secreted VNA can be examined by immunohistochemical staining [39, 40]. To compare the relative activities of VNAs secreted by T3EcN^{VNA} strains, toxin neutralization assays and/or ELISAs with colonic homogenates can be permformed. The behavior of EcN, T3EcN and T3EcN^{VNA} when administered to conventionally reared mice that harbor a diverse gut microbiota can be characterized, as these mice can be used in the STEC/HUS model. Similar subsequent studies can be conducted with the antibiotic-perturbed CDI mouse models, as well as the IBD models, as gut microbiota and host genetics may influence the colonization of EcN-derived strains. These studies are designed to not only confirm that T3EcN can deliver functional VNA into the intestinal lumen, provide guidance in determining the dosing frequency and titers with which the T3EcN^{VNA} strains can be administered when assessed for efficacy in the murine diseases models.

[00262] *Modifications to promote adhesin of T3EcN to intestinal epithelial cells* Wild type EcN is well established to colonize the colons of mice [4, 5], the primary site of pathology observed with CDI, Shiga-toxigenic *E. coli* and IBD. It is possible that the introduction of a functional T3SS into EcN might interfere with its ability to colonize. If this is observed to be an issue when analyzing the behavior of strains in mice using the assays described below, the strains can be engineered to express adhesins that promote attachment to intestinal epithelial cells, including *Salmonella* SiiE [41]. Interestingly, it was recently demonstrated that it is possible to generate synthetic bacterial adhesins that are basically fusion proteins of bacterial adhesins and VHH, such that the VHH is positioned to mediate interactions with defined mammalian cell proteins [42]. This technology can permit targeting of VNA T3EcN secreting bacteria to specific intestinal cell types and/or regions of the gastrointestinal tract.

[00263] VHH can be engineered to be recognized as secreted substrates, permitting the T3EcN^{VNA} platform to be used for the secretion of functional neutralizing VNA into the intestinal lumen. Alternatively VNA can be secreted via the EcN native flagella type 3 secretion system flagella. Furthermore, EcN strains that express synthetic adhesins designed to display the VNA on the outer bacterial surface can be used. In addition, VNA secreting T3EcN strains can be modified to make them autotrophic for amino acids present in the gut, to prevent their survival outside of the intestines. Additonally, "kill switches" can be

introduced into the strains such that we can control their spread between humans as well as eradicate the bacteria from the intestinal lumen, if needed [43, 44].

[00264] Investigate the utility of T3EcN^{VNA} in murine models of infection and inflammation

[00265] Shiga toxin-producing *E. coli* (STEC), a life-threatening infection with no established anti-microbial treatment. STEC, as exemplified by enterohemorrhagic *E. coli* (EHEC) O157:H7, cause both sporadic and major outbreaks of diarrheal disease worldwide. EHEC asymptomatically colonizes cattle, resulting in contaminated beef as well as fecally contaminated foodstuff such as spinach, sprouts, and apple cider [45-49] that are the major sources of human EHEC infection. Shiga toxin (Stx) is the primary EHEC virulence determinant, and is the causative agent of hemolytic uremic syndrome (HUS), a clinical syndrome defined by the triad of hemolytic anemia, thrombotic thrombocytopenia, and uremia. HUS is the leading cause of renal failure in children. Stx, of which there are two major serotypes, Stx1 and Stx2, is encoded by a lysogenic phage and is produced only upon phage induction. Stx produced in the gut enters systemic circulation and targets the endothelium of the glomeruli and the central nervous system, causing HUS. Many antibiotics trigger phage induction, so there are currently no specific therapies for treating life-threatening Stx-associated HUS.

[00266] Towards developing a VHH toxin neutralization therapy for HUS a VHH phage display library derived from the HcAbs of alpacas immunized with catalytically inactive Stx1 and Stx2 [20] has been generated and screened. These experiments led to the identification of VHH that inactivate Stx1 or Stx2 at nM concentrations which they then combined to develop a VNA (VNA^{Stx1,2}), comprised of three VHH, capable of neutralizing either toxin at pM concentrations. Mice were significantly protected from intravenous Stx2 when co-administered VNA^{Stx1,2}, and gnotobiotic piglets were protected from the lethal effects of EHEC 0157:H7 intestinal infection by intramuscular injection of a nonreplicating adenovirus vector that expresses VNA^{Stx1,2} [16]. While the murine and piglet studies demonstrate the efficacy of toxin neutralizing VNA when administered systemically, there is also data to suggest that that intra-intestinal absorption of Stx can prevent the development of HUS, as Patton and colleagues have demonstrated that *E. coli* engineered to express a receptor that sequesters Stx1 and Stx2, prevents the development of lethal renal damage in an antibiotic treated murine STEC model [50]. T3EcN^{VNA} that secrete VNA^{Stx1,2} can not only act as a preventive measure pre-exposure during an outbreak, but also benefit individuals who present

soon after exposure or early in the course of STEC infection, scenarios likely to become more common with ongoing improvements in the tracking of major outbreaks and the development of rapid and sensitive diagnostics.

[00267] *Murine model for STEC infection:* A major obstacle in understanding the pathogenesis of Stx-mediated disease has been the lack of murine model that reflects human disease. EHEC, the STEC of greatest clinical significance, does not efficiently colonize conventional mice, necessitating the use of germ-free or antibiotic-treated mice, which lack a normal microbiota [51, 52]. *Citrobacter rodentium* (CR), a natural murine pathogen and a close relative of EHEC, provides an excellent model to study conserved virulence factors [51, 53-57]. However, CR does not encode Stx, limiting its use as a model for the serious systemic manifestations of EHEC infection such as HUS. CR that express Stx2 have been developed by lysogenizing CR with an Stx-producing phage, termed ϕStx2dact, derived from a naturally occurring STEC strain to generate a strain herein referred to as CR_Stx2 [36]. Upon phage induction, CR_Stx2 produces and secretes Stx2 at levels equivalent to human EHEC isolates. Upon infection of conventional mice, CR_Stx2 not only colonizes the intestine, but also triggers systemic manifestations including weight loss, increased fecal moisture, intestinal inflammation, renal pathology and proteinuria, that reflect human disease [36].

[00268] *T3EcN-mediated prophylaxis from STEC infection:* The murine CR_Stx2 model can be used to investigate the ability of $T3EcN^{VNA\text{-}Stx2}$ to prevent or treat STEC infections. The utility of the engineered probiotic strain to act as a prophylactic intervention can be evaluated. Mice can be gavaged with $T3EcN^{VNA\text{-}Stx2}$ (or, as controls, T3EcN or EcN) for up to 3 days prior to inoculation with CR_Stx2. The mice can then be followed daily for up to 14 days for signs of clinical disease as assessed by weight loss, proteinuria and changes in appearance or behavior, e.g. ruffled fur, hunched posture and lethargy. Colonization of EcN and CR_Stx2 over the course of the experiment can be assessed by fecal shedding. If EcN-derived strain colonization does not reach desired levels, they are be administered both pre- and post-infection with CR_Stx2. Upon necropsy, as dictated by poor clinical condition or at completion of experiment, the intestines and kidneys of mice can be examined for histopathologic evidence of inflammation, bacterial colonization, and tissue damage.

[00269] *T3EcN-mediated treatment of STEC infection:* To assess the potential of using $T3EcN^{VNA}$ administration as a therapeutic measure to resolve CR_Stx2 infections, once a $T3EcN^{VNA}$ strain variant identified as an effective prophylactic, can be used to prevent the

development of systemic disease in mice that are one, three or five days post-inoculation with CR_Stx2,. In addition, the therapeutic value of T3EcN^{VNA} when administered concurrently with antibiotics that target CR, given that high titers of an Stx2-neutralizing probiotic may eliminate the risk of phage-activating (and thus Stx-inducing) antibiotic treatment exacerbating the disease can be evaluated. Indeed, if antibiotic treatment concurrent with T3EcN^{VNA} administration promotes survival and diminishes intestinal and systemic damage treatment, this could lead to changes in treatment strategies, when applied to human infection, that fundamentally alter current guidelines that eschew antibiotic treatment in the setting of an EHEC infection.

[00270] ***Clostridium difficile* infection (CDI), a burgeoning epidemic.** CDI, which is associated with antibiotic administration, is the most common hospital-acquired infection in the U.S., and is also increasingly being recognized as a cause of diarrhea within the community. The yearly incidence of CDI is currently a half million and skyrocketing, with current annual costs approaching 4.8 billion USD. *C. difficile* can cause pseudomembranous colitis, a severe infection, particularly in the elderly, with mortality rates as high as 16.7% [58]. Furthermore, CDI is difficult to treat with relapse rates are as high as 25%. Current therapeutic options for *C. difficile* are limited to three antibiotics that reach high concentrations within the intestinal lumen and there is concern for the emergence of antibiotic resistance strains. While fecal bacteriotherapy has shown promise in preliminary trials, its long-term effects on human health remain unknown. New therapies, particularly those that prevent the occurrence of CDI in patients receiving antibiotics for unrelated life-threatening infections, are greatly needed.

[00271] Two co-regulated and homologous *C. difficile* toxins, TcdA and TcdB, disrupt epithelial tight junctions and concomitant barrier function, as well as induce epithelial cell death, leading to acute inflammation. Notably, the past decade has witnessed the emergence of a more lethal *C. difficile* strain (*C. difficile* BI/NAP1/027), which secretes higher toxin levels [59]. Strains deleted for TcdB demonstrate diminished loss of pathogenicity in mouse models and a small molecule TcdB inhibitor completely blocked CDI development in mice [60]. Merck recently announced that in two phase 3 human trials, bezlotoxumab, a monoclonal antibody directed against TcdB significantly reduced CDI recurrence rates, suggesting a major and perhaps unique role for TcdB in the pathogenesis of CDI.

[00272] By screening a VHH phage display library derived from alpacas immunized with TcdA or TcdB, four anti-TcdA and three anti-TcdB VHH each of which recognizes a unique

toxin epitopes and neutralizes the toxins at nM concentrations, have been identified. $VNA^{TcdB/A}$, a VNA composed of four VHH, two directed against each toxin, completely inhibits toxin-mediated cytopathogenic effects on cultured cells at pM concentrations [19]. Upon intraperitoneal injection, $VNA^{TcdB/A}$ not only blocked a lethal murine TcdA and TcdB challenge upon co-administration, but also fully rescued previously intoxicated mice suffering from severe CDI, including diarrhea and weight loss, with a projected mortality rate of 60% [19]. T3EcN-mediated delivery of VNA, such as $VNA^{TcdB/A}$, that target TcdB and TcdA in the intestinal lumen of infected mice can provide prophylactic as well as therapeutic value in patients with preexisting CDI or at risk for new or recurrent CDI.

[00273] *Murine model for CDI infection:* The indigenous gut microbiota of conventionally raised mice, like that of humans, provides colonization resistance against *C. difficile*, and mice, like humans, do not develop CDI unless first exposed to antibiotics. Mice that are exposed to a broad-spectrum cephalosporin, cefoperazone, in their drinking water for 5 days are highly susceptible to infection with vegetative colonies or spores of *C. difficile* [61, 62]. Mice are typically infected two days after the discontinuation of antibiotics, but the mice remain fully susceptibility for the following seven days. The disease course of mice varies with the infectious dose as well as the strain. Whereas mice infected with strains that secrete lower toxin levels develop only a subclinical infection, those infected with spores from strains such as VPI 10463 that produce high levels of toxin develop severe colitis [62]. After high doses of VPI 10463, mice develop lethargy, diarrhea and a hunched posture within 1 to 2 days post infection and $\geq$ 20% weight loss by day 2 post-infection, necessitating euthanasia. With lower inoculums of VPI 10463, the onset of symptoms is delayed for ~ 2 days. Notably, when treated with oral vancomycin for 5 days, mice appear to control the infection. Although, upon the discontinuation of antibiotics, mice almost uniformly develop recurrent disease, as is often observed in patients after the completion of antibiotics, thus demonstrating the utility of the murine model for studying relapsing CDI [63].

[00274] *T3EcN-mediated prophylaxis from CDI infection:* The ability of $T3EcN^{VNA}$ as a preventive intervention for the development of CDI can be tested. After treating mice with oral cefoperazone for 5 days, we they can be inoculated with $T3EcN^{VNA}$ (or, as controls, EcN or T3EcN) for 5 days before administering VPI 10463 spores via oral gavage. Mice can be observed for seven days post-infection for CDI as characterized by lethargy, diarrhea, changes in posture and weight loss. Fecal shedding of $T3EcN^{VNA}$ and *C. difficile* can be

assessed daily by viable counts. At day seven post-infection, the intestinal inflammation and the integrity of its epithelium can be assessed by histopathology.

[00275] *T3EcN-mediated treatment of CDI infection:* Once conditions under which the daily administration of $T3EcN^{VNA}$ is protective are established, it can be tested whether 12 or 24 hours post-inoculation with the *C. difficile* spores the addition of $T3EcN^{VNA}$ is sufficient to mitigate CDI, using the above disease readouts.

[00276] *T3EcN-mediated prevention of CDI relapse:* Notably, the resolution of CDI of both mice and humans is thought to involve the reconstitution of the indigenous microbiota as well as the clearance of *C. difficile*. For this reason, patients who develop CDI while on extended courses of antibiotics for unrelated infections are maintained on anti-*C. difficile* (typically vancomycin) therapy until 10-14 days after completion of their broad-spectrum antibiotics. In addition, empiric anti-*C. difficile* antibiotics are given to patients with a recent history CDI who are receiving prolonged antibiotic regimens, even if they show no evidence of active CDI. To test whether $T3EcN^{VNA}$ administration can substitute for anti-*C. difficile* antibiotic treatment in preventing CDI relapse, cefoperazone-treated mice will first be inoculated with *C. difficile* to develop a primary CDI, then treated for 5 days with oral vancomycin to suppress disease. This protocol permits the recovery, but upon subsequent cessation of vancomycin treatment, nearly all mice experience relapse of CDI [63]. We can orally inoculate $T3EcN^{TcdB/TcdA}$ one day prior to completion of vancomycin treatment and follow mice using the parameters described above for 10 days to determine if $T3EcN^{VNA}$ can prevent relapse.

[00277] In some embodiments of any of the aspects, T3EcN that secrete a heteromeric VNA that carries VHH that recognize both TcdA and TcdB can be utilized. In some embodiments of any of the aspects, given the growing evidence that TcdB is the prime virulence determinant [60, 64], VNA composed solely of TcdB-neutralizing VHH (VNA^{TcdB}) can be utilized. It is contemplated that when inoculating $T3EcN^{VNA}$ during cefoperazone treatment, stable colonization of the intestine by $T3EcN^{VNA}$ may be hindered by antibiotic treatment, in which case higher titers or more frequent doses of $T3EcN^{VNA}$ can be administered.

[00278] **IBD, a chronic inflammatory disease mitigated by TNFα neutralization.** Inflammatory bowel diseases (IBD) which includes ulcerative colitis (UC) and Crohn's disease (CD) are chronic relapsing intestinal disorders that affect over 3 million individuals in the Westernized world [65]. Patients often present with abdominal pain, diarrhea, and rectal

bleeding, which in severe cases can require surgical interventions [65, 66]. Indeed the current standard of care of severe cases includes surgical resection. Although the etiologies of these diseases are multi-factorial and not fully understood, the inflammation associated with these disorders is often due to a dysregulated immune response to the gut microbiota, and broadly characterized by activation of proinflammatory cytokines including TNFα. Over the past decade, anti-TNFα monoclonal antibodies have transformed the management of both CD and UC and are currently the standard therapy in preventing induction and maintaining remission in patients with moderate to severe disease. Unfortunately, this type of treatment is not always sufficient to relieve symptoms, and is associated with increased risk of developing infection (and even cancers, i.e., lymphomas) due to systemic immunosuppression, reflecting the fact that these systemically administered antibodies are not restricted to the gut [65].

[00279] It is contemplated herein that, similar to anti-TNFα monoclonal antibodies, anti-TNFα VNA have the potential to provide efficacious treatment of chronic inflammatory diseases. A bivalent llama-derived VNA that recognizes murine TNFα, when applied directly at pM concentrations, has been reported to prevent TNFα toxicity to a TNFα-sensitive mouse fibroblast cell line [22]. In addition, in a murine collagen-induced arthritis model, the administration of VNA^{TNF} reduced joint inflammation similarly to a murine anti-TNFα monoclonal antibody [22].

[00280] Similarly, the daily administration of high doses of *Lactococcus lactis* engineered to secrete a VNA^{TNF} composed of a single duplicated VHH via their Sec secretion system moderately suppressed intestinal inflammation (30-40%) in DSS (dextran sulfate sodium) induced intestinal injury and *Il10*$^{-/-}$ mouse models of IBD [24]. These observations support that the direct delivery of VNA into the intestinal lumen is worth pursuing as a novel therapeutic. Notably, unlike EcN, *L. lactis* do not colonize the intestines, thus presumably VNA are released as the bacteria travel through the murine gastrointestinal tract. Furthermore, not only does EcN colonize the intestines, but the bacteria also penetrate and adhere to the intestinal mucosa [62]. Thus, it is contemplated herein that the $T3EcN^{VNA}$ can improve upon the *L. lactis* based therapy by secreting high levels of VNA^{TNF} in close proximity to the intestinal epithelium, the site of disease.

[00281] *Murine models for IBD:* The utility of $T3EcN^{VNA}$ for suppressing inflammation can be assessed using three complementary murine models including TRUC (*T-bet*$^{-/-}$ × *Rag2*$^{-/-}$ ulcerative colitis) and *Il10*$^{-/-}$ mice that spontaneously develop IBD and DSS-treated mice, which display acute intestinal injury and inflammation. TRUC mice lack an adaptive immune

response and develop a spontaneous, highly penetrant, and communicable distal colitis that resembles human ulcerative colitis. TRUC-associated colitis is characterized by diminished colonic barrier function and elevated TNFα levels, resulting in detectable histological inflammatory intestinal damage by 3.5 weeks of age [63]. In contrast, *Il10*$^{-/-}$ mice have intact adaptive and innate immune cell populations but the lack of IL-10 compromises T-regulatory and myeloid cell subsets, resulting in chronic enterocolitis in the presence of a conventional microbiota [64]. The DSS model utilizes mice with intact innate and adaptive immune systems but the injury-induced inflammation leads to bloody stool and histological symptoms within 5 days. All three models exhibit high levels of mucosal pro-inflammatory cytokines such as TNF-α and IL-8 that are suppressed when the mice are treated systemically with anti-TNF-α antibody [68, 70, 71].

[00282] *T3EcN-mediated treatment of IBD:* After optimizing inoculation conditions, TRUC, DSS-treated and *Il10*$^{-/-}$ mice can be orally gavaged with T3EcN^{VNA} (or as controls, EcN or T3EcN). For the TRUC and *Il10*$^{-/-}$ intestinal inflammation can be determined after two and four weeks using multiple complementary assays, while the DSS treated mice can be examined after 7 days. First, the nature and extent of colonic inflammation can be assessed by histopathologically [75]. Second, organ explant cultures can be employed to monitor for changes in the cytokines present in the inflammatory milieu of the treated mice [68]. The distal colon of TRUC mice and transverse and distal colon of *Il10*$^{-/-}$ mice and the entire colon distal to the cecum of DSS-treated wild type mice can be isolated [76] and explant supernatants will be analyzed using multiplex cytokine analysis for IL-1α, IL-1β, IL-2, IL-4, IL-6, KC, TNF-α, IFNγ, IL-10 (not applicable for *Il10*$^{-/-}$), IL-12p40, IL-12p70, IL-13, IL-17A, IL-21, and IL-23 using the Luminex platform. Third, fecal lipocalin can be used to non-invasively monitor intestinal inflammation in the TRUC and *Il10*$^{-/-}$ mice. Finally, the cell populations (cell surface markers and intracellular cytokines) present in the intestines of mice can be examined by flow cytometry.

[00283] Described herein are probiotic strains that secrete single domain antibodies that sequester proteins, including toxins and pro-inflammatory cytokines. Specifically the toxins associated with hemolytic uremic syndrome caused by enterohemorrhagic *E. coli* and the *Clostridium dificile* toxins that cause *C. dificile* colitis can be targeted as described herein. Bacteria to secrete single domain antibodies that bind and sequester TNFalpha, thus developing a treatment for inflammatory bowel disease, are also described herein.

[00284] **EXAMPLE 4**

[00285] Described herein are laboratory and commensal strains of *E. coli* that express modified type 3 secretion systems that act to secrete proteins into their surroundings as opposed to directly into host cells using the following modifications (alone or in combination):

[00286] 1) Removal of genes that encode components of the translocon apparatus (IpaB +/- IpaC)- this is the outer most portion of the type 3 secretion apparatus (T3SA) which is inserted into host cells

[00287] 2) Removal of the gene encode IpaD, the component of the T3SA that serves as the plug that normally holds the machine in a closed state prior to contact with host cells

[00288] 3) Removal of the gene encoding MxiC, a component of the T3SA that serves as the gatekeeper that normally prevents the loading of secreted substrates into the machine until it is activated. In its absence the T3SA constitutively secretes proteins into the media/extracellular milieu

[00289] 4) Introduce mutations in MxiH, the portion of the T3SA that forms the needle, the portion of the machine that extends from the body of the secretion apparatus towards the host cell. When the T3SA contacts host cells, the pressure results in changes in the needle that signal activation of protein secretion. Mutations of the needle protein, MxiH, have been identified which, when present as the sole copy in the cell, result in a secretion system that secretes proteins into the media as opposed to host cells.

[00290] 5) Conduct genetic screens to identify *E. coli* variants that exhibit increased levels of secreted proteins.

[00291] The *E. coli* strains MP (PMID 24563035, Lasaro et al J Bact. 2014) and HS can be used in any of the aspects or embodiments herein.

[00292] These modified bacteria can be engineered to recognize one or more of the following proteins as type 3 secreted substrates

a. single domain antibodies or nanobodies
b. cytokines- e.g., IL-10
c. toxins
d. pro-drug converting enzymes, i.e., cytosine deaminase
e. Other anti-inflammatory cytokines
f. Peptides with anti-inflammatory function or other functions
g. receptor agonists (to modulate immune responses)
h. antigenic proteins- for vaccine development

i. Enzymes - e.g., diagnostic factors, Lactose intolerance relevant enzymes

[00293] Cancer Therapeutics

[00294] Contemplated herein is the targeted secretion of immunostimulatory single domain antibodies (VHH) and/or toxins directly into solid tumors. Nissle *E. coli* homes to tumors and thus can serve as a targeted delivery system. In some embodiments of any of the aspects, single domain antibodies (VHH) that recognize PD-1, PD-L1, CTLA-4 can be utilized for this purpose.

[00295] Inflammatory Bowel disease

[00296] Secretion of cytokines that suppress inflammation, i.e., IL-10 and IL-27 directly into the lumen of the intestines is contemplated herein as a targeted therapy for inflammatory bowel disease

[00297] Secretion of VHH (monomeric and/or multimeric) that bind to cytokines, and/or their receptors, that promote inflammation, including, TNFalpha, IL-6, IL-18, IL-21, Il-33 and IL-13 is contemplated herein as a targeted therapy for IBD.

[00298] Treatment of intestinal infections

[00299] Secretion of VHH or VNA (VHH neutralizing agents = multimeric VHH) that bind Shiga toxin (treatment for hemolytic uremic syndrome linked to EHEC infections, C. dif toxin A and B (treatment for C. dif colitis), cholera toxin (treatment for cholera), anthrax toxin (gastrointestinal anthrax), botulinum toxin (botulism).

[00300] **EXAMPLE 5**

[00301] Development of "secretor" strains of mT3sec_E. *coli*.

[00302] Under physiologic conditions, prior to contact with host cells, T3SSs are fully assembled and held in an "off" but primed condition such that upon contact with host cells type 3 secreted substrates are rapidly injected into host cells. This "off" conformation is maintained by the presence of proteins at the exposed and inner surfaces of the type 3 secretion conduit. In the case of the Shigella T3SS, the system produced by minT3_E. *coli*, the outer proteins are IpaB and IpaD, and the inner "gatekeeper" protein is MxiC (Fig. 4A). Strains that lack any of these three proteins no longer deliver proteins into host cells in a regulated manner, but rather constitutively secrete proteins into the media when grown at 37°C, a condition sufficient to activate expression of the T3SS, when present in either Shigella or E. *coli*. Based on these observations, a recombination platform was used to develop strains of minT3_E. *coli* that no longer encode the translocon apparatus, the outer portion of the T3SA that is composed of IpaB, IpaC and IpaD. As predicted, it is observed

that this strain is capable of constitutive secreting heterologous proteins into the extracellular milieu, as it is capable of secreting type III substrates in the absence of Congo red, a well established in vitro inducer of the secretion of effectors from Shigella (Fig. 11).Development of monomeric and multimeric single domain camelids that are recognized as type III secreted effectors.

[00303] Monoclonal antibody-based "magic bullet" therapeutics with high TNFα specificity are dramatically altering approaches towards the treatment of inflammatory bowel disease. Patients administered such drugs systemically have marked improvement of systems, but unfortunately can suffer from off-target effects including systemic immunosuppression. Monoclonal antibodies are highly complex, composed of multiple subunits linked together by disulfide bonds and thus are highly unlikely to be functional when engineered to be recognized as type III secreted substrates. A new exciting avenue of antibody-mediated therapies is currently emerging that circumvents many of the obstacles of conventional monoclonal antibody-based therapies based on Camelid heavy chain only immunoglobulins (HcAbs). HcAb bind antigens via a single variable-domain heavy-chain region, a VHH. VHH, small ~15 kDa protein domains, bind substrates with Kd's in the nM to pM range and exhibit a predilection for binding protein active sites. Furthermore they can be used as modular building blocks to generate multimeric constructs that exhibit enhanced binding potential, both in terms of binding affinity and breath of epitope recognition. Their remarkable solubility, stability and small size overcome many of the barriers that currently limits the production of monoclonal antibodies, resulting in significant decreases in production costs. Thus, it was next investigated whether functional variants of HcAbs that are recognized as secreted substrates by minT3_E. coli can be developed. As shown in Fig. 12, relatively high levels of secretion of monomeric VHH were observed when fused to an OspC3 or OspG type III secretion signal sequence as well as trimeric VHH when fused to an OspC3 type III secretion signal sequence. It has also been demonstrated that VHH fold correctly and maintain function when secreted by minT3 E. *coli*.

What is claimed herein is:

1. An engineered, non-pathogenic, gram negative microbial cell comprising:
    a) a first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS); wherein the TDESS comprises at least virB; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiD; mxiA; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40; and
    b) a second nucleic acid sequence encoding an T3SS-compatible payload polypeptide.
2. The microbial cell of claim 1, wherein the cell does not comprise or express at least one of:
    a. IpaB;
    b. IpaD; and
    c. MxiC.
3. The microbial cell of claim 2, wherein the cell does not comprise or express at least one of:
    a. IpaB and IpaD; and
    b. MxiC.
4. The microbial cell of claim 3, wherein the cell does not comprise or express IpaB; IpaD; and MxiC.
5. The microbial cell of claim 3, wherein the cell does not comprise or express IpaB; IpaD; IpaC; and MxiC.
6. The microbial cell of any of claims 1-5, wherein the cell has a mutated MxiH.
7. The microbial cell of claim 6, wherein the cell has a D73A mutation in MxiH.
8. The microbial cell of any of claims 1-7, wherein the second nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding an T3SS-compatible payload polypeptide.
9. The microbial cell of claim 8, wherein the inducible promoter sequence is regulated by a master T3SS transcriptional regulator.
10. The microbial cell of any of claims 8-9, wherein the inducible promoter sequence comprises a (T3SS)-associated promoter or promoter element.
11. The microbial cell of claim 10, wherein the T3SS-associated promoter or promoter element is a MxiE recognition sequence.

12. The microbial cell of any of claims 1-11, wherein the cell comprises a third nucleic acid sequence encoding a master T3SS transcriptional regulator.
13. The microbial cell of claim 12, wherein the master T3SS transcriptional regulator is selected from the group consisting of:

    VirB and VirF.
14. The microbial cell of any of claims 12-13, wherein the third nucleic acid sequence comprises 1) an inducible promoter sequence that is operably linked to 2) a sequence encoding a master T3SS transcriptional regulator.
15. The microbial cell of claim 14, wherein the inducible promoter is selected from the group consisting of:

    an arabinose-inducible promoter; pBAD arabinose-inducible promoter; an IPTG-inducible promoter; tumor-induced promoters; ansB promoter; pflE promoter; napF promoter; and an inflammation-induced promoter.
16. The microbial cell of any of claims 1-15, wherein the TDESS comprises at least: virB; acp; ipaA; ipgC; ipgB1; ipgA; icsB; ipgD; ipgE; ipgF; mxiG; mxiH; mxiI; mxiJ; mxiK; mxiN; mxiL; mxiM; mxiE; mxiD; mxiA; spa15; spa47; spa13; spa32; spa33; spa24; spa9; spa29; and spa40.
17. The microbial cell of any of claims 1-16, wherein the TDESS comprises polypeptides endogenous to a bacterium selected from the group consisting of:

    Shigella spp; *Salmonella* spp; enteropathogenic *E. coli*; and *Yersinia* spp.
18. The microbial cell of any of claims 1-17, wherein the first nucleic acid sequence is located on a plasmid.
19. The engineered microbial cell of any of claims 1-17, wherein the first nucleic acid sequence is located on a chromosome.
20. The microbial cell of any of claims 1-19, wherein the second nucleic acid sequence is located on a plasmid.
21. The engineered microbial cell of any of claims 1-19, wherein the second nucleic acid sequence is located on a chromosome.
22. The engineered microbial cell of any of claims 1-21, wherein the first nucleic acid sequence comprising genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS) and/or the genes encoding a type 3 secretion system (T3SS)-derived extracellular secretion system (TDESS) are exogenous to the microbial cell.

23. The engineered microbial cell of any of claims 1-22, wherein the first nucleic acid sequence is no greater than 3kb in size.
24. The engineered microbial cell of any of claims 1-22, wherein the first nucleic acid sequence and third nucleic acid sequence are cumulatively no greater than 3 kb in size.
25. The engineered microbial cell of any of claims 1-24, wherein the cell did not comprise a T3SS prior to being engineered to comprise the first and second nucleic acid sequences.

26. The microbial cell of any of claims 1-25, wherein the T3SS-compatible payload polypeptide comprises a T3SS secretion sequence.
27. The microbial cell of any of claims 1-26, wherein the T3SS-compatible payload polypeptide comprises an N-terminal T3SS secretion sequence.
28. The microbial cell of any of claims 1-27, wherein the T3SS-compatible payload polypeptide comprises a OspC3 T3SS secretion sequence.
29. The microbial cell of any of claims 1-28, wherein the T3SS-compatible payload polypeptide comprises an anti-inflammatory polypeptide.
30. The microbial cell of claim 29, wherein the anti-inflammatory polypeptide is IL-10 or IL-27.
31. The microbial cell of any of claims 1-30, wherein the T3SS-compatible payload polypeptide comprises an antibody reagent.
32. The microbial cell of claim 31, wherein the antibody reagent is selected from the group consisting of:

    a nanobody; a VNA; and a VHH.

33. The microbial cell of any of claims 31-32, wherein the cell comprises at least one further nucleic acid sequence encoding an additional T3SS-compatible payload polypeptide comprising an antibody reagent, VHH, or VNA.
34. The microbial cell of any of claims 31-32, wherein the one or more antibody reagents form a multimeric complex.
35. The microbial cell of claim 34, wherein the multimeric complex is multispecific.

36. The microbial cell of any of claims 31-35, wherein the antibody reagent specifically binds to a cancer cell marker.
37. The microbial cell of any of claims 31-35, wherein the antibody reagent specifically binds to a cancer checkpoint polypeptide.
38. The microbial cell of any of claims 31-37, wherein the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 reagent.
39. The microbial cell of any of claims 31-38, wherein the antibody reagent is an anti-PD-L1; anti-PD-1; or anti-CTLA-4 VNA or VHH.
40. The microbial cell of any of claims 31-39, wherein the antibody reagent specifically binds to an inflammatory cytokine receptor or an inflammatory cytokine.
41. The microbial cell of claim 40, wherein the antibody reagent binds to a molecule selected from the group consisting of:

    TNFα, IL-8; IL-6, IL-18, IL-21, Il-33 and IL-13.
42. The microbial cell of any of claims 31-35, wherein the antibody reagent specifically binds to a bacterial toxin.
43. The microbial cell of claim 42, wherein the bacterial toxin is an *E. coli* or *C. difficile* toxin.
44. The microbial cell of any of claims 42-43, wherein the bacterial toxin is selected from the group consisting of:

    shiga toxin; *C. difficile* toxin A (TcdA); *C. difficile* toxin B (TcdB); cholera toxin; anthrax toxin; and botulinum toxin.

45. The microbial cell of any of claims 1-28, wherein the T3SS-compatible payload polypeptide comprises a toxin.
46. The microbial cell of any of claims 1-28, wherein the T3SS-compatible payload polypeptide comprises an antigen.

47. The microbial cell of any of claims 1-46, wherein the microbial cell is engineered from a microbial cell selected from the group consisting of:

    E. coli NISSLE 1917 (EcN); *E. coli* K12; MP; HS; and derivative strains thereof.
48. The microbial cell of claim 47, wherein the strain which is derivative of *E. coli* K12 is selected from the group consisting of:

E. coli DH10β and *E. coli* DH5α.

49. The microbial cell of any of claims 1-47, wherein the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).
50. The microbial cell of any of claims 1-49, wherein the microbial cell is engineered from a commensal intestinal microbial cell.
51. The microbial cell of claim 50, wherein the commensal intestinal microbial cell is *E. coli* NISSLE 1917 (EcN).
52. The microbial cell of any of claims 1-51, wherein the non-pathogenic microbial cell is engineered from a pathogenic microbial cell organism by deletion or mutation of one or more T3SS components.
53. The microbial cell of claim52, wherein the one or more T3SS components is selected from the group consisting of:

    a toxin; a T3SS effector; a structural T3SS polypeptide; and a master transcriptional regulator of T3SS components.
54. The microbial cell of any of claims 52-53, wherein the pathogenic microbial cell is selected from the group consisting of:

    Salmonella spp.; *Shigella* Spp; and*Yersinia* spp.
55. The microbial cell of claim 52-54, wherein the pathogenic microbial cell is selected from the group consisting of:

    Salmonella typhimurium SPI1and *Shigella felxneri* mxi-spa.

56. The microbial cell of any of claims 1-55, wherein cell has been:
    a. contacted with a mutagenic treatment; and
    b. selected for increased secretion.
57. The microbial cell of any of claims 1-56, wherein cell has been:
    a. contacted with a mutagenic treatment; and
    b. selected for increased secretion of the T3SS-compatible payload polypeptide.

58. The microbial cell of any of claims 1-57, wherein the cell further comprises a nucleic acid sequence encoding one or more polypeptides that increase adhesion to a target cell.
59. The microbial cell of claim 58, wherein the polypeptides that increase adhesion to the target cell comprise Tir and intimin.

60. The microbial cell of claim 59, wherein the polypeptide that increases adhesion to the target cell is selected from a group consisting of:

a bacterial adhesion; Afa1; AIDA; invasion; an antibody reagent specific for an extracellular epitope of a target cell polypeptide; and a single chain antibody specific for an extracellular epitope of a target cell polypeptide.

61. A method of introducing a polypeptide into a target tissue or organism, the method comprising contacting the target tissue or organism with a microbial cell of any of claims 1-60.
62. A method of reducing inflammation in a subject, the method comprising administering an microbial cell of any of claims 29-30 or 40-41 to a subject in need thereof.
63. The method of claim 62, wherein the inflammation is inflammation of the gastrointestinal tract.
64. The method of any of claims 62-63, wherein the subject is in need of treatment for a condition selected from the group consisting of:

asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis.

65. The method of claim 64, wherein the subject is a subject in need of treatment for inflammatory bowel disease.
66. The method of any of claims 62-65, wherein the microbial cell is administered orally.
67. A method of treating cancer in a subject, the method comprising administering an microbial cell of any of claims 30-39 to a subject in need thereof.
68. The method of claim 67, wherein the microbial cell is administered systemically.
69. The method of claim 67, wherein the microbial cell is administered intratumorally.
70. The method of claim 67, wherein the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally.
71. The method of any of claims 67-70, wheiren the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).
72. A method of treating an intestinal infection in a subject, the method comprising administering a microbial cell of any of claims 42-43 to a subject in need thereof.
73. The method of claim 72, wherein the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin.

74. The method of claim 72, wherein the intestinal infection is a *C. difficile* infection and/or the subject has *C. difficile* colitis and the toxin is TcdA and/or TcdB.
75. The method of claim 72, wherein the intestinal infection is cholera and the toxin is cholera toxin.
76. The method of claim 72, wherein the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin.
77. The method of claim 72, wherein the intestinal infection is botulism and the toxin is botulinum toxin.
78. The method of any of claims 72-77, wherein the microbial cell is administered orally.
79. The method of any of claims 61-78, wherein secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master transcriptional regulator.
80. The method of claim 79, wherein the compound is arabinose.

81. A method for delivering a polypeptide into a) the extracellular milieu of a subject's gastrointestinal tract, b) the lumen of a tumor, or c) the extracellular milieu of a subject's tumor , the method comprising contacting administering a microbial cell of any of claims 1-60 to the subject.

82. A kit comprising the microbial cell of any of claims 1-60.

83. The use of a microbial cell of any of claims 29-30 or 40-41 to reduce inflammation in a subject in need thereof.
84. The use of claim 83, wherein the inflammation is inflammation of the gastrointestinal tract.
85. The use of any of claims 83-84, wherein the subject is in need of treatment for a condition selected from the group consisting of:

    asthma; inflammatory bowel disease; Crohn's disease; obesity; and ulcerative colitis.
86. The use of claim 85, wherein the subject is a subject in need of treatment for inflammatory bowel disease.
87. The use of any of claims 83-86, wherein the microbial cell is administered orally.

88. The use of an microbial cell of any of claims 30-39 treat cancer in a subject in need thereof.
89. The use of claim 88, wherein the microbial cell is administered systemically.
90. The use of claim 88, wherein the microbial cell is administered intratumorally.
91. The use of claim 88, wherein the cancer is a cancer of the gastrointestinal tract and the microbial cell is administered orally.
92. The use of any of claims 88-91, wheiren the microbial cell is engineered from *E. coli* NISSLE 1917 (EcN).
93. The use of a microbial cell of any of claims 42-4339 to treat an intestinal infection in a subject in need thereof.
94. The use of claim 93, wherein the intestinal infection is EHEC and/or the subject has hemolytic uremic syndeom and the toxin is shiga toxin.
95. The use of claim 93, wherein the intestinal infection is a *C. difficile* infection and/or the subject has *C. difficile* colitis and the toxin is TcdA and/or TcdB.
96. The use of claim 93, wherein the intestinal infection is cholera and the toxin is cholera toxin.
97. The use of claim 93, wherein the intestinal infection is gastrointestinal anthrax and the toxin is anthrax toxin.
98. The use of claim 93, wherein the intestinal infection is botulism and the toxin is botulinum toxin.
99. The use of any of claims 93-97, wherein the microbial cell is administered orally.
100. The use of any of claims 83-99, wherein secretion of the T3SS-compatible payload polypeptide is induced by further administering the subject a compound to induce expression of the T3SS-compatible payload polypeptide and/or the T3SS master transcriptional regulator.
101. The use of claim100, wherein the compound is arabinose.

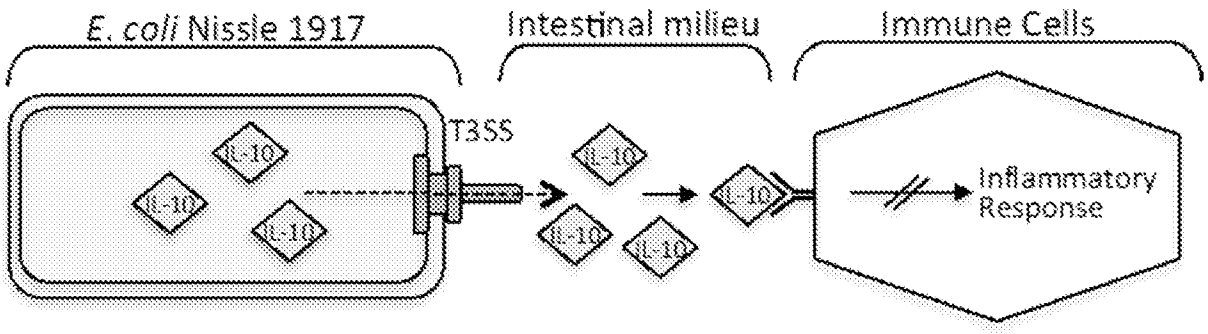


Fig. 1

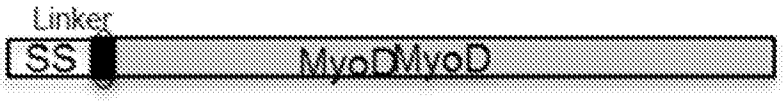


Fig. 2A

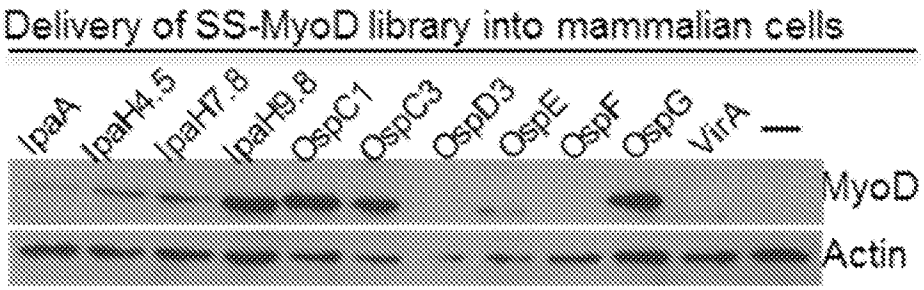


Fig. 2B

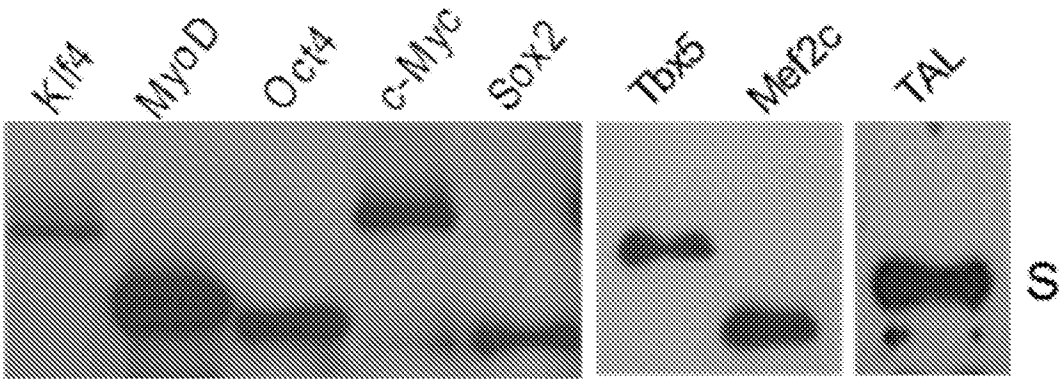


Fig. 3

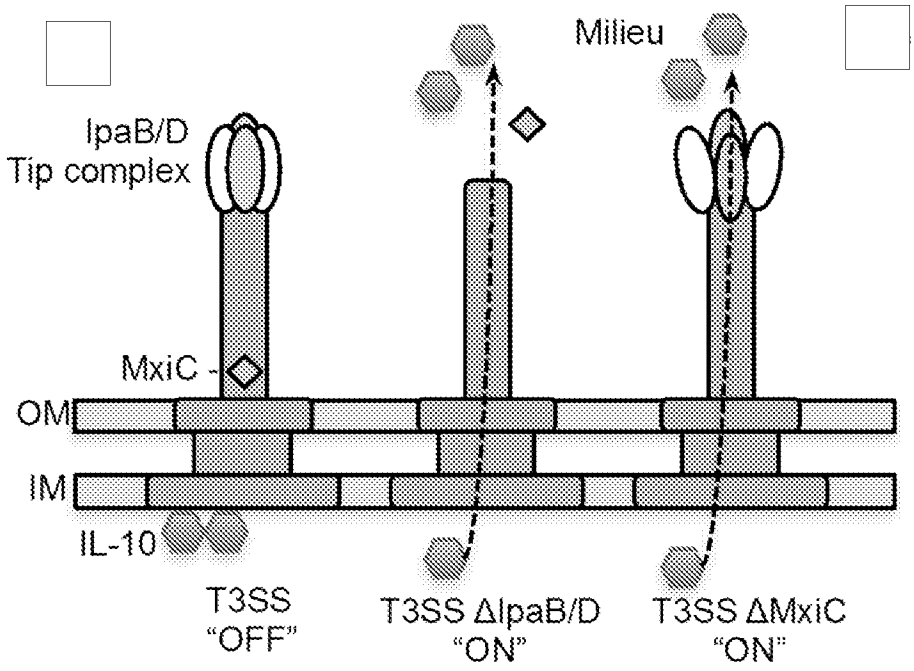


Fig. 4A

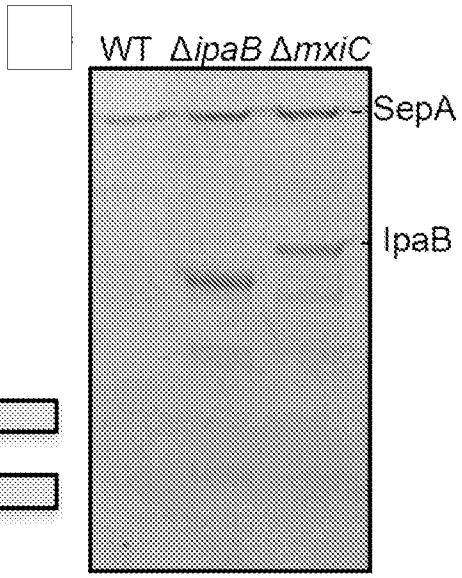
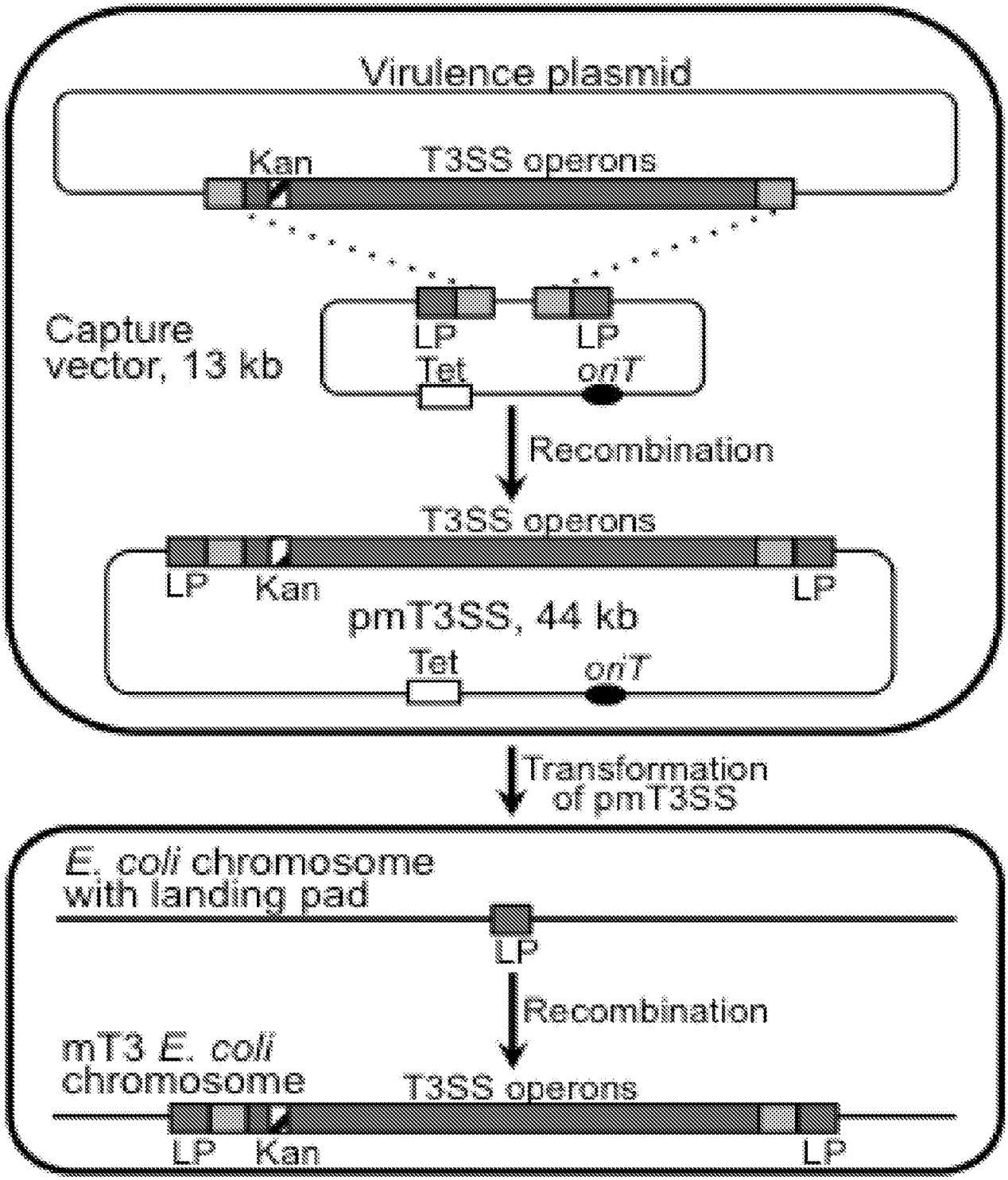


Fig. 4B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Kan
T3SS operons
Capture vector, 13 kb
LP
Tet
LP
oriT
Recombination
T3SS operons
LP
Kan
LP
pmT3SS, 44 kb
Tet
oriT
Transformation of pmT3SS
E. coli chromosome with landing pad
LP
Recombination
mT3 E. coli chromosome
T3SS operons
LP
Kan
LP

Fig. 5

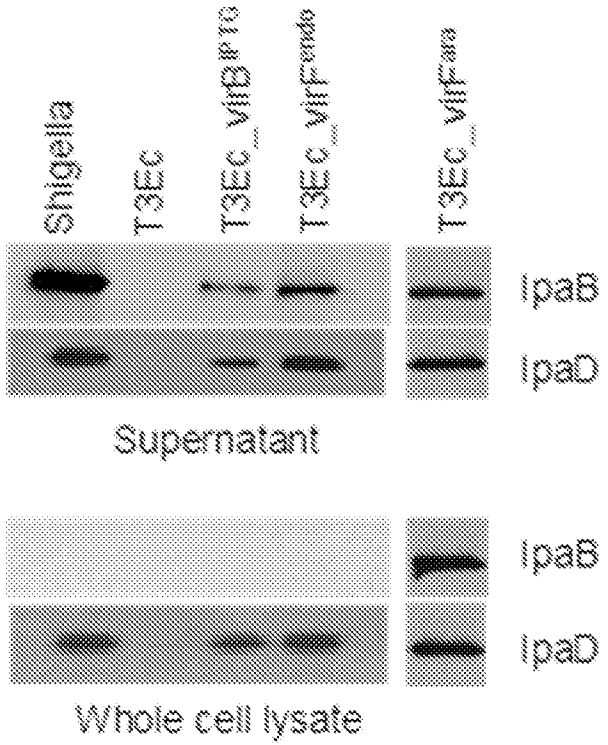
Shigella
T3Ec
T3Ec_VirB
T3Ec_VirF
T3Ec_VirF
IpaB
IpaD
Supernatant
IpaB
IpaD
Whole cell lysate

Fig. 6

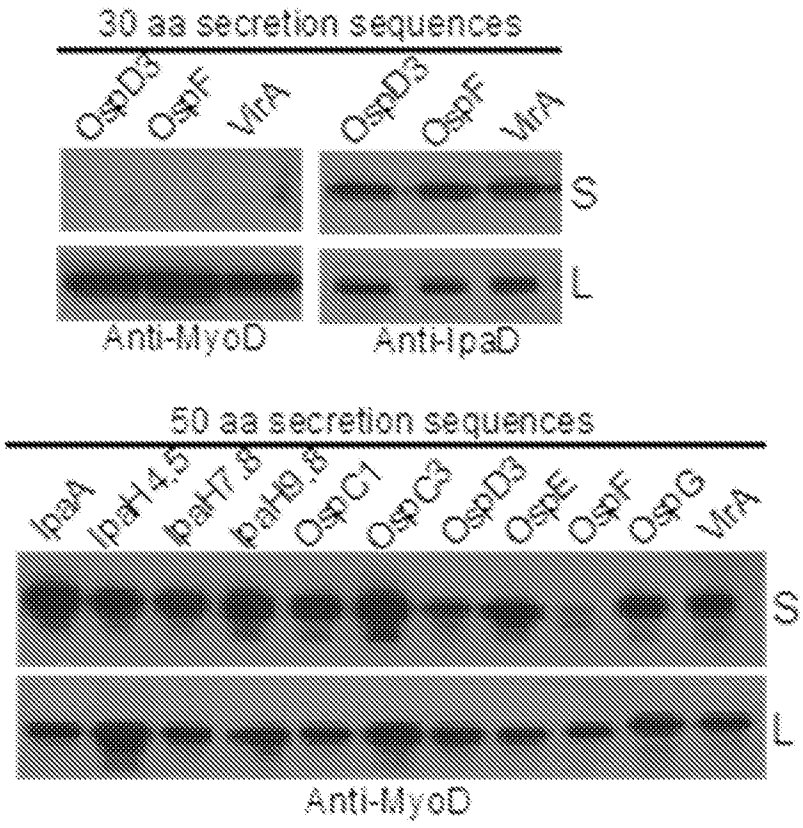
30 aa secretion sequences
OspD3
OspF
VirA
OspD3
OspF
VirA
S
L
Anti-MyoD
Anti-IpaD
50 aa secretion sequences
IpaA
IpaH4.5
IpaH7.8
IpaH9.8
OspC1
OspC3
OspD3
OspE
OspF
OspG
VirA
S
L
Anti-MyoD

Fig.7

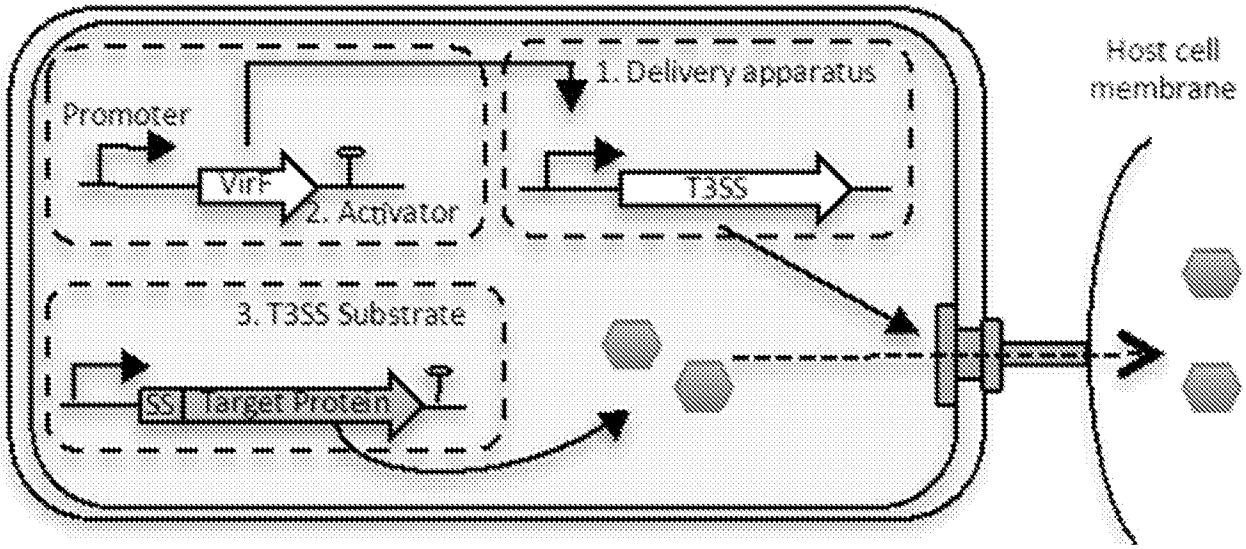

Fig. 8

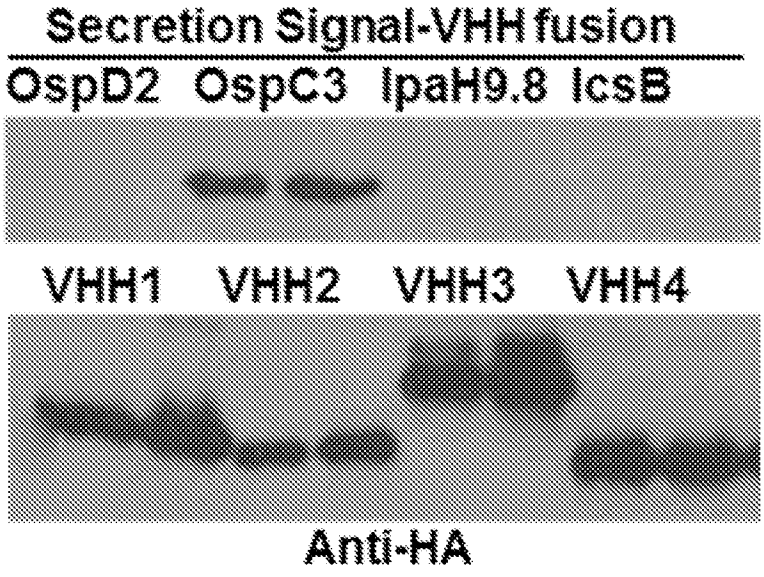

Fig. 9

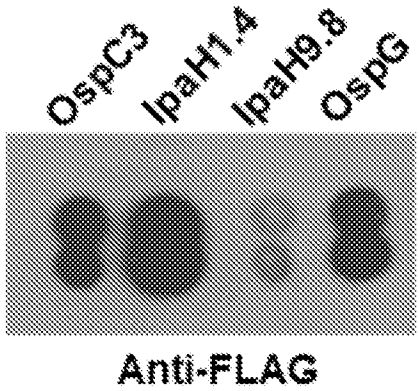

Fig. 10

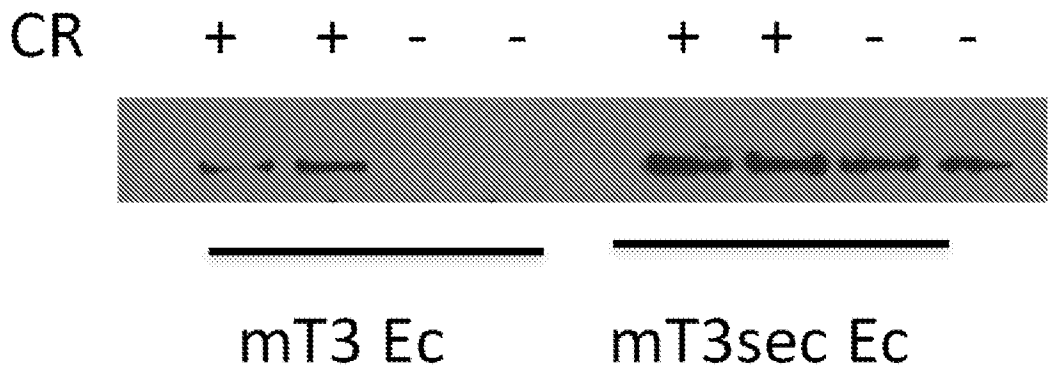
CR
+ + - - + + - -
mT3 Ec
mT3sec Ec

Fig. 11

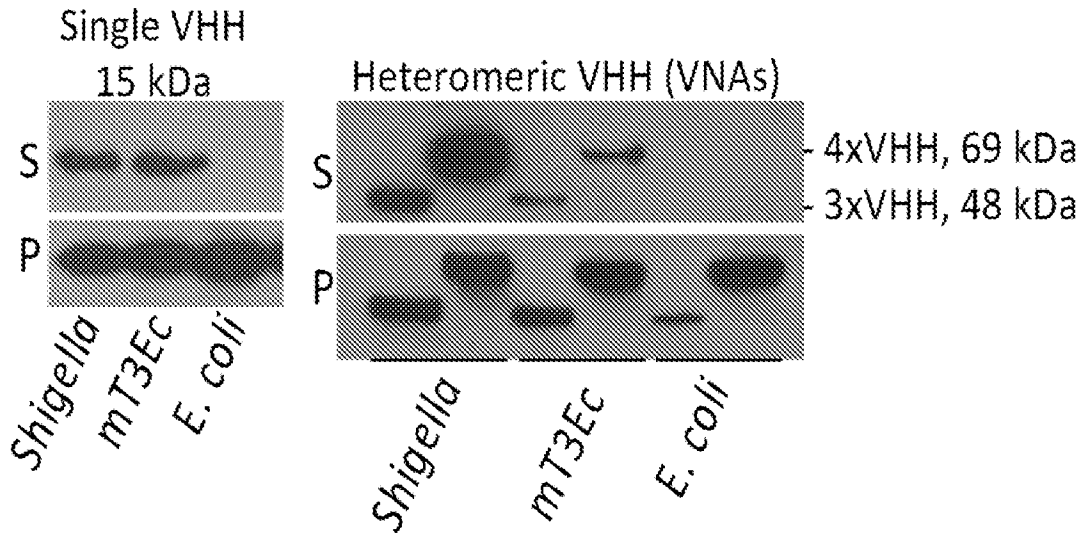
Single VHH
15 kDa
Heteromeric VHH (VNAs)
S
P
S
P
4xVHH, 69 kDa
3xVHH, 48 kDa
Shigella
mT3Ec
E. coli
Shigella
mT3Ec
E. coli

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/016997

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/74; C07K 14/195; C07K 14/25; C12N 1/36; C12N 15/31; C12N 15/74 (2017.01)

CPC - A61K 35/74; A61K 39/0283; A61K 2039/522; C07K 14/195; C07K 14/25; C07K 2319/035; C12N 1/36; C12N 15/74; G01N 33/50; G01N 33/56916 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 424/93.2; 424/200.1; 424/234.1; 435/71.1; 435/252.3; 435/252.1; 536/23.7 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2014/138324 A1 (THE GENERAL HOSPITAL CORPORATION) 12 September 2014 (12.09.2014) entire document	1 ----- 2-7
Y	VEENENDAAL et al. "The type III secretion system needle tip complex mediates host cell sensing and translocon insertion," Mol Microbiol, 22 January 2007 (22.01.2007), Vol. 63, No. 6, Pgs. 1719-1730. entire document	2-7
Y	MARTINEZ-ARGUDO et al. "The Shigella T3SS needle transmits a signal for MxiC release, which controls secretion of effectors," Mol Microbiol, 12 October 2010 (12.10.2010), Vol. 78, No. 6, Pgs. 1365-1378. entire document	4, 5
A	BOTTEAUX et al. "MxiC is secreted by and controls the substrate specificity of the Shigella flexneri type III secretion apparatus," Mol Microbiol, 10 November 2008 (10.11.2008), Vol. 71, No. 2, Pgs. 449-460. entire document	1-7
A	DEANE et al. "Molecular model of a type III secretion system needle: Implications for host-cell sensing," Proc Natl Acad Sci USA, 03 August 2006 (03.08.2006), Vol. 103, No. 33, Pgs. 12529-12533. entire document	1-7
A	KENJALE et al. "The needle component of the type III secreton of Shigella regulates the activity of the secretion apparatus," J Biol Chem, 14 October 2005 (14.10.2005), Vol. 280, No. 52, Pgs. 42929-42937. entire document	1-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2017	09 MAY 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/016997

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-101
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.