(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 December 2010 (16.12.2010)

PCT

(10) International Publication Number
WO 2010/144794 A1

(51) International Patent Classification:
C07K 14/32 (2006.01) *C12N 15/62* (2006.01)

(21) International Application Number:
PCT/US2010/038305

(22) International Filing Date:
11 June 2010 (11.06.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/186,419 12 June 2009 (12.06.2009) US

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

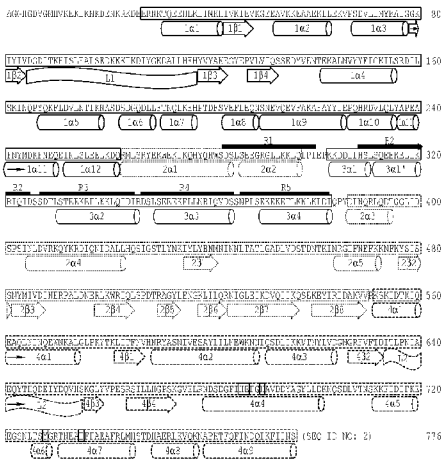
Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) **Title**: BACULOVIRUS-EXPRESSED FUSION POLYPEPTIDE VACCINES WITH ENHANCED IMMUNOGENICITY AND USES THEREOF

FIG. 1



(57) **Abstract**: The present invention is generally related to a method of baculovirus expression of a LFn-fusion protein for use in delivering antigens across the membrane of a cell to elicit a strong response of cell-mediated immunity (CMI) to the antigen portion of the LFn-fusion protein. One aspect of the present invention relates to the methods of use of a baculovirus expression system to produce N-glycosylated forms of LFn and LFn fusion polypeptides, which are more effective than non-glycosylated forms of the fusion proteins for delivering antigens across the membrane of a eukaryotic cell to elicit a strong CMI. Another aspect relates to methods and compositions to elicit a CMI response in a cell to a target antigen, comprising contacting the cell with a target antigen fused to a LFn polypeptide, wherein the target antigen and/or the LFn polypeptide are N-glycosylated.

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BACULOVIRUS-EXPRESSED FUSION POLYPEPTIDE VACCINES WITH ENHANCED IMMUNOGENICITY AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present application is generally directed to methods and compositions for delivering an exogenous protein to the cytosol of a cell, and methods for production of the compositions thereof.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This Application claims benefit under 35 U.S.C. §119(e) to U.S. Provisional Application 61/186,419, filed, June 12, 2009 the entire contents of which is incorporated herein by reference.

BACKGROUND OF INVENTION

[0003] Vaccination is the administration of an antigenic material (the vaccine) to produce immunity to a disease. Vaccines can prevent or ameliorate the effects of infection by a pathogen. Vaccination is considered to be the most effective and cost-effective method of preventing infectious diseases. The material administered as an immunogen can either be live but weakened or attenuated forms of pathogens (bacteria or viruses), killed or inactivated forms of these pathogens, or purified material such as proteins. The immunogen stimulates a response in an animal against the respective pathogen following the initial administration and also in future encounters with the pathogen, thus providing protection against infection by the pathogen in future exposures. Smallpox was the first disease people tried to prevent by purposely inoculating themselves with other types of infections. For example, the cowpox vaccine was used as an immunization for smallpox in humans by the British physician Edward Jenner in 1769.

[0004] However, not all vaccines and the immunogens in them are equally effective in stimulating an effective immune response. For example, poor immunogenicity of vaccines available against tuberculosis (TB), *Streptococcal pneumonia* (SP), measles virus Edmoston-Zagreb strain (EZMV), meningococci, hemagglutinin (HA) of influenza viruses, and hepatitis B virus have been reported. Moreover, some vaccines require an extended period of vaccination regime before immunity is successfully induced. For example, the primary basic vaccination against *Bacillus anthracis* requires six doses, three subcutaneous injections in the deltoid at zero, two, and four weeks, and three vaccinations at six, twelve, and eighteen months followed by annual boosters. For prolonged protection, annual boosters are required.

[0005] One reason accounting for the poor immunogenicity of some vaccines is the inability of some immunogens to enter the cytosol and the major histocompatibility complex pathway in order to stimulate a cell-mediated immune response. A number of bacterial toxins contain domains that share the ability to gain access to the host cell cytosol, where they can exert their effects. Although each toxin may differ in the mechanism or route by which it gains entry to the cytosol, the overall effect is that of a "molecular syringe" that is able to inject the toxic protein into the cell. Several bacterial toxins, including diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE), pertussis toxin, and the pertussis adenylate cyclase have been used in attempts to deliver peptide epitopes to the cell cytosol as internal or amino-terminal fusions. These systems are restricted in their use as potential vaccines because their capacity to deliver larger protein antigens is limited and many individuals have already been immunized against the carrier toxin.

[0006] Although peptides are able to stimulate a cellular immune response, whole protein antigens may be better suited for use in an effective vaccine for two reasons. First, the epitope that is essential for protection in one genetic background may prove to be irrelevant in a different genetic background. Therefore, it is beneficial for a broadly applied T cell vaccine to use the full-length protein from which the various relevant epitopes are derived. Second, peptides recognized by cytotoxic T lymphocytes are processed from the whole protein by specialized degradative

machinery, including the proteasome complex. In certain instances, the processing of the relevant peptide epitopes is dependent on the flanking amino acid sequences. However, flanking residues are not always important for proper processing. Because it currently is not possible to accurately predict which epitopes are dependent on their context for proper processing, it is important to deliver the entire antigen to the cell cytosol for optimal processing and presentation. Therefore, there is a need for new vaccines/ immunogens that are more immunogenic, e. g. immunogens that consist of the whole polypeptide or larger portions thereof and/or novel strategies for introducing the vaccines/ immunogen into cells to elicit an immune response.

SUMMARY OF THE INVENTION

[0007] The inventors have discovered that baculovirus expressed *Bacillus anthracis* lethal factor N-terminal (LFn) fusion protein can be delivered across the membrane of a cell and elicits a strong response of cell-mediated immunity (CMI) to the antigen portion of the LFn-fusion protein. The transmembrane transport is in the absence of the *B. anthracis* protective antigen and the stimulated immune response is greater than that elicited by just the antigen that is not fused to the LFn polypeptide.

[0008] The inventors have also discovered, surprisingly, that N-glycosylated forms of LFn and LFn fusion polypeptides are more effective than non-glycosylated forms of the fusion proteins. This is surprising in that *B. anthracis* lethal factor is, in its native state, a bacterially expressed protein that is not N-glycosylated.

[0009] Accordingly, the present invention provides a fusion polypeptide comprising a *B. anthracis* lethal factor N-terminal (LFn) polypeptide, or conservative substitution variant thereof, that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated. In some embodiments, it is the LFn polypeptide of the fusion polypeptide that is N-glycosylated.

[0010] In one embodiment, the LFn polypeptide of the fusion polypeptide comprises at least the 60 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[0011] In another embodiment, the LFn polypeptide of the fusion polypeptide comprises at least the 104 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[0012] In yet another embodiment, the LFn polypeptide of the fusion polypeptide comprises the amino acid sequence corresponding to SEQ. ID. No. 5, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists of SEQ. ID. No. 5, or a conservative substitution variant thereof.

[0013] In one embodiment, the LFn polypeptide of the fusion polypeptide does not bind *B. anthracis* protective antigen protein.

[0014] In one embodiment, the LFn polypeptide of the fusion polypeptide substantially lacks the amino acids 1-33 of SEQ. ID. No. 3.

[0015] In one embodiment, the target antigen polypeptide of the fusion polypeptide is an intracellular pathogen target antigen polypeptide. The target antigen can be from an antigen from a prokaryotic pathogen, a viral pathogen in which the virus naturally infects mammalian host cells, or a parasitic pathogen.

[0016] Examples of prokaryotic pathogens are: *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella species, Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Clostridium botulinum, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum and Klebsiella pneumoniae, Pseudomonas aeruginosa, Cryptosporidium parvum, Streptococcus pneumoniae, Bordetella pertussis,* and *Neisseria meningitides.*

[0017] Some examples of viral pathogens are: Herpes simplex virus type-1, Herpes simplex virus type-2, HBV, Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus, Human herpes virus 6, Human herpes virus 7, Human

herpes virus 8, Variola virus, Vesicular stomatitis virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, poliovirus, Rhinovirus, Coronavirus, Influenza virus A, Influenza virus B. Measles virus, Polyomavirus, Human Papilomavirus, Respiratory syncytial virus, Adenovirus, Coxsackie virus, Dengue virus, Mumps virus, Poliovirus, Rabies virus, Rous sarcoma virus, Yellow fever virus, Ebola virus, Marburg virus, Lassa fever virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, St. Louis Encephalitis virus, Murray Valley fever virus, West Nile virus, Rift Valley fever virus, Rotavirus A, Rotavirus B. Rotavirus C, Sindbis virus, Rabies virus, Human T-cell Leukemia virus type-l, Hantavirus, Rubella virus and Simian Immunodeficiency virus.

[0018] Some examples of parasitic pathogen are: *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella species, Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum and Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Bordetella pertussis, Neisseria meningitides, Leishmania donovanii, Plasmodium species, Pneumocystis carinii,* and *Trypanosoma* species.

[0019] In a particular embodiment, the pathogen is *M. tuberculosis.* In another particular embodiment, the target antigen polypeptide of the fusion polypeptide is a TB-specific antigen. In a embodiment, the target antigen polypeptide of the fusion polypeptide is a TB1 (CFP-10) polypeptide comprising SEQ ID NO: 7 or a fragment thereof. In another embodiment, the target antigen polypeptide of the fusion polypeptide is a TB2 (ESAT-6) polypeptide comprising SEQ ID NO: 6 or a fragment thereof.

[0020] In one embodiment, target antigen polypeptide or a fragment thereof of the fusion polypeptide is folded in its native conformation.

[0021] In another embodiment, target antigen polypeptide or a fragment thereof of the fusion polypeptide is part of a multi-molecular polypeptide complex.

[0022] In yet another embodiment, target antigen polypeptide or a fragment thereof of the fusion polypeptide is a subunit polypeptide of a multi-molecular polypeptide target antigen.

[0023] In one embodiment, the fusion polypeptide is expressed and purified from a protein expression system using host cells selected from the group consisting of mammalian cells, insect cells, yeast cells, and plant cells. In another embodiment, the fusion polypeptide is isolated from an insect cell. In yet another embodiment, the fusion polypeptide is produced by expression from a recombinant baculovirus vector in an insect cell.

[0024] In one embodiment, the invention provides an isolated polynucleotide encoding the fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide.

[0025] In one embodiment, the invention provides a recombinant vector comprising the polynucleotide encoding a fusion polypeptide described herein. In some embodiments, the recombinant vector is an expression vector that is compatible with a protein expression system using host cells selected from the group consisting of: mammalian cells; insect cells; yeast cells; and plant cells. In one embodiment, the recombinant vector is a viral vector, such as a recombinant baculovirus vector.

[0026] In one embodiment, the invention provides a host cell comprising the expression vector comprising the polynucleotide encoding a fusion polypeptide described herein. In one embodiment, the host cell is an insect cell and the expression vector is a baculovirus vector.

[0027] In one embodiment, the invention provides a method of producing a N-glycosylated fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, the method comprising expressing the fusion polypeptide in a eukaryotic cell.

[0028] In one embodiment, the method producing a N-glycosylated fusion polypeptide comprises infecting lepidopteran insect cells with a recombinant baculovirus vector comprising a polynucleotide encoding the fusion polypeptide, and culturing the insect cells to produce the fusion polypeptide. In one embodiment, the lepidopteran insect cells are *Spodoptera frugiperda* cells.

[0029] In another embodiment, the invention provides a method of preparing a composition for increasing or detecting a cell-mediated immune response to a target antigen polypeptide, the method comprising expressing a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide in an insect cell. In one embodiment, the target antigen polypeptide of the expressed a fusion polypeptide disclosed herein is an intracellular pathogen target antigen polypeptide. In one embodiment, the fusion polypeptide fusion polypeptide is expressed from a recombinant baculovirus vector.

[0030] In one embodiment, the invention provides a method of introducing an intracellular pathogen target antigen polypeptide to a mammalian cell, the method comprising contacting a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, with a mammalian cell. In one embodiment, the cell is a mammalian cell *in vivo* and the method comprises administering a fusion polypeptide to the mammal.

[0031] In one embodiment, the invention provides a method of increasing a cell-mediated immune response to a target antigen polypeptide, the method comprising administering to a mammal, a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated. In some embodiments, the target antigen of the fusion polypeptide is an intracellular pathogen target antigen polypeptide or fragment, and the fusion polypeptide is isolated from an insect cell that has been infected with a recombinant baculovirus vector.

[0032] In one embodiment, the fusion polypeptide is expressed and purified from insect cells.

[0033] In one embodiment, the invention provides a composition comprising a fusion polypeptide *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated and an isolated mammalian cell. In one embodiment, the mammalian cell is an antigen presenting cell which expresses MHC class II molecules.

[0034] In one embodiment, the invention provides a vaccine composition comprising a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated and pharmaceutically acceptable carrier. The fusion polypeptide is expressed and purified from insect cells and the LFn polypeptide of the fusion polypeptide is N-glycosylated.

[0035] In one embodiment, the invention provides a kit for vaccination or conducting CMI assays or screening comprising a fusion polypeptide a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated and packaging materials thereof. In one embodiment, the kit comprises a plurality of fusion polypeptides, wherein individual members of the plurality comprise different fused antigen polypeptides, wherein the members of the plurality each comprise a fused different portion of the same target antigen polypeptide.

BRIEF DESCRIPTION OF THE FIGURES

[0036] Figure 1 shows the domains and secondary structure of the *Bacillus anthracis* lethal factor polypeptide based on the X-ray crystallography data from Andrew D. Pannifer et. al., (2001). Nature 414, 229-233. The N-terminal 1-33

amino acid residues are not shown. The consecutive varying gray-toned regions represent the domains I-IV from N-terminus to C-terminus of the polypeptide.

DETAILED DESCRIPTION OF THE INVENTION

Definitions of terms

[0037] As used herein, the term “comprising” means that other elements can also be present in addition to the defined elements presented. The use of “comprising” indicates inclusion rather than limitation.

[0038] 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.

[0039] 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 of the invention.

[0040] As used herein, the term "fusion polypeptide" or “fusion protein” means a protein created by joining two polypeptide coding sequences together. The fusion polypeptides of this invention are fusion polypeptides formed by joining a coding sequence of a LF polypeptide or fragment or mutant thereof with a coding sequence of a second polypeptide to form a fusion or chimeric coding sequence such that they constitute a single open-reading frame. The fusion coding sequence, when transcribed and translated, expresses a fusion polypeptide. In other words, a "fusion polypeptide" or “fusion protein” is a recombinant protein of two or more proteins which are joined by a peptide bond.

[0041] As used herein, the term "promotes transmembrane delivery" refers to the ability of a first polypeptide to facilitate a second protein to traverse the membrane of an intact, living cell. As used in the inventions described herein, the second protein is physically linked to the first polypeptide, e. g. as a fusion polypeptide.

[0042] As used herein, the term "cytosol" refers to the interior of an intact cell. The "cytosol" comprises the cytoplasm and the organelles inside a cell.

[0043] As used herein, the term " an intact cell " refers to a living cell with an unbroken, uncompromised plasma membrane, and that has a differential membrane potential across the membrane, with the inside of the cell being negative with respect to the outside of the cell.

[0044] As used herein, the term “N-glycosylated” or “N-glycosylation” refers to the covalent attachment of a sugar moiety to asparagine residues in a polypeptide. Sugar moieties can include but are not limited to glucose, mannose, and N-acetylglucosamine. Modifications of the glycans are also included, e. g. siaylation.

[0045] As used herein, the term “N-glycosylated fusion polypeptide” refers to a fusion polypeptide, as defined herein, that has at least one sugar moiety covalently attached to an asparagine residue. For example, Asn-62, Asn-212, and Asn-286 can be glycosylated in an N-glycosylated LFn fusion polypeptide.

[0046] As used herein, the term "substantially lacks amino acids 1-33" in the context of a fusion polypeptide described herein refers to a fusion polypeptide that lacks the signal peptide aa1-33 and the signal peptide activity.

[0047] As used herein, the term "intracellular pathogen" refers to a pathogen or pathogen-derived components thereof that can reside inside an intact cell.

[0048] As used herein, the term “pathogen” refers to an organism or molecule that causes a disease or disorder in a subject. For example, pathogens include but are not limited to viruses, fungi, bacteria, parasites and other infectious organisms or molecules therefrom, as well as taxonomically related macroscopic organisms within the categories algae, fungi, yeast and protozoa or the like.

[0049] As used herein, the term "prokaryotic pathogen" refers to a bacterial pathogen.

[0050] As used herein, the term "viral pathogen" refers to a virus that causes illness or disease, such as HIV.

[0051] As used herein, the term "parasitic pathogen" refers to a microorganism that is parasitic, residing for an extended period inside a host cell or host organism, that gains benefits from the host and at the same time causes illness or disease. A parasitic pathogen can be a bacteria, viruses, fungi, and protists.

[0052] An antigen presenting cell is a cell that express the Major histocompatibility complex (MHC) molecules and can displays foreign antigen complexed with MHC on its surface. Examples of antigen displaying cells are dendritic cells, macrophages, B cells, fibroblasts (skin), thymic epithelial cells, thyroid epithelial cells, glial cells (brain), pancreatic beta cells, and vascular endothelial cells.

[0053] The term "lethal factor" or "LF" as used herein refers generally to a non-PA polypeptide of the bipartite *B. anthracis* exotoxin. Wild-type, intact *B. anthracis* LF polypeptide has the amino acid sequence set out in Genbank Accession Number M29081 (Gene ID No: 143143), which corresponds to SEQ ID NO: 1. SEQ ID NO: 1 corresponds to LF with a signal peptide located at residues 1 to 33 at its N-terminus. Stated another way, immature wild-type LF corresponds to an 809 amino acid protein, which includes a 33 amino acid signal peptide at the N-terminus. The amino acid sequence of immature wild-type LF (SEQ ID NO: 1) with the signal peptide highlighted in bold is as follows:

[0054] **MNIKKEFIKVISMSCLVTAITLSGPVFIPLVQG**AGGHGDVGMHVKEKEKNKDENKRKDEERNKT
QEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIK
DIYGKDALLHEHYVYAKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKN
ASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEI
NLSLEELKDQRMLSRYEKWEKIKQHYQHWSDSLSEEGRGLLKKLQIPIEPKKDDIIHSLSQEEKELLKRIQIDSS
DFLSTEEKEFLKKLQIDIRDSLSEEEKELLNRIQVDSSNPLSEKEKEFLKKLKLDIQPYDINQRLQDTGGLIDSPSI
NLDVRKQYKRDIQNIDALLHQSIGSTLYNKIYLYENMNINNLTATLGADLVDSTDNTKINRGIFNEFKKNFKYS
ISSNYMIVDINERPALDNERLKWRIQLSPDTRAGYLENGKLILQRNIGLEIKDVQIIKQSEKEYIRIDAKVVPKSK
IDTKIQEAQLNINQEWNKALGLPKYTKLITFNVHNRYASNIVESAYLILNEWKNNIQSDLIKKVTNYLVDGNG
RFVFTDITLPNIAEQYTHQDEIYEQVHSKGLYVPESRSILLHGPSKGVELRNDSEGFIHEFGHAVDDYAGYLLD
KNQSDLVTNSKKFIDIFKEEGSNLTSYGRTNEAEFFAEAFRLMHSTDHAERLKVQKNAPKTFQFINDQIKFIINS
(SEQ ID NO: 1)

[0055] Cleavage of the immature LF protein results in a mature wild-type LF polypeptide of 776 amino acids in length. The 776 amino acid polypeptide sequence of mature wild-type LF polypeptide (i.e. lacking the N-terminal signal peptide) corresponds to SEQ ID NO: 2, as follows:

[0056] AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLE
KVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVYAKEGYEPVLVIQSSEDYV
ENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEV
QEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLSLEELKDQRMLSRYEKWEKIKQHYQHWSDSL
SEEGRGLLKKLQIPIEPKKDDIIHSLSQEEKELLKRIQIDSSDFLSTEEKEFLKKLQIDIRDSLSEEEKELLNRIQVD
SSNPLSEKEKEFLKKLKLDIQPYDINQRLQDTGGLIDSPSINLDVRKQYKRDIQNIDALLHQSIGSTLYNKIYLYE
NMNINNLTATLGADLVDSTDNTKINRGIFNEFKKNFKYSISSNYMIVDINERPALDNERLKWRIQLSPDTRAGY
LENGKLILQRNIGLEIKDVQIIKQSEKEYIRIDAKVVPKSKIDTKIQEAQLNINQEWNKALGLPKYTKLITFNVHN
RYASNIVESAYLILNEWKNNIQSDLIKKVTNYLVDGNGRFVFTDITLPNIAEQYTHQDEIYEQVHSKGLYVPES
RSILLHGPSKGVELRNDSEGFIHEFGHAVDDYAGYLLDKNQSDLVTNSKKFIDIFKEEGSNLTSYGRTNEAEFF
AEAFRLMHSTDHAERLKVQKNAPKTFQFINDQIKFIINS (SEQ ID NO: 2).

[0057] The term "LF polypeptide" applies not only to full length, wild-type LF (with or without the signal sequence), but also to fragments thereof that mediate intracellular delivery of fused or physically associated

polypeptides to an intact cell, such as, an immune cell. Also included in the term "LF polypeptide" are conservative substitution variants of LF, including conservative substitution variants that mediate such intracellular delivery.

[0058] The term "LFn polypeptide" refers to an N-terminal fragment of *B. anthracis* LF that does not display zinc metalloproteinase activity and does not inactivate mitogen-activated kinase activity, yet does mediate intracellular or transmembrane delivery of fused polypeptides. LFn polypeptides as defined and described herein are preferred. In one aspect, "LFn polypeptide" includes SEQ ID NO: 3, which corresponds to a 288 amino acid immature LFn protein; this LFn protein is "immature" in that it includes a signal peptide located at residues 1 to 33 of the N-terminus. Stated another way, immature LFn corresponds to a 288 amino acid protein, which includes a 33 amino acid signal peptide at the N-terminus. Cleavage of the immature LFn protein of SEQ ID NO: 3 results in a mature LFn polypeptide of 255 amino acids in length. It should be emphasized that, for the purposes of the methods and compositions described herein, the LF and/or LFn polypeptides can either include or lack the signal peptide – that is, the presence or absence of the signal peptide is not expected to influence the activity of LF polypeptides as transmembrane transport facilitators in the methods described herein. The amino acid sequence of immature LFn (SEQ ID NO: 3) with the signal peptide highlighted in bold is as follows:

[0059] **MNIKKEFIKVISMSCLVTAITLSGPVFIPLVQG**AGGHGDVGMHVKEKEKNKDENKRKDEERNKT QEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIK DIYGKDALLHEHYVYAKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKN ASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEI NLS (SEQ ID NO: 3)

[0060] The polypeptide sequence of a mature LFn polypeptide (which lacks the N-terminal signal peptide) is 255 amino acids in length and corresponds to SEQ ID NO: 4 is as follows:

[0061] AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLE KVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVYAKEGYEPVLVIQSSEDYV ENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEV QEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLS (SEQ ID NO: 4)

[0062] The term "functional fragment" as used in the context of a "functional fragment of LFn" refers to a fragment of an LFn polypeptide that mediates, effects or facilitates transport of an antigen across an intact, alive immune cell's membrane. One example of such a fragment of an LFn polypeptide is a 104 amino acid C-terminal fragment of LFn corresponding to SEQ ID NO: 5 as follows (this sequence is also disclosed as SEQ ID NO: 3 in U.S. Patent Application 10/473190, which is incorporated herein by reference):

[0063] GKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAK AFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLS (SEQ ID NO: 5)

[0064] The term "LFn polypeptide" as used herein encompasses each of the "immature" LFn and "mature" LFn nolecules described herein, as well as fragments, variants (including conservative substitution variants) and derivatives thereof that mediate, effect or facilitate transport of a physically associated, e.g., fused, polypeptide across the membrane of an intact, living cell. Additional fragments of LFn polypeptides specifically contemplated for use in the methods, compositions and kits described herein include a fragment comprising, or optionally, consisting essentially of the C-terminal 60, 80, 90, 100 or 104 amino acids of SEQ ID NO: 3 or a conservative substitution variant thereof that mediates, effects or facilitates transfer of a physically associated, e.g., fused polypeptide across an intact membrane of a living cell.

[0065] The term "adjuvant" as used herein refers to any agent or entity which increases the antigenic response by a cell to a target antigen.

[0066] The terms "protective antigen" or "PA" are used interchangeably herein to refer to part of the *B. anthracis* exotoxin bipartite protein which binds to a mammalian cell's surface by cellular receptors. A "PA," as the term is used herein has its receptor binding site intact and functional. U. S. Patents 5,591,631 and 5,677,274 (incorporated by reference in their entirety) describe PA fusion proteins that target PA to particular cells, such as cancer cells and HIV-infected cells, using as fusion partners ligands for receptors on the targeted cells.

[0067] A "fragment" of a target antigen as that term is used herein will be at least 15 amino acids in length, and can be, for example, at least 16, at least 17, at least 18, at least 19, at least 20 or at least 25 amino acids or greater. Thus, in instances where, for example, a panel of target antigen fragment-LF polypeptides are prepared, the target antigen fragments will be at least 15 amino acids in length.

[0068] The terms "Cytotoxic T Lymphocyte" or "CTL" refers to lymphocytes which induce apoptosis in targeted cells. CTLs form antigen-specific conjugates with target cells via interaction of TCRs with processed antigen (Ag) on target cell surfaces, resulting in apoptosis of the targeted cell. Apoptotic bodies are eliminated by macrophages. The term "CTL response" is used to refer to the primary immune response mediated by CTL cells.

[0069] The term "cell mediated immunity" or "CMI" as used herein refers to an immune response that does not involve antibodies or complement but rather involves the activation of, for example, macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes (T-cells), and the release of various cytokines in response to a target antigen. Stated another way, CMI refers to immune cells (such as T cells and lymphocytes) which bind to the surface of other cells that display a target antigen (such as antigen presenting cells (APS)) and trigger a response. The response may involve either other lymphocytes and/or any of the other white blood cells (leukocytes) and the release of cytokines. Cellular immunity protects the body by: (i) activating antigen-specific cytotoxic T-lymphocytes (CTLs) that are able to destroy body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells and cells with intracellular bacteria; (2) activating macrophages and NK cells, enabling them to destroy intracellular pathogens; and (3) stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses. Without wishing to be bound by theory and by way of background, the immune system was separated into two branches: humoral immunity, for which the protective function of immunization could be found in the humor (cell-free bodily fluid or serum) and cellular immunity, for which the protective function of immunization was associated with cells.

[0070] The term "immune cell" as used herein refers to any cell which can release a cytokine in response to a direct or indirect antigenic stimulation. Included in the term "immune cells" herein are lympocytes, including natural killer (NK) cells, T-cells (CD4+ and/or CD8+ cells), B-cells, macrophages and monocytes, Th cells; Th1 cells; Th2 cells; Tc cells; leukocytes; dendritic cells; macrophages; mast cells and monocytes and any other cell which is capable of producing a cytokine molecule in response to direct or indirect antigen stimulation. Typically, an immune cell is a lymphocyte, for example a T-cell lymphocyte.

[0071] The term "cytokine" as used herein is used interchangeably with the term "effector molecule," and refers to a molecule released from an immune cell in response to stimulation with an antigen. Examples of such cytokines include, but are not limited to: GM-CSF; IL-1α; IL-1β; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-8; IL-10; IL-12; IFN-α; IFN-β; IFN-γ; MIP-1α; MIP-1β; TGF-β; TNFα and TNFβ.

[0072] The term "complex" as used herein refers to a collection of two or more molecules, whereby they are connected spatially by means other than a covalent interaction; for example they can be connected by electrostatic interactions such as van der Waals forces etc.

[0073] The term "translocated into a cell" refers to the movement of a moiety, such as a target antigen, and optionally a fusion polypeptide described herein from a location outside a cell, across the plasma membrane to the inside of an intact, living cell.

[0074] The term "*in vivo*" refers to assays or processes that occur in an animal.

[0075] The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to humans; primates such as apes, monkeys, orangutans, and chimpanzees; canids such as dogs and wolves; felids such as cats, lions, and tigers; equids such as horses, donkeys, and zebras, food animals such as cows, pigs, and sheep; ungulates such as deer and giraffes; rodents such as mice, rats, hamsters and guinea pigs; and bears. In some embodiments, a mammal is a human.

[0076] The term "subject" as used herein refers to any animal in which it is useful to raise or detect a CMI response. The subject can be a mammal, for example a human, or can be a wild, domestic, commercial or companion animal. In some embodiments, the subject is an experimental animal or animal substitute as a disease model. The subject may be a subject in need of veterinary treatment, including treatment of companion animals such as dogs and cats, and domestic animals such as horses, ponies, donkeys, mules, llama, alpaca, pigs, cattle and sheep, or zoo animals such as primates, felids, canids, bovids, and ungulates, or livestock animals such as pigs, cattle and sheep.

[0077] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues linked by peptide bonds, and for the purposes of the claimed invention, have a minimum length of at least 15 amino acids. Oligopeptides, oligomers multimers, and the like, typically refer to longer chains of amino acids and are also composed of linearly arranged amino acids linked by peptide bonds, whether produced biologically, recombinantly, or synthetically and whether composed of naturally occurring or non-naturally occurring amino acids, are included within this definition. Both full-length proteins and fragments thereof greater than 15 amino acids are encompassed by the definition. The terms also include polypeptides that have co-translational (e.g., signal peptide cleavage) and post-translational modifications of the polypeptide, such as, for example, disulfide-bond formation, glycosylation, acetylation, phosphorylation, proteolytic cleavage (e.g., cleavage by furins or metalloproteases), and the like. Furthermore, as used herein, a "polypeptide" refers to a protein that includes modifications, such as deletions, additions, and substitutions (generally conservative in nature as would be known to a person in the art) to the native sequence, as long as the protein maintains the desired activity. These modifications can be deliberate, as through site-directed mutagenesis, or can be accidental, such as through mutations of hosts that produce the proteins, or errors due to PCR amplification or other recombinant DNA methods. For the methods, kits and compositions described herein, the term "peptide" refers to a sequence of peptide-bond linked amino acids containing at least two and less than 15 amino acids in length.

[0078] It will be appreciated that proteins or polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, can be modified in a given polypeptide, either by natural processes such as glycosylation and other post-translational modifications, or by chemical modification techniques which are well known in the art. Known modifications which can be present in polypeptides of the present invention include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a polynucleotide or polynucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formulation, gamma-carboxylation, glycation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic

processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0079] As used herein, the terms "homologous" or "homologues" are used interchangeably, and when used to describe a polynucleotide or polypeptide, indicate that two polynucleotides or polypeptides, or designated sequences thereof, when optimally aligned and compared, for example using BLAST, version 2.2.14 with default parameters for an alignment (see herein) are identical, with appropriate nucleotide insertions or deletions or amino-acid insertions or deletions, in at least 70% of the nucleotides, usually from about 75% to 99%, and more preferably at least about 98 to 99% of the nucleotides. For a polypeptide, there should be at least 50% of amino acid identity in the polypeptide. The term "homolog" or "homologous" as used herein also refers to homology with respect to structure and/or function. That is, a polypeptide that performs the same function as a given polypeptide in another species can be viewed as a homolog of that given polypeptide. Determination of homologs of genes or polypeptides can be easily ascertained by the skilled artisan.

[0080] As used herein, the term "heterologous" reference to nucleic acid sequences, proteins or polypeptides mean that these molecules are not naturally occurring in that cell. For example, the nucleic acid sequence coding for a fusion LFn-target antigen polypeptide described herein that is inserted into a cell, e. g. in the context of a protein expression vector, is a heterologous nucleic acid sequence.

[0081] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0082] Where necessary or desired, optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482 (1981), which is incorporated by reference herein), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443-53 (1970), which is incorporated by reference herein), by the search for similarity method of Pearson and Lipman (Proc. Natl. Acad. Sci. USA 85:2444-48 (1988), which is incorporated by reference herein), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection. (See generally Ausubel et al. (eds.), Current Protocols in Molecular Biology, 4th ed., John Wiley and Sons, New York (1999)).

[0083] One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show the percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (J. Mol. Evol. 25:351-60 (1987), which is incorporated by reference herein). The method used is similar to the method described by Higgins and Sharp (Comput. Appl. Biosci. 5:151-53 (1989), which is incorporated by reference herein). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a

reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

[0084] Another example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described by Altschul et al. (J. Mol. Biol. 215:403-410 (1990), which is incorporated by reference herein). (See also Zhang et al., Nucleic Acid Res. 26:3986-90 (1998); Altschul et al., Nucleic Acid Res. 25:3389-402 (1997), which are incorporated by reference herein). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information internet web site. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al. (1990), supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-9 (1992), which is incorporated by reference herein) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0085] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-77 (1993), which is incorporated by reference herein). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, an amino acid sequence is considered similar to a reference amino acid sequence if the smallest sum probability in a comparison of the test amino acid to the reference amino acid is less than about 0.1, more typically less than about 0.01, and most typically less than about 0.001.

[0086] The term "variant" as used herein refers to a polypeptide or nucleic acid that differs from the naturally occurring polypeptide or nucleic acid by one or more amino acid or nucleic acid deletions, additions, substitutions or side-chain modifications, yet retains one or more specific functions or biological activities of the naturally occurring molecule. Amino acid substitutions include alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as “conservative,” in which case an amino acid residue contained in a polypeptide is replaced with another naturally occurring amino acid of similar character either in relation to polarity, side chain functionality or size. Substitutions encompassed by variants as described herein may also be “non conservative,” in which an amino acid residue which is present in a peptide is substituted with an amino acid having different properties (e.g., substituting a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid. Also encompassed within the term “variant,” when used with reference to a polynucleotide or polypeptide, are variations in primary, secondary, or tertiary structure, as compared to a reference polynucleotide or polypeptide, respectively (e.g., as compared to a wild-type polynucleotide or polypeptide). A "variant" of an LFn polypeptide refers to a molecule substantially similar in structure and function to that of a polypeptide of SEQ. ID. NO: 3, where the function is the ability to mediate, effect or facilitate transport of an associated or fused polypeptide across a cell

membrane of a living cell from a subject. In some embodiments, a variant of SEQ. ID. NO: 3 or SEQ. ID. NO: 4 is a fragment of SEQ. ID. NO: 3 or 4 as disclosed herein, such as SEQ. ID. NO: 5.

[0087] The term "substantially similar," when used in reference to a variant of LFn or a functional derivative of LFn as compared to the LFn protein encoded by SEQ. ID. NO: 3 means that a particular subject sequence, for example, an LFn fragment or LFn variant or LFn derivative sequence, varies from the sequence of the LFn polypeptide encoded by SEQ. ID. NO: 3 by one or more substitutions, deletions, or additions relative to SEQ. ID. NO: 3, but retains at least 50% of the transmembrane transport facilitation activity, and preferably higher, e.g., at least 60%, 70%, 80%, 90% or more exhibited by the LFn protein of SEQ. ID. NO: 3. (It is acknowledged that LFn does not occur naturally – reference to a "native" or "natural" LFn sequence is intended to convey that the sequence is identical to the portion of naturally-occurring LF polypeptide designated as LFn herein.) In determining polynucleotide sequences, all subject polynucleotide sequences capable of encoding substantially similar amino acid sequences are considered to be substantially similar to a reference polynucleotide sequence, regardless of differences in codon sequence. A nucleotide sequence is "substantially similar" to a given LFn nucleic acid sequence if: (a) the nucleotide sequence is hybridizes to the coding regions of the native LFn sequence, or (b) the nucleotide sequence is capable of hybridization to nucleotide sequence of LFn encoded by SEQ. ID. NO: 1 under moderately stringent conditions and has biological activity similar to the native LFn protein; or (c) the nucleotide sequences are degenerate as a result of the genetic code relative to the nucleotide sequences defined in (a) or (b). Substantially similar proteins will typically be greater than about 80% similar to the corresponding sequence of the native protein.

[0088] Variants can include conservative or non-conservative amino acid changes, as described below. Polynucleotide changes can result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. Variants can also include insertions, deletions or substitutions of amino acids, including insertions and substitutions of amino acids and other molecules) that do not normally occur in the peptide sequence that is the basis of the variant, for example but not limited to insertion of ornithine which do not normally occur in human proteins. "Conservative amino acid substitutions" result from replacing one amino acid with another having similar structural and/or chemical properties. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (See also Creighton, Proteins, W. H. Freeman and Company (1984).)

[0089] The choice of conservative amino acids may be selected based on the location of the amino acid to be substituted in the peptide, for example if the amino acid is on the exterior of the peptide and exposed to solvents, or on the interior and not exposed to solvents. Selection of such conservative amino acid substitutions is within the skill of one of ordinary skill in the art and is described, for example by Dordo et al., J. Mol Biol, 1999, 217, 721-739 and Taylor et al., J. Theor. Biol. 119(1986);205-218 and S. French and B. Robson, J. Mol. Evol. 19(1983)171. Accordingly, one can select conservative amino acid substitutions suitable for amino acids on the exterior of a protein or peptide (i.e. amino acids exposed to a solvent). These substitutions include, but are not limited to the following: substitution of Y with F, T with S or K, P with A, E with D or Q, N with D or G, R with K, G with N or A, T with S or K, D with N or E, I with L or V, F with Y, S with T or A, R with K, G with N or A, K with R, A with S, K or P.

[0090] In alternative embodiments, one can also select conservative amino acid substitutions suitable for amino acids on the interior of a protein or peptide (i.e. the amino acids are not exposed to a solvent). For example, one can use the following conservative substitutions: where Y is substituted with F, T with A or S, I with L or V, W with Y, M

with L, N with D, G with A, T with A or S, D with N, I with L or V, F with Y or L, S with A or T and A with S, G, T or V. In some embodiments, LF polypeptides including non-conservative amino acid substitutions are also encompassed within the term "variants." A variant of an LFn polypeptide, for example a variant of SEQ ID NO: 3 or 4 is meant to refer to any molecule substantially similar in structure (i.e., having at least 50% homology as determined by BLASTp analysis using default parameters) and function (i.e., at least 50% as effective as a polypeptide of SEQ. ID. NO: 3 in transmembrane transport) to a molecule of SEQ. ID. NO: 3 or 4.

[0091] As used herein, the term "non-conservative" refers to substituting an amino acid residue for a different amino acid residue that has different chemical properties. Non-limiting examples of non-conservative substitutions include aspartic acid (D) being replaced with glycine (G); asparagine (N) being replaced with lysine (K); and alanine (A) being replaced with arginine (R).

[0092] The term "derivative" as used herein refers to peptides which have been chemically modified, for example by ubiquitination, labeling, pegylation (derivatization with polyethylene glycol) or addition of other molecules. A molecule is also a "derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half life, etc. The moieties can alternatively decrease the toxicity of the molecule, or eliminate or attenuate an undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., MackPubl., Easton, PA (1990).

[0093] The term "functional" when used in conjunction with "derivative" or "variant" refers to a protein molecule which possesses a biological activity that is substantially similar to a biological activity of the entity or molecule of which it is a derivative or variant. By "substantially similar" in this context is meant that the biological activity, e.g., transmembrane transport of associated polypeptides is at least 50% as active as a reference, e.g., a corresponding wild-type polypeptide, and preferably at least 60% as active, 70% as active, 80% as active, 90% as active, 95% as active, 100% as active or even higher (i.e., the variant or derivative has greater activity than the wild-type), e.g., 110% as active, 120% as active, or more.

[0094] The term "recombinant" as used herein to describe a nucleic acid molecule, means a polynucleotide of genomic, cDNA, viral, semisynthetic, and/or synthetic origin, which, by virtue of its origin or manipulation, is not associated with all or a portion of the polynucleotide sequences with which it is associated in nature. The term recombinant as used with respect to a protein or polypeptide, means a polypeptide produced by expression from a recombinant polynucleotide. The term recombinant as used with respect to a host cell means a host cell into which a recombinant polynucleotide has been introduced. Recombinant is also used herein to refer to, with reference to material (e.g., a cell, a nucleic acid, a protein, or a vector) that the material has been modified by the introduction of a heterologous material (e.g., a cell, a nucleic acid, a protein, or a vector).

[0095] The term "vectors" refers to a nucleic acid molecule capable of transporting or mediating expression of a heterologous nucleic acid to which it has been linked to a host cell; a plasmid is a species of the genus encompassed by the term "vector." The term "vector" typically refers to a nucleic acid sequence containing an origin of replication and other entities necessary for replication and/or maintenance in a host cell. Vectors capable of directing the expression of genes and/or nucleic acid sequence to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility are often in the form of "plasmids" which refer to circular double stranded DNA molecules which, in their vector form are not bound to the chromosome, and typically comprise entities for stable or transient expression or the encoded DNA. Other expression vectors that can be used in the methods as disclosed herein include, but are not limited to plasmids, episomes, bacterial artificial chromosomes, yeast artificial chromosomes, bacteriophages or viral vectors, and such vectors can integrate into the host's genome or replicate

autonomously in the particular cell. A vector can be a DNA or RNA vector. Other forms of expression vectors known by those skilled in the art which serve the equivalent functions can also be used, for example self replicating extrachromosomal vectors or vectors which integrates into a host genome. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked.

A fusion polypeptide comprising a B. anthracis lethal factor (LF) N-terminal (LFn) polypeptide

[0096] The inventors have discovered that baculovirus expressed LFn-fusion protein can be delivered across the membrane of a cell and elicits a strong response of cell-mediated immunity (CMI) to the antigen portion of the LFn-fusion protein. The transmembrane transport is in the absence of the *Bacillus anthracis* protective antigen (PA) and the stimulated immune response is greater than that elicited by just the antigen that is not fused to the LFn polypeptide. While not wishing to be bound by theory, the LFn portion of the LFn-fusion protein facilitates the passage of the antigen across cell plasma membrane as well as the membrane of the endoplasmic reticulum (ER). Once inside the ER, the antigen portion of the LFn-fusion protein can complex with MHC class I molecules and become displayed with the MHC complexes to cytotoxic T cells. As a result, the CMI elicited with a LFn-fusion protein is stronger than with a non-LFn-fused antigen.

[0097] The inventors have also discovered, surprisingly, that N-glycosylated forms of LFn and LFn fusion polypeptides are more effective than non-glycosylated forms of the fusion proteins. This is surprising in that *B. anthracis* lethal factor is, in its native state, a bacterially expressed protein that is not N-glycosylated. In fact, it is unexpected that N-glycosylation of any bacterial polypeptide would enhance the activity of that polypeptide, particularly where, as here, the function of the bacterial polypeptide, LF and its fragment LFn, is to permit access of the polypeptide to the cytosol of a eukaryotic cell.

[0098] Accordingly, the invention provides a fusion polypeptide comprising a *B. anthracis* lethal factor (LF) N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated. The fusion polypeptide comprises amino acid sequences from the LFn polypeptide and the amino acid sequences from a target antigen polypeptide that is not a LFn polypeptide and not a LF polypeptide. A polypeptide comprising the amino acid residues 1-288 of the LF polypeptide is capable of traversing cell membranes in the absence of the *B. anthracis* protective antigen (PA). This is an LFn polypeptide. In addition, when a second protein is attached to an LFn or LF polypeptide, this second protein is also transported across membranes into the cytosol along with the LFn or LF polypeptide. However, by itself, as a non-LF fused protein, the second protein is inefficient in traversing the cell membrane and entering the interior of an intact cell. The LFn or LF polypeptide therefore facilitates and promotes the transmembrane delivery of other proteins in the context of a LFn or LF fusion polypeptide.

[0099] The inventors have also discovered, surprisingly, that N-glycosylated forms of LFn and LFn fusion polypeptides are more effective than non-glycosylated forms of the fusion proteins. For example, N-glycosylated forms of LFn and LFn fusion polypeptides are about at least 10%, or at least about 15%, or at least about 20%, or at least about 25%, or at least about 30%, or at least about 35%, or at least about 40%, or at least about 45%, or at least about 50%, or at least about 55%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 100%, or 1.5-fold, or 2-fold, or about 3-fold or about 5-fold or more than 5-fold more effective at delivering a target antigen to the cytosol of a cell as compared to non-glycosylated forms of the LFn-fusion proteins.

[00100] In one embodiment, the fusion polypeptide is N-linked glycosylated. N-glycosylation is important for the folding of some eukaryotic proteins, providing a co-translational and post-translational modification mechanism that modulates the structure and function of membrane and secreted proteins. Glycosylation is the enzymatic process that links saccharides to produce glycans, and attaches them to proteins and lipids. In N-glycosylation, glycans are attached

to the amide nitrogen of asparagine side chain during protein translation. The three major saccharides forming glycans are glucose, mannose, and N-acetylglucosamine molecules. The N-glycosylation consensus is Asn-Xaa-Ser/Thr, where Xaa can be any of the known amino acids. One skilled in the art can use bioinformatics software such as NetNGlyc 1.0 Prediction software from the Technical University of Denmark to find the N-glycosylation sites in a fusion polypeptide of the present invention. The NetNglyc server predicts N-Glycosylation sites in proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons. The NetNGlyc 1.0 Prediction software can be accessed at the EXPASY website. In one embodiment, N-glycosylation occurs in the target antigen polypeptide of the fusion polypeptide described herein. In another embodiment, N-glycosylation occurs in the LFn polypeptide of the fusion polypeptide described herein, for example, at asparagine positions 62, 212, and/or 286, all of which have the potential of > 0.51 according to the NetNGlyc 1.0 Prediction software. Various combinations of N-glycosylation in the fusion polypeptide of the present invention are possible. In some embodiments, the fusion polypeptide described herein has a single N-glycosylation at one of these three sites: asparagine positions 62, 212, and 286. In some other embodiments, the fusion polypeptide described herein is N-glycosylated at two of these three sites: asparagine positions 62, 212, and 286. In another embodiment, the fusion polypeptide described herein is N-glycosylated at all three sites: asparagine positions 62, 212, and 286. In yet another embodiment, N-glycosylation occurs in both the target antigen polypeptide and the LFn polypeptide. In some embodiments, the glycans of the fusion polypeptide described herein are modified, for example, sialyated or asialyated. Glycosylation analysis of proteins is known in the art. For example, via glycan hydrolysis (using enzymes such as N-glycosidase F, EndoS endoglycosidase, sialidase or with 4N trifluroacetic acid), derivitization, and chromatographic separation such as LC-MS or LC-MS/MS (Pei Chen et. al., 2008, J. Cancer Res. Clin.Oncology, 134: 851-860; Kainz,E. et. al., 2008, Appl Environ Microbiol., 74: 1076–1086).

[00101] In one embodiment, the intact cell is a living cell with an unbroken, uncompromised plasma membrane. An living cell would generally have a defined differential membrane potential across the membrane, with the inside of the cell being negative with respect to the outside of the cell. In one embodiment, the intact cell is a mammalian cell, including, for example, an antigen-presenting cell.

[00102] While the whole of the N-terminal amino acid residues 1-288 (i. e. domain I, see Fig. 1) of the LF polypeptide promotes the transmembrane delivery of other proteins, it should be understood that smaller fragments of domain I can be sufficient to translocate across cell membrane and promote the transmembrane delivery of other proteins when fused together as a fusion polypeptide. The x-ray crystal structure of domain I shows 12 alpha helices and four beta sheet secondary protein structure (Fig. 1). Smaller fragments of domain I that preserve these alpha helices and/or beta sheet secondary protein structures of domain I can translocate across cell membrane and promote the transmembrane delivery of other proteins when fused together as a fusion polypeptide. One skilled in the art can determine the presence of alpha helices and beta sheet secondary protein structure in the LFn polypeptide of the fusion polypeptide using methods known in the art, such as circular dichroism (CD).

[0100] In one embodiment, the LFn polypeptide of the fusion polypeptide comprises at least the 60 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists essentially of 60 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof. In yet another embodiment, the LFn polypeptide of the fusion polypeptide consists of 60 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[0101] In one embodiment, the LFn polypeptide of the fusion polypeptide comprises at least the 80 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists essentially of 80 carboxy-terminal amino acids of SEQ. ID. No. 3, or a

conservative substitution variant thereof. In yet another embodiment, the LFn polypeptide of the fusion polypeptide consists of 80 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[0102] In one embodiment, the LFn polypeptide of the fusion polypeptide comprises at least the 104 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists essentially of 104 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof. In yet another embodiment, the LFn polypeptide of the fusion polypeptide consists of 104 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[0103] In one embodiment, the LFn polypeptide of the fusion polypeptide consists of the amino acid sequence corresponding to SEQ. ID. No. 5, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists essentially of the amino acid sequence corresponding to SEQ. ID. No. 5, or a conservative substitution variant thereof. In yet another embodiment, the LFn polypeptide of the fusion polypeptide comprises of the amino acid sequence corresponding to SEQ. ID. No. 5, or a conservative substitution variant thereof.

[0104] In one embodiment, the LFn polypeptide of the fusion polypeptide comprises the amino acid sequence corresponding to SEQ. ID. No. 4, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists essentially of the amino acid sequence corresponding to SEQ. ID. No. 4, or a conservative substitution variant thereof. In yet another embodiment, the LFn polypeptide of the fusion polypeptide consists of the amino acid sequence corresponding to SEQ. ID. No. 4, or a conservative substitution variant thereof.

[0105] In one embodiment, the LFn polypeptide of the fusion polypeptide comprises the amino acid sequence corresponding to SEQ. ID. No. 3, or a conservative substitution variant thereof. In another embodiment, the LFn polypeptide of the fusion polypeptide consists essentially of the amino acid sequence corresponding to SEQ. ID. No. 3, or a conservative substitution variant thereof. In yet another embodiment, the LFn polypeptide of the fusion polypeptide consists of the amino acid sequence corresponding to SEQ. ID. No. 3, or a conservative substitution variant thereof.

[0106] In one preferred embodiment, the LFn polypeptide of any of fusion polypeptides described herein or a conservative substitution variant thereof promotes transmembrane delivery.

[0107] In one embodiment, the LFn polypeptide of the fusion polypeptide does not bind *B. anthracis* protective antigen (PA) protein. The PA protein is the natural binding partner of LF, forming bipartite protein exotoxin, lethal toxin (LT). The PA protein is a 735-amino acid polypeptide, a multi-functional protein that binds to cell surface receptors, mediates the assembly and internalization of the complexes, and delivers them to the host cell endosome. Once PA is attached to the host receptor, it is cleaved by a host cell surface (furin family) protease before it is able to bind LF. The cleavage of the N-terminus of PA enables the C-terminal fragment to self-associate into a ring-shaped heptameric complex (prepore) that can bind LF and delivers LF into the cytosol. The N-terminal fragment (residues 1–288, domain I) can be expressed as a soluble folded domain that maintains the ability to bind PA and enables the translocation of heterologous fusion proteins into the cytosol. Smaller fragments of this residue 1–288 N-terminal fragment have been shown to also translocate heterologous fusion proteins into the cytosol in the absence of PA. Hence, in one embodiment, smaller fragments described herein can translocate across membranes but do not bind PA. Methods of measuring or detecting protein-protein interaction are known to one skilled in the art.

[0108] In one embodiment, the LFn polypeptide of any of the fusion polypeptides described herein substantially lacks the amino acids 1-33 of SEQ. ID. No. 3. Amino acids 1-33 of SEQ. ID. No. 3 encompass the signal peptide that is predicted to direct the post-translational transport of the LF protein. In some embodiments, the LFn polypeptide of

any of the fusion polypeptides described herein lacks a signal peptide that functions to direct the post-translational transport of the fusion polypeptide. In other embodiments, the LFn polypeptide of any of the fusion polypeptides described herein comprises a signal peptide for co-translation on the ER. The signal peptide is also called a leader peptide in the N-terminus, which may or may not be cleaved off after the translocation through the ER membrane. One example of a signal peptide is MAPFEPLASGILLLLWLIAPSRA (SEQ. ID. No. 47). Other examples of signal peptides can be found at SPdb, a Signal Peptide Database, which is found at the world wide web site of http colon "forward slash" "forward slash" proline "period" bic "period" nus "period" edu "period" sg "forward slash" spdb "forward slash".

[0109] In one embodiment, the target antigen polypeptide of the fusion polypeptide described herein is an intracellular pathogen target antigen polypeptide. A pathogen has been defined as a microorganism capable of causing damage to the host. An intracellular pathogen is a microorganism that can gain entry into the interior of a cell, live inside host cells and cause damage to the host and/or host cells. For example, the pathogen can be phagocytosed and/or endocytosed by a host cell, followed by the pathogen's escape from the phagosome or endosome. The pathogen then resides intracellular to evade other/subsequent host defense, such as antibodies, and to multiply. Phagocytosis by macrophages is a primary frontline host defense mechanism against pathogens. When a pathogen fails to escape from the phagosome or endosome, the phagocytosed or engulfed pathogen is digested by the enzymes coming from the lysosomes. The digested, smaller peptides derived from pathogen proteins are complexed with host cell MHC molecules and displayed extracellularly to other immune cells in the host so as to stimulate the immune system of the host to respond to that particular pathogen. An intracellular pathogen target antigen polypeptide is a pathogen-encoded protein, which, when displayed and presented to host immune cells in the context of MHC molecules as described herein, can stimulate an immune response in the host that can involve numerous cellular processes known in the art of immunology. Aspects of such a response include an increase in cytokine production, increased antibody production, and increased B-cell multiplication. Intracellular pathogens include but not limited to viruses, bacteria and protozoa. They cause a range of human diseases and ailments: tuberculosis, leprosy, typhoid fever, bacillary dysentery, plague, brucellosis, pneumonia, typhus; Rocky Mountain spotted fever, chlamydia, trachoma, gonorrhea, Listeriosis, scarlet/rheumatic fever, "strep" throat, hepatitis, AIDS, congenital viral infections, mononucleosis, Burkitts lymphoma and other lymphoproliferative diseases, cold sores, genital herpes, genital warts, cervical cancer, leishmaniasis, malaria, and trypanosomiasis to name but a few.

[0110] In one embodiment, the target antigen polypeptide of the fusion polypeptide described herein is an intracellular pathogen target antigen polypeptide from a prokaryotic pathogen. A prokaryotic pathogen is a bacterium. In one embodiment, the prokaryotic pathogen includes but not limited to *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella* species, *Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Clostridium botulinum, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum, Klebsiella pneumoniae, Pseudomonas aeruginosa, Cryptosporidium parvum, Streptococcus pneumoniae, Bordetella pertussis,* and *Neisseria meningitides.*

[0111] In one embodiment, the target antigen polypeptide of the fusion polypeptide described herein is an intracellular pathogen target antigen polypeptide from a viral pathogen, in which the virus naturally infects mammalian host cells. In one embodiment, the viral pathogen includes but is not limited to Herpes simplex virus type-1, Herpes simplex virus type-2, HBV, Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus, Human herpes virus 6, Human herpes virus 7, Human herpes virus 8, Variola virus, Vesicular stomatitis virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, poliovirus, Rhinovirus, Coronavirus, Influenza virus A, Influenza virus B. Measles virus, Polyomavirus, Human Papilomavirus, Respiratory syncytial virus, Adenovirus,

Coxsackie virus, Dengue virus, Mumps virus, Poliovirus, Rabies virus, Rous sarcoma virus, Yellow fever virus, Ebola virus, Marburg virus, Lassa fever virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, St. Louis Encephalitis virus, Murray Valley fever virus, West Nile virus, Rift Valley fever virus, Rotavirus A, Rotavirus B. Rotavirus C, Sindbis virus, Rabies virus, Human T-cell Leukemia virus type-l, Hantavirus, Rubella virus and Simian Immunodeficiency virus.

[0112] In one embodiment, the target antigen polypeptide of the fusion polypeptide described herein is an intracellular pathogen target antigen polypeptide of a parasitic pathogen. In one embodiment, the parasitic pathogen includes but is not limited to *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella* species, *Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum,Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Bordetella pertussis, Neisseria meningitides, Leishmania donovanii, Plasmodium* species, *Pneumocystis carinii, Trypanosoma* species, Herpes simplex virus type-1, Herpes simplex virus type-2, HBV, Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus, Human herpes virus 6, Human herpes virus 7, Human herpes virus 8, Variola virus, Vesicular stomatitis virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, poliovirus, Rhinovirus, Coronavirus, Influenza virus A, Influenza virus B. Measles virus, Polyomavirus, Human Papilomavirus, Respiratory syncytial virus, Adenovirus, Coxsackie virus, Dengue virus, Mumps virus, Poliovirus, Rabies virus, Rous sarcoma virus, Yellow fever virus, Ebola virus, Marburg virus, Lassa fever virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, St. Louis Encephalitis virus, Murray Valley fever virus, West Nile virus, Rift Valley fever virus, Rotavirus A, Rotavirus B. Rotavirus C, Sindbis virus, Rabies virus, Human T-cell Leukemia virus type-l, Hantavirus, Rubella virus and Simian Immunodeficiency virus.

[0113] In one embodiment, the target antigen polypeptide of the fusion polypeptide described herein is an antigen of *M. tuberculosis.*

[0114] In one embodiment, the target antigen polypeptide of the fusion polypeptide described herein is a TB-specific antigen.

[0115] In a further embodiment, the TB-specific antigen is a TB1 (CFP-10) polypeptide comprising SEQ ID NO: 7 or a fragment thereof.

[0116] In another embodiment, the TB-specific antigen is a TB2 (ESAT-6) polypeptide comprising SEQ ID NO: 6 or a fragment thereof.

[0117] In a preferred embodiment, the target antigen polypeptide of any of the fusion polypeptides described herein is an intracellular pathogen target antigen polypeptide and the target antigen is at least 15 amino acids long. For example, the target antigen can be the 91 amino acid fragment of (amino acids 27–117) of *P. falciparum* circumsporozoite protein, a predominant surface protein, that is involved in invasion of liver cells by *Plasmodium* sporozoites, which leads to malaria.

[0118] In other embodiments, the target antigen polypeptide of any of the fusion polypeptides described herein is a protein associated with a disease or disorder. For example, cancer, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), prion diseases, Creutzfeldt-Jakob disease (CJD), variant Creutzfeldt-Jakob disease (vCJD), 'Kuru' and scrapie. These diseases are often characterized by common cellular and molecular mechanisms including protein aggregation, inclusion body formation, and unregulated constitutively active form of protein. The aggregates usually consist of fibers containing misfolded protein with a beta-sheet conformation, termed amyloid. Examples of some proteins that are missed fold and form protein aggregates, or mutant rouge proteins include but not limited to p53, Ras GTPases, retinoblastoma, mutant c-myc, prion protein,

huntingtin, hyper-phosphorylation of tau, beta-amyloid, α-synuclein, and synphilin 1. Some proteins are expressed specifically in and/or on cancer cells but are not on healthy, non-cancer cells, e. g. tyrosinase (abundant on melanoma cells), cancer antigen 125 (ovarian cancer cells), MART-1 (also known as Melan-A) (an antigen expressed by melanocytes), carcinoembryonic antigen (CEA) (in high levels on tumors in people with colorectal, lung, breast and pancreatic cancer), and prostate specific antigen (PSA) (on prostate cancer cells). The proteins on the surface of cancer cells are either unique or more abundant than those found on normal or non-cancerous cells. These proteins can act as antigens, meaning that they can stimulate the immune system to make a specific immune response. A fusion polypeptide comprising a cancer-specific target antigen can be used as a therapeutic vaccine to be injected into a patient. The cancer-specific antigens will stimulate the immune system to attack cancer cells without harming normal cells. In some embodiments, the target antigen polypeptides are fragments of the proteins that induces protein aggregation.

[0119] In one embodiment, the target antigen polypeptide of any of the fusion polypeptides described herein is folded in its native conformation.

[0120] In one embodiment, the target antigen polypeptide of any of the fusion polypeptides described herein is part of a multi-molecular polypeptide complex.

[0121] In one embodiment, the target antigen polypeptide of any of the fusion polypeptides described herein is a subunit polypeptide of a multi-molecular polypeptide target antigen.

[0122] In one embodiment, any of the fusion polypeptide described herein is expressed and purified from a protein expression system using host cells selected from the group consisting of: mammalian cells, insect cells, yeast cells, and plant cells. The cloning, protein expression, and purification of recombinant proteins are known. One skilled in the art can use modern molecular techniques to construct an isolated polynucleotide encoding any of the fusion polypeptides described herein, and ligate the isolated polynucleotide into a vector to form a recombinant vector, wherein the recombinant vector is an expression vector that is compatible with a protein expression system using host cells selected from the group consisting of: mammalian cells; insect cells; yeast cells; and plant cells. It is preferred that the host cell can N-glycosylate the recombinant fusion polypeptide. There are many options for an expression vector depending on the choice of protein expression system and the types of host cells used. In one embodiment, the recombinant vector is a viral vector, such as, a recombinant baculovirus vector, an adeno-associated virus (AAV) vector or a lentivirus vector. Viral vectors provide ease of introducing the coding polynucleotide construct into the desired host cells. For example, adeno-associated virus (AAV) vector or a lentivirus vector infects mammalian cells and baculovirus vectors infect lepidopteran insect cells, such as, *Spodoptera frugiperda* cells. Expression of any of the fusion polypeptides described herein in eukaryotic host cells, e. g, mammalian cells and insect cells can result in N-glycosylation of the fusion polypeptide thus expressed.

[0123] In one embodiment, the invention provides an insect host cell expressing any of the fusion polypeptides described herein. In one embodiment, the insect host cell is infected with a baculovirus vector comprising a polynucleotide that encodes the fusion polypeptides described herein. In one embodiment, the insect host cell is a *S. frugiperda* cell.

[0124] Reports on the construction of LFn-fusion polypeptides can be found, for example, in WO/2002/079417, WO/2008/048289, U. S. Patent No. 20040166120, Huyen Cao, et. al., 2002, The Journal of Infectious Diseases;185:244–251; N. Kushner, et. al., 2003, Proc Natl. Acad. Sci. U S A. 100: 6652–6657; Ballard, J. D., et. al., 1996, Proc. Natl. Acad. Sci. USA 93, 12531-12534; and Goletz, T. J. et al., 1997, Proc. Natl. Acad. Sci. USA 94, 12059-12064, all of which are incorporated hereby reference in their entirety.

Membrane translocation assays

[0125] Methods of determining membrane translocation are well known in the art, for example, in Wesche, J., et. al., 1998, Biochemistry 37: 15737–15746 and Sellman, B. R.,et. al., 2001 J. Biol. Chem. 276: 8371–8376. For example, CHO-K1 cells in a 24-well plate are chilled on ice, washed, and incubated on ice for 2 h with any of the LFn-target antigen fusion polypeptides described herein or a conservative substitution variant thereof or fragments of domain I that have been labeled with [^{35}S]methionine in an *in vitro* transcription/translation system (Promega). The cells then are washed with ice-cold PBS at pH 5.0 or 8.0, incubated at 37°C for 1 min, and either treated with pronase to digest residual untranslocated ^{35}S at the cell surface or left untreated as controls. The cells are then lysed, and ^{35}S liberated into the lysis buffer is assayed. The percent translocation is defined as dpm protected from pronase/dpm bound to cells × 100. The cell lysate of cells incubated with fusion polypeptides or fragments of domain I that facilitate transmembrane delivery would have higher percent translocation.

[0126] Alternatively, green fluorescent protein fused to LFn, LF or smaller fragments of domain I (e. g. LFn-GFP) can be used to assay for membrane translocation capability, such as described in N. Kushner, et. al., 2003, Proc. Natl. Acad. Sci. U S A. 100: 6652–6657. Briefly, HeLa cells (American Type Culture Collection) are grown on collagen-treated chamber slides (BD Science) to reach ~80% confluence and incubated with 40 μg/ml purified GFP or LFn-GFP at 37°C for 1 or 2 h. After washing, GFP fluorescence is compared between GFP and GFP-LFn treated samples. Membrane translocation is evidenced by GFP signal greater in the LFn-GFP-treated cells than in cells treated with GFP alone. Some incubations can also be performed in the presence of 100 μg/ml Texas red-conjugated transferrin (INVITROGEN™ Inc., Molecular Probes) as a marker for the endocytic pathway. For the transferrin experiments, cells are washed four times with cold DMEM and then fixed for 15 min in 4% paraformaldehyde in cold PBS. For antibody labeling, slides are then incubated on ice for 15 min in 50 mM NH_4Cl in PBS and then in PBS containing 0.1% saponin for 20 min on ice. After further washing in PBS, slides are incubated at room temperature for 1 hr in a moisture chamber with PBS containing 4% donkey serum and the following primary antibodies: mouse anti-early endosome antigen 1 (EEA-1) (BD Laboratory) to stain early endosomes, mouse anti-Lamp1 and anti-Lamp2 (Developmental Studies Hybridoma Banks, University of Iowa, Iowa City) to stain late endosomes and lysosome, mouse Ab-1 (Oncogene) to stain the Golgi apparatus, mouse anti-mitochondrial antibody from CALBIOCHEM®, and rabbit anti-calreticulin (STRESSGEN® Biotechnologies, Victoria, Canada). Cells are then processed for secondary antibody staining and microscopy. Fusion LFn-GFP that promotes transmembrane delivery would be visualized in the interior of the cell. The antibody markers will further indicate sub-cellular localization of the translocated GFP.

Methods of measuring or detecting protein-protein interaction

[0127] Methods of measuring or detecting protein-protein interaction are well known. One skilled in the art can determine PA binding activity, for example, by mixing and incubating PA63 with LFn for a period of time, chemically cross-linking of any complex formed and analysis of the covalently linked complex by gel electrophoresis or by radioactivity counting as described by Quinn CP. et. al., 1991, J. Biol. Chem. 266:20124-20130. Briefly, the binding assay is determined at 5°C by competition with radiolabeled ^{125}I-LFn. Native LF or full-length N-terminal (amino acid 1-288) LFn is radiolabeled (~7.3 x 10^6 cpm/μg protein) using Bolton-Hunter reagent (Amersham Corp). For binding studies, J774A.1 cells cultured in 24-well tissue culture plates are cooled by incubating at 4°C for 60 min and then placing the plates on ice. The medium is then replaced with cold (4°C) minimal essential medium containing Hanks' salts (GIBCO®/BRL) supplemented with 1% (w/v) bovine serum albumin and 25 mM HEPES (binding medium). Native PA (0.1 g/ml) is added with radiolabeled native LF (^{125}I-LF, 0.1 μg/ml, 7.3 x 10^6 cpm/μg) and the plates incubated for 14 h on wet ice. Mutant LF proteins were assayed at varying concentrations for their ability to compete with native ^{125}I-LF. For quantitation of bound, radiolabeled LF, cells were gently washed twice in cold binding

medium, once in cold Hanks' balanced salt solution, solubilized in 0.50 ml of 0.1 M NaOH, and counted in a gamma counter (Beckman Gamma 9000).

Zinc metalloproteinase activity by FRET analysis

[0128] Assays of LF peptidolytic activity based on cleavage of the FRET-quenched substrate MAPKKide can be carried out according to a modification of the method of Cummings et al. (2002, Proc. Natl. Acad. Sci. USA 99:6603-6606.). MAPKKide (*o*-aminobenzoyl [o-ABZ]/2,4-dinitrophenyl [DNP]), a synthetic peptide containing the *o*-ABZ donor and DNP acceptor groups separated by a cleavage site specific for anthrax LF, was purchased from List Biological Labs. Digestion of MAPKKide by LF was carried out in Dulbecco's phosphate-buffered saline (DPBS) (HyClone, Logan Utah), pH 8.2, as recommended by the manufacturer and was followed in a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) or in a, LS-5 fluorescence spectrophotometer (Perkin-Elmer, Wellesley, MA) using a λ excitation (ex) value of 320 nm and a λ emission (em) value of 420 nm. LF was preincubated with indicated concentrations of putative inhibitors for 10 min at room temperature, and the reaction was initiated by addition of indicated concentrations of the substrate to a 100-µl or 500-µl reaction mixture.

Lethal factor (LF) of Bacillus anthracis and the N-terminal fragment (LFn)

[0129] By way of background and without wishing to be limited by theory, *B. anthracis* is the causative agent of anthrax in animals and humans. The toxin produced by *B. anthracis* consists of two bipartite protein exotoxins, lethal toxin (LT) and edema toxin. LT is composed of protective antigen (PA) and lethal factor (LF), whereas edema toxin consists of PA and edema factor (EF). None of these three components, PA, LF, and EF, alone is toxic. Once combined however, edema toxin causes edema and LT causes death by systemic shock in animals and humans. Consistent with its critical role in forming both toxins, PA has been identified as the protective component in vaccines against anthrax. The molecular mechanism of anthrax toxin action is currently hypothesized as follows: PA is a 735-amino acid polypeptide, a multi-functional protein that binds to cell surface receptors, mediates the assembly and internalization of the complexes, and delivers them to the host cell endosome. Once PA is attached to the host receptor, it must then be cleaved by a host cell surface (furin family) protease before it is able to bind LF. The cleavage of the N-terminus of PA enables the C-terminal fragment to self-associate into a ring-shaped heptameric complex (prepore) that can bind LF or EF competitively. The cleaved PA is a 63-kDa molecule (PA63) capable of forming a ring-shaped heptamer in the plasma membrane of the targeted cell (Milne et al., (1994) J. Biol. Chem. 269, 20607–20612, Petosa, et al., (1997) Nature (London) 385, 833–838). The PA heptamer then binds either EF or LF, which are internalized by endocytosis into intracellular compartments called endosomes. Natural cellular processes lead to acidification of the endosome, which triggers a conformational change in PA63, causing it to insert into the endosomal membrane and translocates the toxic enzymes into the host cell interior (cytosol), presumably by means of a pore formed by the heptamer. Once inside the cytosol, LF and EF do their damage to the host cell defense system. LF is a metalloproteinase that cleaves six members of the MAPKK family (Vitale G, . et. al., 2000, Biochem. J. 352, 739-745) of intracellular signaling proteins, removing the specific fragment from individual MAPKKs that are crucial for immediate interaction with other signaling proteins. This action by LF rapidly blocks the signals that would normally recruit other immune cells to fight the infection (Duesbury NS,. et. al., 1998, Science 280, 734-737). EF, on the other hand, is a calmodulin-activated adenylyl cyclase that increases the concentration of a messenger molecule (cAMP) needed for regulated cell functions (Leppla SH., et. al., 1982, Proc. Natl. Acad. Sci. USA 79, 3162-3166) to abnormal levels, causing accumulation of fluids within and between cells, and hence edema. The disruption of normal signaling pathways result in cell lysis, the sudden release of messenger molecules, and toxic shock.

[0130] Anthrax lethal factor or LF is a protein, encoded by GenBank Accession Number M29081 (Gene ID No: 143143), that is naturally produced by *B. anthracis* and that has MAPKK protease activity. Deletion analysis of LF shows that the PA binding domain is located within the amino-terminus of LFn. Mutational studies demonstrate the PA binding domain is located within the region of 34 to 254 of the LF polypeptide of SEQ. ID. NO: 1, and within the region of 34 to 288 of the LF polypeptide of SEQ. ID. NO: 2 (Arora et al., J. Biol. Chem. 268:3334 3341 (1993); Milne, et al., (1995) Mol. Microbiol. 15, 661–66). The three-dimensional atomic resolution structures of LF have now been solved by X-ray crystallography. Andrew D. Pannifer et. al., describes the crystal structure of LF and its complex with a 16-amino acid residue (16-mer) peptide representing the N-terminus of its natural substrate, MAPKK-2, in Nature vol. 414, pg. 229-233 (2001) as a protein that comprises four structural domains: domain I binds the membrane-translocating component of anthrax toxin, the protective antigen (PA); domains II, III and IV together create a long deep groove that holds the 16-residue N-terminal tail of MAPKK-2 before cleavage. Domain I is perched on top of the other three domains, which are intimately connected and comprise a single folding unit. The only contacts between domain I and the rest of the molecule are with domain II, and these chiefly involve charged polar and water-mediated interactions. The nature of the interface is consistent with the ability of a recombinant N-terminal fragment (residues 1–254, excluding the signal peptide) to be expressed as a soluble folded domain that maintains the ability to bind PA and enables the translocation of heterologous fusion proteins into the cytosol (Ballard, J. D., et. al., 1996, Proc. Natl Acad. Sci. USA 93, 12531-12534; Goletz, T. J. et al., 1997, Proc. Natl Acad. Sci. USA 94, 12059-12064). Moreover, deletion of the first 36 residues of LFn had no effect on its binding to PA or LF ability to be translocated across membranes (D. Borden Lacy, et.al., 2002, J. Biol. Chem., 277:3006-3010). Domain I consists of a 12-helix bundle that packs against one face of a mixed four-stranded β-sheet, with a large (30-residue) ordered loop, L1, between the second and third -strands forming a flap over the distal face of the sheet (see Fig. 1). The exact docking site on domain I for PA is unknown, but the integrity of the folded domain seems to be required, because a series of insertion and point mutants of buried residues in domain I that presumably disrupt the fold abrogate binding of PA and toxicity (Quinn, C. P., et. al., 1991, J. Biol. Chem., 266: 20124-20130; Gupta, P., et. al., 2001, Biochem. Biophys. Res. Comm., 280:158-163). In addition, LFn has been shown to deliver exogenous protein antigens to the major histocompatibility complex class I pathway in the cytosol of B-cells, CTL-cells and macrophages in the absence of PA (Huyen Cao, et. al., 2002, The Journal of Infectious Diseases;185:244–251; N. Kushner, et. al., 2003, Proc Natl Acad Sci U S A. 100: 6652–6657). The PA-independent LFn delivery of LFn-fusion proteins depends on functional transport-associated proteins for intracellular antigen processing and transport into the endoplasmic reticulum for binding to MHC class I molecules.

[0131] An abrupt turn at the end of the last helix of domain I leads directly into the first helix of domain II (residues 263–297 and 385–550). Although sequence-based comparisons failed to yield any homology, the structural similarity with the catalytic domain of the B. cereus toxin, VIP2 (Protein Data Bank accession code 1QS2), is outstanding. Domain II and VIP2 superimpose with an RMSD of 3.3 Å and a sequence identity of 15%, as determined by DALI (Holm, L. & Sander, 1997, Nucleic Acids Res. 25, 231-234). VIP2 contains an NAD-binding pocket and conserved residues involved in NAD binding and catalysis. Domain II lacks these conserved residues; moreover, a critical glutamic acid that is conserved throughout the family of ADP ribosylating toxins (Carroll, S. F. & Collier, R. J., 1984, Proc. Natl. Acad. Sci. USA 81, 3307-3311) is replaced by a lysine (K518). We therefore expect that domain II does not have ADP-ribosylating activity.

[0132] Domain III is a small α-helical bundle with a hydrophobic core (residues 303–382), inserted at a turn between the second and third helices of domain II. Sequence analysis has revealed the presence of a 101-residue segment comprising five tandem repeats (residues 282–382), and suggested that repeats 2–5 arose from a duplication of repeat 1. The crystal structure reveals that repeat 1 actually forms the second helix-turn element of domain II, whereas

repeats 2–5 form the four helix-turn elements of the helical bundle, suggesting a mechanism of creating a new protein domain by the repeated replication of a short segment of the parent domain. Domain III is required for LF activity, because insertion mutagenesis and point mutations of buried residues in this domain abrogate function (Quinn, C. P., et. al., 1991, J. Biol. Chem. 266, 20124-20130). It makes limited contact with domain II, but shares a hydrophobic surface with domain IV. Its location is such that it severely restricts access to the active site by potential substrates such as the loops of a globular protein; that is, it contributes towards specificity for a flexible 'tail' of a protein substrate. It also contributes sequence specificity by making specific interactions with the substrate (see below).

[0133] Domain IV (residues 552–776) consists of a nine-helix bundle packed against a four-stranded -sheet. Sequence comparisons had failed to detect any homology with other proteins of known structure beyond the HExxH motif. The three-dimensional structure reveals that the β-sheet and the first six helices can be superimposed with those of the metalloprotease thermolysin, with an RMSD of 4.9 Å over 131 residues. Large insertions and deletions occur elsewhere within the loops connecting these elements, so that the overall shapes of the domains are quite different. In particular, a large ordered loop (L2) inserted between strands 42 and 43 of the sheet partly obscures the active site, packs against domain II, and provides a buttress for domain III.

[0134] A zinc ion (Zn^{2+}) is coordinated tetrahedrally by a water molecule and three protein side chains, in an arrangement typical of the thermolysin family. Two coordinating residues are the histidines from the HExxH motif (His 686 and His 690) lying on one helix (44), as expected. The structure reveals that the third coordinating residue is Glu 735 from helix 46. Glu 687 from the HExxH motif lies 3.5 Å from the water molecule, well positioned to act as a general base to activate the zinc-bound water during catalysis. The hydroxyl group of a tyrosine residue (Tyr 728) forms a strong hydrogen bond (O–O distance 2.6 Å) to the water molecule, on the opposite side of Glu 687, and probably functions as a general acid to protonate the amine leaving group.

[0135] The action of LF in the cytosol causes the death of host cells by a mechanism that is not well understood. LF induces over-production of a number of lymphokines (Klimpel, et al., 1994, Mol. Microbiol. 13, 1093–1100), contributing to lethal systemic shock in host animals. Recent studies also show that LF has two enzymatic activities: it can act as a zinc metalloprotease (Duesbery, et al., 1998, Science 280, 734–737), and it inactivates mitogen-activated protein kinase (Hanna, et al.,. 1994, Mol. Med. 1, 7–18). The zinc metalloprotease activity is found at the C-terminus of LF and it inactivates members of the mitogen-activated protein kinase kinase or MEK family through proteolysis of their $NH_{(2)}$ termini (Vitale G. et. al., 2000, Biochem. J. 352:739-45; Chopra AP el. al., J. Biol. Chem. 2003, 278:9402-6). It has previously been reported that anthrax toxin B moieties may be used to deliver eptiopes which in turn elicit an antibody response by the immune system, in the presence of PA (WO 97/23236).

[0136] The gene encoded *B. anthracis* LF is a 809 amino acid polypeptide while the mature *B. anthracis* LF is a 796 amino acid polypeptide after cleavage of the N-terminal leader peptide. There are functional domains for both enzymatic activities is located between amino acids 383 and 796 of SEQ ID NO: 1. The N-terminal truncated LF without this catalytic domain completely lacks any toxic effect when mixed with PA and added to cultured macrophages or when injected into animals. It does, however, still bind to PA effectively. The PA binding domain of LFn occurs within residues 34-288 of SEQ ID NO:2 (Milne, et al., (1995) Mol. Microbiol. 15, 661–66).

[0137] The gene encoded 809 amino acid polypeptide *B. anthracis* LF has seven potential N-glycosylation sites located at asparagine positions 62, 212, 286, 478, 712 736, and 757. Within the LFn (1-288), there are three potential N-glycosylation sites, at asparagine positions 62, 212, and 286, all of which have the potential of > 0.51 according to the NetNGlyc 1.0 Prediction software from the Technical University of Denmark. The NetNglyc server predicts N-Glycosylation sites in proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons.

[0138] The gene encoded 809-aa polypeptide *B. anthracis* LF is not predicted to have any O- glycosylation sites according to the NetOGlyc 3.1 Prediction software from the Technical University of Denmark. The NetOglyc server produces neural network predictions of mucin type GalNAc O-glycosylation sites in proteins.

[0139] "LFn polypeptides" include LF polypeptide fragments represented by SEQ ID NOs. 3 and 4, as well as recombinant LFn, and functional LFn, fragments and variants that retain the function to deliver an LFn-fused target antigen polypeptide to the cytosol of an intact cell, preferably a living cell. The term "LFn polypeptide" therefore includes functional LFn homologues such as polymorphic variants, alleles, mutants, and closely related interspecies variants that have at least about 60% amino acid sequence identity to LFn and have the function to deliver a fused polypeptide target antigen to the cytosol of a cell, as determined using the assays described herein. In particular embodiments, the LFn polypeptides are substantially identical to LFn of SEQ. ID. NO: 3 and SEQ. ID. NO: 4 as disclosed herein. In other embodiments, the LFn polypeptides are conservative substitution mutants of LFn of SEQ. ID. NO: 3 and SEQ. ID. NO: 4 as disclosed herein. These conservative substitution mutants of LFn can also function to deliver a fused polypeptide target antigen to the cytosol of a cell, as determined using the assays described herein. In some embodiments, some functional polymorphic variants, alleles, mutants, and closely related interspecies variants of LFn that function to deliver a target antigen polypeptide to an intact cell can be determined by the methods and assays as disclosed in U.S. Patent Application 10/473,190 which is incorporated herein by reference.

[0140] The inventors have discovered that a fragment of LFn which is about 250 amino acids or less, or about 150 amino acids or less, or about 104 amino acids or less, is able to deliver the fused target antigen to a cell and is useful in the methods and compositions described herein.

[0141] In one embodiment, a fusion polypeptide as described herein comprises a non-functional binding site for PA, and thus is a mutant of LFn which does not result in functional binding with PA. Such mutants include, but are not limited to mutants altered at one or more of the residues critical for interacting with PA, such as a mutation in one or more of the following residues: Y22; L188; D187; Y226; L235; H229 (see Lacy et al., J. Biol. Chem., 2002; 277; 3006-3010); D106A; Y108K; E135K; D136K; N140A and K143A (see Melnyk et al., J. Biol. Chem., 2006; 281; 1630-1635 and Cunningham et al., PNAS, 2002; 99; 70497052), which are incorporated by reference in their entirety.

[0142] In another embodiment, a fusion polypeptide as described herein comprises a LF polypeptide, fragments, variants, conservative substitution mutants thereof, and a target antigen polypeptide. In some embodiments, the target antigen polypeptide is from an intracellular pathogen. As used herein, the term "is from" in this context means the polypeptide is coded by a gene found in the genome of an intracellular pathogen.

The target antigen polypeptide from an intracellular pathogen

[0143] A target antigen polypeptide of any fusion polypeptides described herein can be any target antigen, including, but not limited to pathogenic peptides, toxins, toxoids, subunits thereof, or combinations thereof (e.g., tetanus toxoid, diphtheria toxoid, cholera subunit B and Protein D from *H. influenza*).

[0144] A target antigen polypeptide of any fusion polypeptides described herein can be any antigen associated with an infectious disease, or cancer or immune disease, for example. In some embodiments, a target antigen polypeptide of any fusion polypeptides described herein can be an antigen expressed by any of a variety of infectious agents, including a pathogen, virus, bacterium, fungus or parasite.

[0145] As described herein, an intact (i.e. an entire or whole or complete) target antigen can be included in the fusion polypeptides described herein. By "intact" in this context is meant that the target antigen is the full length target antigen as that antigen polypeptide occurs in nature. This is in direct contrast to delivery of only a small portion or peptide of the target antigen. By delivering an intact target antigen to a cell, the fusion polypeptide enables or facilitates the translocation of the whole target antigen across the cell membrane and the display of a full range of epitopes of the

intact target antigen in complexes with MHC molecules. Moreover, this also facilitates detection of a cell mediated immune (CMI) response to a full range of epitopes of the intact target antigen, rather than just a single or selected few peptide epitopes. CMI occurs when T cells (lymphocytes) bind to the surface of other cells that display the antigen and trigger a response, e. g. production and release of cytokines. The response can involve other lymphocytes and any of the other white blood cells (leukocytes). Accordingly, the use of the fusion polypeptide comprising an intact target antigen in CMI assays results in CMI responses which are more sensitive and have higher specificity as compared to use of a peptide-based target antigen, in that when a whole target antigen is used to assay a CMI response, a response that was raised against essentially any epitope of the whole antigen would be more likely to be detected. CMI assays are known in the art and described, for example, in U. S. Patent Application No. 20050014205, WO/1987/005400, U. S. Patent No. 5,674,698 and commercially available kits such as IMMUNKNOW® CYLEX Immune cell function assay Product No. 4400.

[0146] In some embodiments, the intact target antigen can be divided into fragments, or parts, of the whole target antigen, for example, at least two, or at least 3, or at least 4, or a least 5 or more target antigen fragments, depending on size of the intact target antigen protein. These fragments of the whole target antigen can be used, for example, as a quality control to filter out false positives of a positive CMI response. By way of an example only, a positive CMI response to a whole target antigen fused to LFn or LF can be confirmed by assessing a CMI response to a panel of target antigens fused to LFn which are fragments of the whole target antigen. A true CMI response is confirmed if one or two of the fragments give a positive response, but not all fragments. If a positive CMI response is detected for all fragments, it is likely that the positive CMI response was a false positive.

[0147] In some embodiments, an intact target antigen can be divided into many parts, depending on the size of the initial target antigen, for use as a panel of sub-target antigens. Typically, where a whole target antigen is a multimer polypeptide, the whole target protein can be divided into sub-units and/or domains which can each individually be fused to the LFn or LF polypeptide, to create a panel of LFn-fusion or LF-fusion polypeptides of the target antigen which can be used in assay methods and compositions as disclosed herein. Alternatively, an intact target antigen can be divided into fragments, or parts of the whole target antigen, for example, at least two, or at least 3, or at least 4, or a least 5, or at least 6, or at least 7, or at least 8, or at least about 9, or at least about 10, or at least about 11, or at least about 12, or at least about 13, or at least about 15, or at least about 20, or at least about 25, or more than 25 fragments, and each fragment is individually fused to an LFn or LF polypeptide to create a panel of LFn-fusion or LF-fusion polypeptides which can be used in assay methods and compositions as disclosed herein.

[0148] The fragmentation or division of a full length target antigen polypeptide can be an equal division of the full length target antigen polypeptide, or alternatively, in some embodiments, the fragmentation is asymmetrical or unequal. As a non-limiting example, where a target antigen is divided into two overlapping fragments, a target antigen can be divided into fragments of approximately the same (equal) size, or alternatively one fragment can be about 45% of the whole target antigen and the other fragment can be about 65%. As further non-limiting examples, a whole target antigen can be divided into a combination of differently sized fragments, for example, where a target antigen is divided into two fragments, fragments can be divided into about 40% and about 70%, or about 45% and about 65%; or about 35% and about 75%; or about 25% and about 85% of the whole target antigen. Any combination of overlapping fragments of a full length whole target antigen is encompassed for use in the generation of a panel of overlapping LFn-fusion or LF-fusion polypeptides. As an illustrative example only, where a target antigen is divided into 5 portions, the portions can be divided equally (i.e. each overlapping fragment is about 21 to 25% of the entire full length if the target antigen) or unequally (i.e. a target antigen can be divided into the following 5 overlapping fragments; fragment 1 is

about 25%, fragment 2 is about 5%, fragment 3 is about 35%, fragment 4 is about 10% and fragment 5 is about 25% of the size of the full length target antigen, provided each fragment overlaps with at least one other fragment).

[0149] Typically, a panel of fusion polypeptides, e.g., LFn-fusion polypeptides, with fragments of a target antigen substantially covers the entire length of the whole (or intact) target antigen polypeptide. Accordingly, they can be used to identify which polypeptide fragments of a whole target antigen elicit a CMI response.

[0150] As discussed herein, using *overlapping proteins* of a target antigen fused to an LF polypeptide, e.g., LFn, increases the specificity of the CMI response. For example, a CMI assay enables one to distinguish the difference between a subject that has been immunized against a pathogen and a subject who is infected with or has been exposed to a pathogen. In another example, the CMI assay can distinguish between the T cell targets. Accordingly, the diagnostic methods and CMI assay as disclosed herein provide a simple, cheap and rapid method to detect a CMI response to a target antigen and/or detect a subject affected with a pathology (such as a pathogen infection).

[0151] One of ordinary skill in the art can divide a whole target antigen into *overlapping proteins* of a target antigen, the coding sequences of which are fused to that of an LF polypeptide, e.g., LFn, to express a panel of LFn-fusion polypeptides comprising different but overlapping target antigens. By way of an illustrative example only, the TB-specific target antigen TB1 (CFP-10 also known as culture filtrate-10) comprising SEQ. ID. NO: 7 can be divided into, for example at least 17 fragments, such as those of SEQ. ID. NO: 30-46 shown in Table 1 to generate a panel of 17 different LFn-fusion polypeptides, the coding sequences of each comprising a different but overlapping TB-specific target antigen TB1 (CFP-10) fragment fused to the coding sequence of LFn. In another example, the TB-specific target antigen TB2 (ESAT-6, also known as "early secreted antigenic target-6 polypeptide") comprising SEQ. ID. NO: 6 can be divided into, for example at least 23 fragments, such as those of SEQ. ID. NO: 8-29 shown in Table 1 to generate a panel of 23 different LFn-fusion polypeptides, the coding sequences of each comprising a different but overlapping TB-specific target antigen TB2 (ESAT-6) fragment fused to the coding sequence of LFn.

[0152] In some embodiments, a fragment of a whole target antigen is substantially about half the size of the full length target antigen, or substantially about a third the size of the full length target antigen, or substantially about a quarter the size of the full length target antigen or less than substantially a quarter the size the full length target antigen. In all cases, fragments of a full length whole target antigen must overlap with at least one, but preferably more than one other fragment of the whole target antigen. By way of an example, the panel of 17 different LFn-fusion polypeptides comprising different fragments of the TB1 (CFP-10) polypeptide, each fragment overlaps with at least two or three other fragments. The amount of overlap can vary depending on the size of the fragments and the number of the fragments that the full length target antigen has been divided into. A typical amount of overlap between fragments can be about 10%, or about 20% or about 30% or about 40% or about 50% or about 60% or about 70% or about 80% or more, or any overlap in between about 10% and about 80%. By way of an example, for each of the different fragments of the TB1 (CFP-10) target antigen polypeptide shown in Table 1, approximately 73% of each fragment overlaps with each adjacent fragment (i.e. each fragments overlaps with the fragment before it and after it by about 73%).

[0153] Early secreted antigenic target (ESAT-6) is a 95 amino acid residue protein fragment from *M. tuberculosis*. The amino acid sequence is MTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWDATATELNNALQN LARTISEAGQAMASTEGNVTGMFA (SEQ. ID. NO. 6).

[0154] Culture filtrate protein (CFP-10) (Genbank AAC83445) is a 10 kDa,100 amino acid residue protein fragment from *M. tuberculosis*. It is also known as L45 antigen homologous protein (LHP). The amino acid sequence is MAEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQAAVVRFQEAANKQKQELD EISTNIRQAGVQYSRADEEQQQALSSQMGF (SEQ. ID. NO. 7).

[0155] Table 1. Fragments of whole TB-specific target antigens TB-1 (CFP-10) and TB-2 (EAST-6).

ESAT-6 (TB2)	SEQ ID NO:	6
CFP-10 (TB1)	SEQ ID NO:	7
RPMTEQQWNFAGIEA	SEQ ID NO:	8
EQQWNFAGIEAAASA	SEQ ID NO:	9
NFAGIEAAASAIQGN	SEQ ID NO:	10
IEAAASAIQGNVTSI	SEQ ID NO:	11
ASAIQGNVTSIHSLL	SEQ ID NO:	12
IQGNVTSIHSLLDEGK	SEQ ID NO:	13
TSIHSLLDEGKQSLT	SEQ ID NO:	14
SLLDEGKQSLTKLAA	SEQ ID NO:	15
EGKQSLTKLAAAWGG	SEQ ID NO:	16
SLTKLAAAWGGSGSE	SEQ ID NO:	17
LAAAWGGSGSEAYQG	SEQ ID NO:	18
WGGSGSEAYQGVQQK	SEQ ID NO:	19
GSEAYQGVQQKWDAT	SEQ ID NO:	20
YQGVQQKWDATATEL	SEQ ID NO:	21
VQQKWDATATELNNAL	SEQ ID NO:	22
DATATELNNALQNLA	SEQ ID NO:	23
TELNNALQNLARTIS	SEQ ID NO:	24
NALQNLARTISEAGQ	SEQ ID NO:	25
NLARTISEAGQAMAS	SEQ ID NO:	26
TISEAGQAMASTEGN	SEQ ID NO:	27
AGQAMASTEGNVTGM	SEQ ID NO:	28
MASTEGNVTGMFALE	SEQ ID NO:	29
RPLKNDAATLAQEAG	SEQ ID NO:	30
NDAATLAQEAGNFER	SEQ ID NO:	31
TLAQEAGNFERISGD	SEQ ID NO:	32
EAGNFERISGDLKTQ	SEQ ID NO:	33
FERISGDLKTQIDQV	SEQ ID NO:	34
SGDLKTQIDQVESTA	SEQ ID NO:	35
KTQIDQVESTAGSLQ	SEQ ID NO:	36
DQVESTAGSLQAQWR	SEQ ID NO:	37
STAGSLQAQWRGAAG	SEQ ID NO:	38
SLQAQWRGAAGTAAQ	SEQ ID NO:	39
AQWRGAAGTAAQAAVV	SEQ ID NO:	40
AAGTAAQAAVVRFQE	SEQ ID NO:	41
AAQAAVVRFQEAANK	SEQ ID NO:	42
AVVRFQEAANKQKAE	SEQ ID NO:	43
FQEAANKQKAELDEI	SEQ ID NO:	44
ANKQKAELDEISTNI	SEQ ID NO:	45
KAELDEISTNIRLE	SEQ ID NO:	46

[0156] In some embodiments, a target antigen polypeptide in any of fusion polypeptides described herein, e.g., LFn-fusion polypeptides, is any antigen associated with a pathology, for example an infectious disease or pathogen, or cancer or an immune disease such as an autoimmune disease.

[0157] One example of an infectious disease antigen is TbH9 (also known as Mtb 39A), a tuberculosis antigen. Other tuberculosis antigens include, but are not limited to, DPV (also known as Mtb8.4), 381, Mtb4l, Mtb40, Mtb32A, Mtb9.9A, Mtb9.8, Mtb16, Mtb72f, Mtb59f, Mtb88f, Mtb7lf, Mtb46f and Mtb31f ("f" indicates that it is a fusion of two or more proteins).

[0158] Unless otherwise explained, 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 disclosure belongs. Definitions of common terms in molecular biology can be found in Benjamin Lewin, Genes IX, published by Jones & Bartlett Publishing, 2007 (ISBN-13: 9780763740634); Kendrew et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[0159] Unless otherwise stated, the present invention can be performed using standard procedures, as described, for example in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Sambrook et al., Molecular Cloning: A Laboratory Manual (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (1989); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987));Current Protocols in Molecular Biology (CPMB) (Fred M. Ausubel, et al. ed., John Wiley and Sons, Inc.); Current Protocols in Protein Science (CPPS) (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.); Baculovirus Expression Protocols (Methods in Molecular Biology, Vol 39) by Christopher D. Richardson (Editor); Hardcover - 450 pages Spiral edition (March 1998) Humana Pr; ISBN: 0896032728; Baculovirus Expression Vectors : A Laboratory Manual by David R. O'Reilly, Lois Miller, Verne A. Luckow; Paperback Spiral edition (June 1994) Oxford Univ Press; ISBN: 0195091310; and The Baculovirus Expression System : A Laboratory Guide by Linda A. King, R.D. Possee; Hardcover (May 1992) Chapman & Hall; ISBN: 0412371502, which are all incorporated by reference herein in their entirety.

[0160] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may 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.

[0161] 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. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. 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."

[0162] All patents and other publications identified 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 present invention. 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.

Production of fusion polypeptide using a baculovirus system

[0163] In one embodiment, any of the fusion polypeptides described herein is produced by expression from a recombinant baculoviruses vector. In another embodiment, any of the fusion polypeptides described herein is expressed by an insect cell. In yet another embodiment, any of the fusion polypeptides described herein is isolated from an insect cell. There are several benefits of protein expression with baculovirus in insect cells, including high

expression levels, ease of scale-up, production of proteins with post-translational modifications, and simplified cell growth. Insect cells do not require CO_2 for growth and can be readily adapted to high-density suspension culture for large-scale expression. Many of the post-translational modification pathways present in mammalian systems are also utilized in insect cells, allowing the production of recombinant protein that is antigenically, immunogenically, and functionally similar to the native mammalian protein.

[0164] In one embodiment, the invention provides a method of preparing a composition for raising or detecting a cell-mediated immune (CMI) response to a target antigen polypeptide, the method comprising expressing a fusion polypeptide comprising a *B. anthracis* lethal factor N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to the target antigen polypeptide in an insect cell.

[0165] In one embodiment, the target antigen polypeptide is an intracellular pathogen polypeptide.

[0166] Baculoviruses are DNA viruses in the family *Baculoviridae*. These viruses are known to have a narrow host-range that is limited primarily to Lepidopteran species of insects (butterflies and moths). The baculovirus *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV), which has become the prototype baculovirus, replicates efficiently in susceptible cultured insect cells. AcNPV has a double-stranded closed circular DNA genome of about 130,000 base-pairs and is well characterized with regard to host range, molecular biology, and genetics.

[0167] Many baculoviruses, including AcNPV, form large protein crystalline occlusions within the nucleus of infected cells. A single polypeptide, referred to as a polyhedrin, accounts for approximately 95% of the protein mass of these occlusion bodies. The gene for polyhedrin is present as a single copy in the AcNPV viral genome. Because the polyhedrin gene is not essential for virus replication in cultured cells, it can be readily modified to express foreign genes. The foreign gene sequence is inserted into the AcNPV gene just 3' to the polyhedrin promoter sequence such that it is under the transcriptional control of the polyhedrin promoter.

[0168] The Baculovirus Expression Vector System (BEVS) is a safe and rapid method for the abundant production of recombinant proteins in insect cells and insects pioneered in the laboratory of Dr. Max D. Summers.

[0169] Baculovirus expression systems are powerful and versatile systems for high-level, recombinant protein expression in insect cells. Expression levels up to 500 mg/l have been reported using the baculovirus expression system, making it an ideal system for high-level expression. Recombinant baculoviruses that express foreign genes are constructed by way of homologous recombination between baculovirus DNA and chimeric plasmids containing the gene sequence of interest. Recombinant viruses can be detected by virtue of their distinct plaque morphology and plaque-purified to homogeneity.

[0170] Baculoviruses are particularly well-suited for use as eukaryotic cloning and expression vectors. They are generally safe by virtue of their narrow host range which is restricted to arthropods. The U.S. Environmental Protection Agency (EPA) has approved the use of three baculovirus species for the control of insect pests. AcNPV has been applied to crops for many years under EPA Experimental Use Permits.

[0171] AcNPV wild type and recombinant viruses replicate in a variety of insect cells, including continuous cell lines derived from the fall armyworm, *Spodoptera frugiperda* (Lepidoptera; *Noctuidae*). *S. frugiperda* cells have a population doubling time of 18 to 24 hours and can be propagated in monolayer or in free suspension cultures.

[0172] Recombinant fusion proteins described herein can be produced in insect cells including, but not limited to, cells derived from the Lepidopteran species *S. frugiperda*. Other insect cells that can be infected by baculovirus, such as those from the species *Bombyx mori*, *Galleria mellanoma*, *Trichplusia ni*, or *Lamanthria dispar*, can also be used as a suitable substrate to produce recombinant proteins described herein.

[0173] Baculovirus expression of recombinant proteins is well known in the art and is described in U. S. Patent Nos. 4,745,051, 4,879,236, 5,179,007, 5,516,657, 5,571,709, and 5,759,809, which are all incorporated by reference herein

in their entirety. It will be understood by those skilled in the art that the expression system is not limited to a baculovirus expression system. What is important is that the expression system directs the N-glycosylation of expressed recombinant proteins. The recombinant proteins described herein can also be expressed in other expression systems such as Entomopox viruses (the poxviruses of insects), cytoplasmic polyhedrosis viruses (CPV), and transformation of insect cells with the recombinant gene or genes constitutive expression.

[0174] The most common expression vector system is from the insect baculovirus *A. californica* nuclear polyhedrosis virus (AcNPV). AcNPV has a genome of ca. 130 kilobases (kb) of double-stranded, circular DNA and it is the most extensively studied baculovirus. Miller, L.K., J Virol. 1981, 39:973-976. AcNPV has a biphasic replication cycle and produces a different form of infectious virus during each phase. Between 10 and 24 h postinfection (p.i.), extracellular virus is produced by the budding of nucleocapsids through the cytoplasmic membrane. By 15 to 18 h p.i., nucleocapsids are enveloped within the nucleus and embedded in a paracrystalline protein matrix, which is formed from a single major protein called polyhedrin. In infected *S. frugiperda* (fall armyworm, Lepidoptera, *Noctuidae*) cells, AcNPV polyhedrin accumulates to high levels and constitutes 25% or more of the total protein mass in the cell; it may be synthesized in greater abundance than any other protein in a virus-infected eukaryotic cell.

[0175] In one embodiment, provided herein is a method of producing a fusion polypeptide comprising a *B. anthracis* lethal factor N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated, using a Baculovirus Expression Vector System (BEVS), comprising infecting lepidopteran insect cells with a recombinant baculovirus vector comprising a polynucleotide encoding the fusion polypeptide and culturing the insect cells to produce the fusion polypeptide.

[0176] In some embodiments, the method of producing fusion polypeptides using a Baculovirus Expression Vector System (BEVS) in lepidopteran insect cells uses *S. frugiperda* cells.

[0177] The gene encoding LF has been cloned and sequenced, and has been assigned Genbank accession no. M29081 (Robertson and Leppla, Gene 44:71 78 (1986); Bragg and Robertson, Gene 81:45 54 (1989); see also U. S. Patent Nos. 5,591,631 and 5,677,274; see generally Leppla, Anthrax Toxins, in Bacterial Toxins and Virulence Factors in Disease (Handbook of natural toxins, Vol. 8. Moss et al., eds., 1995).

[0178] The coding DNA sequences are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically cloning plasmids, (e. g. pPUC19, pBLUESCRIPT®-SK) or shuttle vectors that can be propagated in a number of different hosts and to allow more efficient manipulation of DNA (e. g. the pRS YCp and pRS Yip vectors can shuttle between bacteria and *Saccharomyces cerevisiae*).

[0179] The first step in the production of recombinant proteins from a BEVS is the construction of a recombinant baculovirus vector, either by homologous recombination or by site specific transposition. To obtain a recombinant baculovirus vector by homologous recombination, a baculovirus transfer vector is needed. A baculovirus transfer vector is a temporary vector whose sole purpose is to enable the insertion of foreign coding DNA, under an appropriate gene promoter, into the baculovirus genome at a site that would not affect normal viral replication. The baculovirus transfer vector comprises a portion of the baculovirus genomic sequence that spans the intended insertion site of the foreign coding DNA. The most common regions contain the polyhedrin or p10 gene. Both are dispensible for viral replication in cell culture and insect larvae and the production of infectious extracellular virus. Both proteins are highly expressed at the very late phase of viral replication and effect high level of transcription of the foreign gene when inserted back into the viral genome. A typical baculovirus transfer vector comprises a promoter, a transcriptional terminator, and most often native viral sequences and regions flanking both sides of the promoter that are homologous

to the target genes in the viral genome. The region between the promoter and the transcriptional terminator can have multiple restriction enzyme digestion sites for facilitating cloning of the foreign coding sequence, in this instance, the coding DNA sequence for an LF polypeptide, e.g., an LFn polypeptide and a target antigen. Additional sequences can be included, e.g., signal peptides and/or tag coding sequences,such as His-tag, MAT-Tag, FLAG tag, recognition sequence for enterokinase, honeybee melittin secretion signal, beta-galactosidase, glutathione S-transferase (GST) tag upstream of the MCS for facilitating the secretion, identification, proper insertion, positive selection of recombinant virus, and/or purification of the recombinant protein. Subsequent to the construction of the baculovirus transfer vector, it is mixed with AcNPV viral DNA and co-transfected into insect cells to establish an infection. The native polyhedrin gene is removed by a double-cross over homologous recombination event and replaced by the foreign coding sequence to be expressed in the insect cells. Inactivation of the polyhedrin gene by deletion or by insertion results in mutants that do not produce occlusions in infected cells. These occlusion-negative viruses form plaques that are different from plaques produced by wild-type viruses, and this distinctive plaque morphology is useful as a means to screen for recombinant viruses.

[0180] A good number of baculovirus transfer vectors and the corresponding appropriately modified host cells are commercially available, for example, pAcGP67, pAcSECG2TA, pVL1392, pVL1393, pAcGHLT, and pAcAB4 from BD Biosciences; pBAC-3, pBAC-6, pBACgus-6, and pBACsurf-1 from NOVAGEN®, and pPolh-FLAG and pPolh-MAT from SIGMA ALDRICH®. One skilled in the art would be able to clone and ligate the coding region of the *B. anthracis* lethal factor N-terminal (LFn) portion with the coding region of a target antigen polypeptide or fragment thereof to construct a chimeric coding sequence for a fusion polypeptide comprising LFn and the target antigen polypeptide or fragment thereof using specially designed oligonucleotide probes and polymerase chain reaction (PCR) methodologies that are well known in the art. One skilled in the art would also be able to clone and ligate the chimeric coding sequence for a fusion protein into a selected baculovirus transfer vector. The coding sequences of LFn and the target antigen polypeptide or fragment thereof should be ligated in-frame and the chimeric coding sequence should be ligated downstream of the promoter, and between the promoter and the transcription terminator. Subsequent to that, the recombinant baculovirus transfer vector is transfected into regular cloning *Escherichia coli,* such as XL1Blue. Tranformants *E. coli* harboring the recombinant transfer vector DNA is then selected by antibiotic resistance to remove any *E. coli* harboring the non-recombinant plasmid DNA. The selected *E. coli* is grown, and the the recombinant transfer vector DNA is purified for transfection into *S. frugiperda* (SF) cells.

[0181] As an example, the oligonucleotide 5'-GGAGGAACATATGGCGGGCGGTCATGGTGATG-3' (SEQ. ID. No. 48) can be used to introduce an *Nde*I site and serve as a forward primer in the amplification of the coding DNA sequence for LFn-(amino acids 1-263) and the oligonucleotide 5'-CTAGGATCCTTACCGTTGATCTTTAAGTTCTTCC-3' (SEQ. ID. No. 49) can be used to introduce a *Bam*HI site and act as the reverse primer. PCR amplification is performed using the cDNA template according to GenBank Accession No. M29081. The forward primers for LFn-(28-263), LFn-(33-263), LFn-(37-263), LFn-(40-263), and LFn-(43-263) can be designed accordingly permit the PCR amplification of the coding sequence of the appropriate truncated LFn and also introduce an *Nde*I site. If the target antigen is HIV gp120, for example, the oligonucleotide 5′-CCGCGTAAGTCCATGAGAGTGAAGGAGAAATATCAG-3′ (SEQ. ID. No. 50) can be used to introduce a *Bam*HI site and 5′-TTCGAGCTCGAGTTATCTTTTTTCTCTCTGCACCAC-3′ (SEQ. ID. No. 52) can be used to introduce a *Xho*I site when both are used in the amplification of the coding DNA sequence for gp120. The gene for gp120 is Genbank Accession No. AY775283 (Arora N, et. al., 1992, J Biol Chem 267:15542–15548). The sequences can introduce a stop sequence (TAA) after the gp120 coding sequence. The common *Bam*HI site at the end of the amplified coding sequence of LFn and at the beginning of the amplified coding sequence of gp120 facilitates the ligation of the

two separate amplified coding sequences into a chimeric or fusion coding sequence. The ligation of the two separate amplified coding sequences should be such that the gp120 is in frame with LFn and there is no translation stop codon around the ligation site. The fusion coding sequence can then be digested with *Nde*I and *Xho*I and ligated into a selected baculovirus transfer vector that has *Nde*I and *Xho*I sites with the appropriate orientation. The newly constructed baculovirus transfer vector can be transformed into *E.coli* DH5. *E. coli* transformants can be screened by digestion and verified by sequencing. After that, the baculovirus transfer vector can be isolated for co-transfection into insect cells for homologous recombination.

[0182] To obtain a recombinant baculovirus vector by site specific transposition, e. g. with Tn7 to insert foreign genes into bacmid DNA propagated in *E. coli.,* INVITROGEN™ Inc. provides the pFASTBAC™ plasmid and bacmid containing DH10BAC™ competent *E. coli* for constructing a recombinant baculovirus vector by site specific transposition. The coding sequence is cloned into a pFASTBAC™ plasmid and the recombinant plasmid is transformed into an DH10BAC™ competent *E. coli* harboring bacmid, a baculovirus shuttle vector, with a mini-*att*Tn7 target site and a helper plasmid. The mini-*att*Tn7 element on the pFASTBAC™ plasmid can transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by antibiotics selection and by blue/white screening, since the transposition results in the disruption of the LacZα gene that is flanked by the mini-*att*Tn7 target site on the bacmid. The bacmid is then harvested for transfection of insect cells.

[0183] In one embodiment, the fusion polypeptide described herein has a spacer peptide, e. g., a 14-residue spacer (GSPGISGGGGGILE) (SEQ. ID. No. 51) separating the LFn polypeptide from the target antigen polypeptide. The coding sequence of such a short spacer can be constructed be annealing a complementary pair of primers. One of skill in the art can design and synthesize oligonucleotides that will code for the selected spacer. Spacer peptides should generally have non-polar amino acid residues, such as, glycine and prolines.

[0184] In some instances, specific site-directed mutagenesis of the chimeric coding sequence in the baculovirus transfer vector can be performed to create specific amino acid mutations and substitutions to further promote transmembrane delivery, protein expression or protein folding. An example of an amino acid substitution is glutamate for aspartate. Site-directed mutagenesis can be carried out, e.g., using the QUIKCHANGE® site-directed mutagenesis kit from STRATAGENE® according to manufacture's instructions or any methods known in the art.

[0185] Standard viral DNA is used to co-transfect *S. frugiperda* (SF) cells. Putative recombinant viruses containing the recombinant molecules are isolated from the virus yield from these transfected monolayers. Because the polyhedrin structural gene has been removed, plaques containing the recombinant viruses can be easily identified since they lack occlusion bodies. Confirmation that these recombinants contain the desired chimeric coding sequence is established by methods well known in the art, such as hybridization with specific gene probes, plaque assays, and end point dilution.

[0186] A preferred host cell line for protein production from recombinant baculoviruses described herein is Sf900+. Another preferred host cell line for protein production from recombinant baculoviruses is Sf9. Sf900+ and Sf9 are non-transformed, non-tumorigenic continuous cell lines derived from the fall armyworm, *S. frugiperda* (Lepidoptera; Noctuidae).

[0187] Sf900+ and Sf9 cells are propagated at 28 ± 2°C without carbon dioxide supplementation. The culture medium used for Sf9 cells is TNMFH, a simple mixture of salts, vitamins, sugars and amino acids, supplemented with 10% fetal bovine serum. Aside from fetal bovine serum, no other animal derived products (i.e, trypsin, etc.) are used in cell propagation. Serum free culture medium (available as Sf900 culture media, GIBCO® BRL, Gaithersburg, Md.) can also be used to grow Sf9 cells and is preferred for propagation of Sf900+ cells. Sf9 cells have a population doubling

time of 18-24 hours and can be propagated in monolayer or in free suspension cultures. *S. frugiperda* cells have not been reported to support the replication of any known mammalian viruses.

[0188] Plaque assays of baculovirus transfected monolayers SF cells are well known in the art. Below is a standard protocol.

[0189] Reagents needed: Grace's Insect Medium, 2X (e.g. BD Biosciences GIBCO® catalog No. 11667), fetal bovine serum (heat inactivated), (e.g. BD Biosciences GIBCO® catalog No. 16140), 3% SEAPLAQUE® or other low-melting agarose in ddH_2O, sterile water, 50ml sterile conical screw-top tubes, and 37°C water bath microwave

[0190] Step one: Prepare infected monolayer of cells

1. Grow a suspension culture of Sf9 cells to a density of less than $3x10^6$.
2. Dilute this culture to a density of 5 to $6x10^5$.
3. For a 6-well culture dish, transfer 2 ml of this cell suspension to each well. For 6 cm dishes, double all volumes in this protocol. Scale by surface area. This cell number will depend somewhat on the cell line and can be adjusted up or down according to your results. If there is no confluent monolayer by day 3, increase the cell number. There should be space available on Day 2.
4. Let the cells settle for at least 30 min to ensure the cells are firmly attached.
5. Meanwhile, dilute the plaque virus to 1 ml aliquots at dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.
6. After SF cells have attached well to the plates, aspirate off the media.
7. Quickly add 1 ml of diluted virus to each well of the 6-well plate.
8. Transfer the plates to a rocking platform and slowly rock for at least two hours, four hours is preferred, though after this the benefit diminishes substantially.

[0191] Step Two. Prepare the overlay agarose just before use.

9. Mix as follows: 1 part 2X Grace's Medium supplemented with 20% Fetal Calf Serum and 1 part 3% SEAPLAQUE® Agarose in double distilled water (ddH_2O).
10. Melt the agarose completely.
11. Allow the agarose to cool slightly, to near 70°C and then aliquot 20 ml to each 50 ml conical tube.
12. Add 20 ml of room temp or warmer 2X Graces/FCS to each 20 ml aliquot of agarose, then place in a 35-37°C water bath.
13. Remove the overlay agarose from the water bath one tube at a time and check the temperature. Let it cool to at least 38°C, but preferably less than 37°C.

[0192] Step Three. Overlay agarose onto infected cell monolayer

14. When ready, tip up the plates and let the media inside drain to one side. Aspirate all the medium off.
15. Return the plate to level and quickly add approximately 3 ml of molten overlay mix to each well by allowing it to slide down the far wall of the well and onto the plate.
16. After overlaying the cells let the plates sit level in the hood for 30 or so minutes to dry a bit and solidify.
17. Place in a 27°C, 98% humidity controlled incubator for at least 3 days.

[0193] Step Four. Staining the Plates

18. Prepare a solution of 1% Neutral red. Ten mls of this will stain about 1000 assays.
19. Prepare an overlay agarose solution as above, but only prepare 1 ml for each assay.
20. Add 1/100th volume of the 1% Neutral Red solution to the molten agarose (e.g. 100 microliters to 10 ml).
21. Add approximately 1 ml of the Red Agarose to each well of a 6-well dish. Be sure the plate is level until the agarose sets up.
22. Add enough Red Agarose to cover the surface evenly.

23. Return the plate to the incubator for at least 4 hours. After several hours the plaques will begin to appear as clear spots among stained cells.
24. The plates can be left overnight before counting.
25. Controls can verify that longer incubations do not give higher titer results with the medium and cells used.

[0194] In one embodiment, the positive plaques can be identified by end point dilution assay (EPDA). A 96-well plate EPDA can be used to replace the plaque assay and plaque purification as a method for either determining viral titer or identifying and purifying recombinant virus. A modified 12-well plate EPDA can be used as a routine method for determining viral titer; it is useful for estimating the efficiency of the initial co-transfection, identifying infected cells, approximating viral titers, and amplifying viral titer. In the 12-well EPDA, individual wells containing equal amounts of insect cells are inoculated with 100, 10, 1 or 0 µl aliquots of the original transfection supernatant, wild-type virus, or recombinant XylE positive control viral supernatant. A visual comparison between cells in wells inoculated with 100, 10, 1 and 0 µl is used to estimate the viral titer.

[0195] For example, if cells receiving 100 µl of the initial co-transfection supernatant look infected in the EPDA, but cells receiving 10, 1 and 0 µl do not, then it is likely that the viral titer is low and should be amplified to produce a high titer stock. If wells receiving 100 µl of the original co-transfection supernatant look similar to those receiving 0 µl, it is likely that the original co-transfection did not result in a significant viral titer and must be repeated. When assaying the efficiency of a co-transfection or estimating the titer of a virus stock, if the EPDA shows a 10 fold decrease in the number of infected cells between dilutions, amplify the virus once or twice more to generate a high titer stock for protein production. However, if all three wells (100, 10, 1 µl) show equal signs of infection, the viral titer is high, ~ 2 x 10^8 plaque-forming units (pfu) /ml. High titer recombinant virus stocks are used for infection of cells at optimal multiplicity of infection (MOI = # of virus / # of cells) resulting in maximum protein production.

[0196] If the EPDA is used as an amplification step to generate a high titer stock, cross contamination between wells containing different viruses, e. g., the highly infectious wild-type virus used as a positive control, can be avoided by using separate 12 well plates.

[0197] EPDA controls are recommended. The recombinant virus from a pVL1392-XylE transfection is a particularly useful positive control. Infected cells producing the the XylE protein turn yellow in the presence of catechol and are easily identifiable. An example of a protocol for EPDA follows:

[0198] Protocol

1. Dilute log-phase Sf9 cells (with greater than 98% viability) to 1 x 10^5 cells /ml with fresh TNM-FH medium. Seed 1 x 10^5 Sf9 cells per well on a 12-well plate (BD FALCON™, Cat. No. 353043). Allow cells to attach firmly, approximately 10 min. Confirm 30% confluency by visualization on a light microscope. Replace medium with 1 ml fresh TNM-FH.
2. Add 100, 10, 1 and 0 µl of the recombinant virus supernatant obtained 5 days after the start of co-transfection (or other virus stock), to separate wells. Do the same for the positive control, e.g., pVL1392-XylE supernatant.
3. Incubate the cells at 27°C for three days. Examine the cells for signs of infection.
4. A successful transfection should result in uniformly large infected cells in the 100, 10, and 1 µl experimental wells.
5. If only the 100 µl and 10 µl wells seem to have infected cells and the 1 µl well looks more like the control, the titer of virus supernatant is low. Amplify the virus an additional time before proceeding with protein production.

[0199] Protein production can be analyzed by western blot analysis (if antibodies are available) or by Coomassie blue-stained SDS-PAGE gel by harvesting cells from the 100 µl well and lysing in appropriate lysing buffer.

[0200] The virus supernatant from the 100 µl well can be kept as the first viral amplification stock, however care should be taken to avoid cross-contamination between wells containing different virus.

[0201] To further purify the virus population, a plaque assay purification of the co-transfection supernatant can be performed using the approximate titer obtained from the EPDA.

[0202] Once recombinant baculoviral vectors that express the proteins are established, then the virus can be amplified and purified for infection of SF cells.

[0203] Purification of Virus. Viral particles produced from the first passage are purified from the media using a known purification method such as sucrose density gradient centrifugation. For example, virus is harvested 24-48 hours post infection by centrifuging media of infected cells. The resulting viral pellet is resuspended in buffer and centrifuged through a buffered sucrose gradient. The virus band is harvested from the 40-45% sucrose region of the gradient, diluted with buffer and pelleted by centrifugation at 100,000×g. The purified virus pellet is resuspended in buffer and stored at -70°C or used in large scale infection of cells for protein production.

[0204] The infection process, including viral protein synthesis, viral assembly and partial cell lysis can be complete by approximately 72 hours post-infection. This can be protein dependent and thus can occur earlier or later. The proteins produced in infected cells can be radiolabeled with ^{35}S-methionine, ^{3}H-leucine, or ^{3}H-mannose and both cell-associated and cell-free polypeptides can be analyzed by electrophoresis on polyacrylamide gels to determine their molecular weight. The expression of these products can also be examined at different times post-infection, prior to cell lysis.

[0205] Immunological identification of expressed fusion polypeptides can be examined, e.g., by either direct immunoprecipitation or by Western blots. For Western blots, cell-associated proteins or the proteins in the media are separated on SDS polyacrylamide gels, transferred onto nitrocellulose or nylon filters, and identified with antiserum to the LF polypeptide or target antigen proteins or to the polyhedrin. Specifically bound antibody is detected by incubating the filters with ^{125}I-labeled protein A or enzyme conjugated anti-antibody, and followed by exposure to X-ray film at -80°C with intensifying screens or colorimetic reaction with enzyme substrate.

[0206] Having confirmed the identity of the expressed fusion polypeptides, the next step is to purify the proteins for uses and compositions described herein, e. g. evaluation for use as vaccines (e. g. protective/prophylactic or therapeutic vaccination) or screening agents. If the fusion polypeptides described herein are designed with secretion signal peptides, the encoded polypeptides are often released into the cell culture medium. Media from these infected cells can be concentrated and the proteins purified using standard methods. Salt precipitation, sucrose gradient centrifugation and chromatography, high or fast pressure liquid chromatography (HPLC or FPLC) can be used because these methods allow rapid, quantitative and large scale purification of proteins, and do not denature expressed products.

[0207] The efficiency of synthesis of the desired gene product is dependent on multiple factors including: (1) the choice of an expression vector system; (2) the number of gene copies that will be available in the cells as templates for the production of mRNA; (3) the promoter strength; (4) the stability and structure of the mRNA; (5) the efficient binding of ribosomes for the initiation or translation; (6) the properties of the protein product, such as, the stability of the gene product or lethality of the product to the host cells; and (7) the ability of the system to synthesize and export the protein from the cells, thus simplifying subsequent analysis, purification and use.

Production of fusion polypeptide using other expression systems

[0208] The fusion polypeptides described herein can all be synthesized and purified by protein and molecular methods that are well known to one skilled in the art. Preferably molecular biology methods and recombinant heterologous protein expression systems are used. For example, recombinant protein can be expressed in mammalian, insect, yeast, or plant cells.

[0209] Some examples of recombinant cloning and truncation of LF, LFn, their expression, and specific site mutations and insertions are described, for example, in WO/2002/079417, WO/2008/048289, U. S. Patent application No. 20040166120, Huyen Cao, et. al., 2002, The Journal of Infectious Diseases;185:244–251; N. Kushner, et. al., 2003, Proc. Natl. Acad. Sci. U S A. 100: 6652–6657; Ballard, J. D., et. al., 1996, Proc. Natl. Acad. Sci. USA 93, 12531-12534; and Goletz, T. J. et al., 1997, Proc. Natl. Acad. Sci. USA 94, 12059-12064, all of which are incorporated herein by reference in their entirety. Approaches similar to those described in these references can be used to produce the fusion polypeptides as described herein.

[0210] In one embodiment, provided herein is an isolated polynucleotide encoding a fusion polypeptide described herein. Conventional polymerase chain reaction (PCR) cloning techniques can be used to construct a chimeric or fusion coding sequence encoding a fusion polypeptide as described herein. A fusion coding sequence can be cloned into a general purpose cloning vector such as pUC19, pBR322 , p BLUESCRIPT® vectors (STRATAGENE® Inc.) or pCR TOPO® from INVITROGEN™ Inc. The resultant recombinant vector carrying the nucleic acid encoding a fusion polypeptide as described herein can then be used for further molecular biological manipulations such as site-directed mutagenesis to create a variant fusion polypeptide as described herein or can be subcloned into protein expression vectors or viral vectors for protein synthesis in a variety of protein expression systems using host cells selected from the group consisting of mammalian cell lines, insect cell lines, yeast, bacteria, and plant cells.

[0211] Each PCR primer should have at least 15 nucleotides overlapping with its corresponding templates at the region to be amplified. The polymerase used in the PCR amplification should have high fidelity such as STRATAGENE®'s *Pfu*ULTRA® polymerase for reducing sequence mistakes during the PCR amplification process. For ease of ligating several separate PCR fragments together, for example in the construction of a fusion polypeptide, and subsequently inserting into a cloning vector, the PCR primers should also have distinct and unique restriction digestion sites on their flanking ends that do not anneal to the DNA template during PCR amplification. The choice of the restriction digestion sites for each pair of specific primers should be such that the fusion polypeptide coding DNA sequence is in-frame and will encode the fusion polypeptide from beginning to end with no stop codons. At the same time the chosen restriction digestion sites should not be found within the coding DNA sequence for the fusion polypeptide. The coding DNA sequence for the fusion polypeptide can be ligated into cloning vector pBR322 or one of its derivatives, for amplification, verification of fidelity and authenticity of the chimeric coding sequence, substitutions/or specific site-directed mutagenesis for specific amino acid mutations and substitutions in the fusion polypeptide.

[0212] Accordingly, provided herein is a recombinant vector comprising a fusion polypeptide described herein.

[0213] Alternatively the coding DNA sequence for the fusion polypeptide can be PCR cloned into a vector using for example, INVITROGEN™ Inc.'s TOPO® cloning method comprising topoisomerase-assisted TA vectors such as pCR-TOPO®, pCR-Blunt II-TOPO®, pENTR/D-TOPO®, and pENTR/SD/D-TOPO®. Both pENTR/D-TOPO®, and pENTR/SD/D-TOPO® are directional TOPO entry vectors which allow the cloning of the DNA sequence in the 5'→3' orientation into a GATEWAY® expression vector. Directional cloning in the 5'→3' orientation facilitates the unidirectional insertion of the DNA sequence into a protein expression vector such that the promoter is upstream of the 5' ATG start codon of the fusion polypeptide coding DNA sequence, enabling promoter driven protein expression. The recombinant vector carrying the coding DNA sequence for the fusion polypeptide can be transfected into and propagated in general cloning *E. coli* such as XL1Blue, SURE® (STRATAGENE®) and TOP-10 cells (INVITROGEN™ Inc.).

[0214] Standard techniques known to those of skill in the art can be used to introduce mutations (to create amino acid substitutions in the polypeptide sequence of the fusion polypeptide described herein, e. g., in the LFn polypeptide,

i. e. SEQ. ID. No. 3 or 4 or 5) in the nucleotide sequence encoding the fusion polypeptide described herein, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, the variant fusion polypeptide has less than 50 amino acid substitutions, less than 40 amino acid substitutions, less than 30 amino acid substitutions, less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions relative to the fusion polypeptides described herein.

[0215] Certain silent or neutral missense mutations can also be made in the DNA coding sequence that do not change the encoded amino acid sequence or the capability to promote transmembrane delivery. These types of mutations are useful to optimize codon usage, or to improve recombinant protein expression and production.

[0216] Specific site-directed mutagenesis of a coding sequence for the fusion polypeptide in a vector can be used to create specific amino acid mutations and substitutions. Site-directed mutagenesis can be carried out using, e. g. the QUIKCHANGE® site-directed mutagenesis kit from STRATAGENE® according to the manufacturer's instructions.

[0217] In one embodiment, disclosed herein are expression vectors comprising the coding DNA sequence for the fusion polypeptides described herein for the expression and purification of the recombinant fusion polypeptide produced from a protein expression system using host cells selected from, e.g., mammalian, insect, yeast, or plant cells. The expression vector is, preferably, a vector having the transcription promoter selected from a group consisting of CMV (cytomegalovirus) promoter, RSV (Rous sarcoma virus) promoter, β-actin promoter, SV40 (simian virus 40) promoter and muscle creatine kinase promoter, and the transcription terminator selected from a group consisting of SV40 poly (A) and BGH terminator; more preferably, an expression vector having the early promoter/enhancer sequence of cytomegalovirus and the adenovirus tripartite leader/intron sequence and containing the replication orgin and poly (A) sequence of SV40. The expression vector should have the necessary 5' upstream and 3' downstream regulatory elements such as promoter sequences, ribosome recognition and TATA (SEQ. ID. NO. 53) box, and 3' UTR AAUAAA (SEQ. ID. NO. 54) transcription termination sequence for efficient gene transcription and translation in its respective host cell. The expression vector can have additional sequence such as 6X-histidine, V5, thioredoxin, glutathione-S-transferase, c-Myc, VSV-G, HSV, FLAG, maltose binding peptide, metal-binding peptide, HA and "secretion" signals (Honeybee melittin, α-factor, PHO, Bip), which are incorporated into the expressed fusion polypeptide. In addition, there can be enzyme digestion sites incorporated after these sequences to facilitate enzymatic removal of them after they are not needed. These additional sequences are useful for the detection of fusion polypeptide expression, for protein purification by affinity chromatography, enhanced solubility of the recombinant protein in the host cytoplasm, and/or for secreting the expressed fusion polypeptide out into the culture media or the spheroplast of the yeast cells. The expression of the fusion polypeptide can be constitutive in the host cells or it can be induced, e.g., with copper sulfate, sugars such as galactose, methanol, methylamine, thiamine, tetracycline, infection with baculovirus, and (isopropyl-beta-D-thiogalactopyranoside) IPTG, a stable synthetic analog of lactose.

[0218] In one embodiment, the recombinant vector comprising a fusion polypeptide described herein is an expression vector that facilitates protein expression. In another embodiment, the expression vector comprising a fusion polypeptide described herein is a viral vector, such as adenovirus, adeno-associated virus (AAV), retrovirus, baculovirus and lentivirus vectors, among others. The expression vectors can be viral or non-viral vectors. Recombinant viruses provide a versatile system for gene expression studies and therapeutic applications.

[0219] The fusion polypeptides described herein can be expressed in a variety of expression host cells e.g., yeasts, mammalian cells, insect cells and plant cells such as *Chlamadomonas,* or even in cell-free expression systems. From the cloning vector, the nucleic acid can be subcloned into a recombinant expression vector that is appropriate for the

expression of fusion polypeptide in mammalian, insect, yeast, or plant cells or a cell-free expression system such as a rabbit reticulocyte expression system. Some vectors are designed to transfer coding nucleic acid for expression in mammalian cells, insect cells and year in one single recombination reaction. For example, some of the GATEWAY® (INVITROGEN™ Inc.) destination vectors are designed for the construction of baculovirus, adenovirus, adeno-associated virus (AAV), retrovirus, and lentiviruses, which upon infecting their respective host cells, permit heterologous expression of fusion polypeptides in the appropriate host cells. Transferring a gene into a destination vector is accomplished in just two steps according to manufacturer's instructions. There are Gateway® expression vectors for protein expression in insect cells, mammalian cells, and yeast. Following transformation and selection in *E. coli,* the expression vector is ready to be used for expression in the appropriate host.

[0220] Examples of other expression vectors and host cells are the strong CMV promoter-based pcDNA3.1 (INVITROGEN™ Inc.) and pCIneo vectors (Promega) for expression in mammalian cell lines such as CHO, COS, HEK-293, Jurkat, and MCF-7; replication incompetent adenoviral vector vectors pADENO™-X, pAd5F35, pLP-ADENO™-X-CMV (CLONTECH®), pAd/CMV/V5-DEST, pAd-DEST vector (INVITROGEN™ Inc.) for adenovirus-mediated gene transfer and expression in mammalian cells; pLNCX2, pLXSN, and pLAPSN retrovirus vectors for use with the RETRO-X™ system from CLONTECH® for retroviral-mediated gene transfer and expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (INVITROGEN™ Inc.) for lentivirus-mediated gene transfer and expression in mammalian cells; adenovirus-associated virus expression vectors such as pAAV-MCS, pAAV-IRES-hrGFP, and pAAV-RC vector (STRATAGENE®) for adeno-associated virus-mediated gene transfer and expression in mammalian cells; BACpak6 baculovirus (CLONTECH®) and pFastBac™ HT (INVITROGEN™ Inc.) for the expression in *Spodopera frugiperda* 9 (Sf9), Sf11, Tn-368 and BTI-TN-5B4-1 insect cell lines; pMT/BiP/V5-His (INVITROGEN™ Inc.) for the expression in *Drosophila Schneider* S2 cells; Pichia expression vectors pPICZα, pPICZ, pFLDα and pFLD (INVITROGEN™ Inc.) for expression in *Pichia pastoris* and vectors pMETα and pMET for expression in *P. methanolica*; and pYES2/GS and pYD1 (INVITROGEN™ Inc.) vectors for expression in yeast *S. cerevisiae.* Recent advances in the large scale expression heterologous proteins in *Chlamydomonas reinhardtii* are described by Griesbeck C. et. al. 2006 Mol. Biotechnol. 34:213-33 and Fuhrmann M. 2004, Methods Mol Med. 94:191-5. Foreign heterologous coding sequences are inserted into the genome of the nucleus, chloroplast and mitochondria by homologous recombination. The chloroplast expression vector p64 carrying the most versatile chloroplast selectable marker aminoglycoside adenyl transferase (aadA), which confer resistance to spectinomycin or streptomycin, can be used to express foreign protein in the chloroplast. The biolistic gene gun method can be used to introduce the vector in the algae. Upon its entry into chloroplasts, the foreign DNA is released from the gene gun particles and integrates into the chloroplast genome through homologous recombination.

[0221] In some embodiments, the fusion polypeptides described herein are expressed from viral infection of mammalian cells. The viral vectors can be adenovirus, adeno-associated virus (AAV), retrovirus, and lentivirus. A simplified system for generating recombinant adenoviruses is presented by He TC. et. al. Proc. Natl. Acad. Sci. USA 95:2509-2514, 1998. The gene of interest is first cloned into a shuttle vector, e.g. pAdTrack-CMV. The resultant plasmid is linearized by digesting with restriction endonuclease *Pme* I, and subsequently cotransformed into E. coli. BJ5183 cells with an adenoviral backbone plasmid, e.g. pADEASY-1 of STRATAGENE®'s ADEASY™ Adenoviral Vector System. Recombinant adenovirus vectors are selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analyses. Finally, the linearized recombinant plasmid is transfected into adenovirus packaging cell lines, for example HEK 293 cells (E1-transformed human embryonic kidney cells) or 911 (E1-

transformed human embryonic retinal cells) (Human Gene Therapy 7:215-222, 1996). Recombinant adenovirus are generated within the HEK 293 cells.

[0222] Recombinant lentivirus has the advantage of delivery and expression of fusion polypeptides in either dividing and non-dividing mammalian cells. The HIV-1 based lentivirus can effectively transduce a broader host range than the Moloney Leukemia Virus (MoMLV)-base retroviral systems. Preparation of the recombinant lentivirus can be achieved using, for example, the pLenti4/V5-DEST™, pLenti6/V5-DEST™ or pLenti vectors together with VIRAPOWER™ Lentiviral Expression systems from INVITROGEN™ Inc.

[0223] Recombinant adeno-associated virus (rAAV) vectors are applicable to a wide range of host cells including many different human and non-human cell lines or tissues. rAAVs are capable of transducing a broad range of cell types and transduction is not dependent on active host cell division. High titers, > 10^8 viral particle/ml, are easily obtained in the supernatant and 10^{11} -10^{12} viral particle/ml with further concentration. The transgene is integrated into the host genome so expression is long term and stable.

[0224] Large scale preparation of AAV vectors is made by a three-plasmid cotransfection of a packaging cell line: AAV vector carrying the coding nucleic acid, AAV RC vector containing AAV rep and cap genes, and adenovirus helper plasmid pDF6, into 50 x 150 mm plates of subconfluent 293 cells. Cells are harvested three days after transfection, and viruses are released by three freeze-thaw cycles or by sonication.

[0225] AAV vectors can be purified by two different methods depending on the serotype of the vector. AAV2 vector is purified by the single-step gravity-flow column purification method based on its affinity for heparin (Auricchio, A., et. al., 2001, Human Gene therapy 12:71-6; Summerford, C. and R. Samulski, 1998, J. Virol. 72:1438-45; Summerford, C. and R. Samulski, 1999, Nat. Med. 5: 587-88). AAV2/1 and AAV2/5 vectors are currently purified by three sequential CsCl gradients.

[0226] The fusion polypeptides described herein can be expressed and purified by a variety methods known to one skilled in the art, for example, the fusion polypeptides described herein can be purified from any suitable expression system. Fusion polypeptides can be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (see, e.g., Scopes, Protein Purification: Principles and Practice (1982); U. S. Patent No. 4,673,641; Ausubel et al., supra; and Sambrook et al. supra).

[0227] A number of procedures can be employed when recombinant proteins are purified. For example, proteins having established molecular adhesion properties can be reversibly fused to the protein of choice. With the appropriate ligand, the protein can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, the protein of choice can be purified using affinity or immunoaffinity columns.

[0228] After the protein is expressed in the host cells, the host cells can be lysed to liberate the expressed protein for purification. Methods of lysing the various host cells are featured in "Sample Preparation-Tools for Protein Research" EMD Bioscience and in the Current Protocols in Protein Sciences (CPPS). A preferred purification method is affinity chromatography such as metal-ion affinity chromatograph using nickel, cobalt, or zinc affinity resins for histidine-tagged fusion polypeptides. Methods of purifying histidine-tagged recombinant proteins are described by CLONTECH® using their TALON® cobalt resin and by NOVAGEN® in their pET system manual, 10th edition. Another preferred purification strategy is immuno-affinity chromatography, for example, anti-myc antibody conjugated resin can be used to affinity purify myc-tagged fusion polypeptides. When appropriate protease recognition sequences are present, fusion polypeptides can be cleaved from the histidine or myc tag, releasing the fusion polypeptide from the affinity resin while the histidine-tags and myc-tags are left attached to the affinity resin.

[0229] Standard protein separation techniques for purifying recombinant and naturally occurring proteins are well known in the art, e. g. solubility fractionation, size exclusion gel filtration, and various column chromatography.

[0230] Solubility fractionation: Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

[0231] Size exclusion filtration: The molecular weight of the protein of choice can be used to isolate it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, AMICON® or MILLIPORE® membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

[0232] Column chromatography: The protein of choice can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against recombinant or naturally occurring proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech). For example, LFn can be purified using a PA63 heptamer affinity column (Singh et al., J. Biol. Chem. 269:29039 29046 (1994)).

[0233] In some embodiments, a combination of purification steps comprising, for example: (i) anion exchange chromatography, (ii) hydroxyapatite chromatography, (iii) hydrophobic interaction chromatography, and (iv) size exclusion chromatography can be used to purify the fusion polypeptides described herein.

[0234] Cell-free expression systems are also contemplated. Cell-free expression systems offer several advantages over traditional cell-based expression methods, including the easy modification of reaction conditions to favor protein folding, decreased sensitivity to product toxicity and suitability for high-throughput strategies such as rapid expression screening or large amount protein production because of reduced reaction volumes and process time. The cell-free expression system can use plasmid or linear DNA. Moreover, improvements in translation efficiency have resulted in yields that exceed a milligram of protein per milliliter of reaction mix. Commercially available cell-free expression systems include the TNT coupled reticulocyte lysate systems (Promega) which uses rabbit reticulocyte-based in-vitro system.

Applications of fusion polypeptides

[0235] The fusion polypeptides described herein, e.g., LFn fusion polypeptides, have many uses. The fusion polypeptide can used in a vaccine composition for immunization of a subject against a specific pathogen (protective vaccination) or for therapeutic treatment for ailments such as cancer, Alzheimer's disease, Creutzfeldt-Jakob disease

(CJD), variant Creutzfeldt-Jakob disease (vCJD), 'Kuru' or scrapie. The fusion polypeptide can be also used in screening for exposure to pathogens, for example, in a CMI response assay. The fusion polypeptides provide a strategy of introducing an antigen into an intact cell. The fusion polypeptides also provide a strategy to induce an immune response in a subject. The fusion polypeptides permit an antigen to enter into a cell, facilitate the display of the antigen or fragments thereof by MHC molecules so as to induce an immune response in a subject and thereby produce immunity against a pathogen having that antigen.

[0236] In one embodiment, the invention provides a method of introducing an intracellular pathogen target antigen polypeptide to a mammalian cell, the method comprising contacting a fusion polypeptide described herein with a mammalian cell. In one embodiment, the intracellular pathogen target antigen polypeptide is fused to a *B. anthracis* lethal factor N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell. In one embodiment, the cell is a mammalian cell *in vivo* and the method comprises administering a fusion polypeptide to the mammal. A polypeptide normally cannot traverse the plasma membrane and enter an intact cell on its own. There are several contributing factors, the size of the polypeptide for one. Proteins in aqueous solutions have their polar amino acid residues on the outside of the folded structure and the non-polar amino acid residues on the inside. The lipid bi-layer of the plasma membrane, being non-charged, repels the externally charged protein, prevents translocation of the protein across the membrane. Protein can enter a cell by a number of ways, via protein channels which require expenditure of energy or via specific cell surface receptor mediated phagocytosis and/or endocytosis, both of which also require expenditure of energy. Since the LFn polypeptide can itself traverse the plasma membrane and enter an intact cell on its own, any polypeptide fused to LFn can also be transported into the cell by virtue of the physical linkage to LFn polypeptide. This method is applicable to any target antigen polypeptide, including, e.g., any intracellular pathogen antigen polypeptide. In other words, as long as a protein is to be delivered in an intact cell, this method can be used to achieve that goal, i. e., making a fusion polypeptide comprising a *B. anthracis* lethal factor N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide. No special knowledge of specific protein channels or specific cell surface receptor for the target antigen polypeptide is needed. In one embodiment, the fusion polypeptide is N-glycosylated. To introduce that antigen polypeptide to a mammalian cell, the fusion polypeptides described herein comprising the antigen polypeptide can be simply mixed and contacted with the mammalian cell. In a mammalian subject, the fusion polypeptide comprising the target antigen polypeptide can be administered to the subject. Topical and systemic routes of administration are possible, e. g., parenteral, nasal inhalation, intratracheal, intrathecal, intracranial, intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-arterial, intrasynovial, oral, and intrarectal. A composition comprising a fusion polypeptide described herein in a sterile saline solution can be administered intravenously.

[0237] In one embodiment, the invention provides a method of raising a cell-mediated immune (CMI) response to a target antigen polypeptide, the method comprising administering to a mammal a fusion polypeptide comprising a *B. anthracis* lethal factor N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated. Preferably, the fusion polypeptide described herein is formulated as a vaccine composition for administering to a mammal. In some aspects, any of the LFn-fusion polypeptide described herein can be used in a vaccine composition for immunization of a subject against a specific pathogen., Plotkin and Mortimer (In 'Vaccines', 1994, W.B. Saunders Company; 2nd edition (1994)) provide antigens which can be used to vaccinate animals or humans to induce an immune response specific for particular pathogens, as well as methods of preparing antigen, determining a suitable dose of antigen, assaying for induction of an immune response, and treating infection by a pathogen (e.g., bacterium, virus, fungus, or parasite). Accordingly, in one embodiment, provided herein is a vaccine composition comprising a fusion polypeptide

comprising a *B. anthracis* lethal factor N-terminal (LFn) polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein the fusion polypeptide is N-glycosylated.

[0238] In one embodiment, the vaccine composition comprises a fusion polypeptide that is expressed and purified from insect cells. In one embodiment, the vaccine composition comprises a plurality of fusion polypeptides that are expressed and purified from insect cells, wherein the target antigen polypeptide of each of the plurality of fusion polypeptides is different but all are from a single intracellular pathogen. In one embodiment, the target antigen polypeptide of each of the plurality of fusion polypeptides are all from a single polypeptide from a single intracellular pathogen. In one embodiment, the vaccine composition comprises a plurality of fusion polypeptides that are expressed and purified from insect cells, wherein the target antigen polypeptide of each of the plurality of fusion polypeptides is different but all are from several intracellular pathogens. For example, a vaccine composition raising a cell-mediated immune (CMI) response to mumps, measles and rubella viruses can have at least three different fusion polypeptides, each having target antigen polypeptide to the mumps, measles and rubella viruses.

[0239] In another embodiment, the vaccine composition comprises the fusion polypeptide wherein the LFn polypeptide is N-glycosylated. The N-glycosylation can be at asparagine 62, 212 and/or 286.

[0240] In some embodiments, the vaccine composition described herein further comprises an adjuvant. Adjuvants are a heterogeneous group of substances that enhance the immunological response against an antigen that is administered simultaneously. In some instances, adjuvants are added to a vaccine to improve the immune response so that less vaccine is needed. Adjuvants serve to bring the antigen—the substance that stimulates the specific protective immune response—into contact with the immune system and influence the type of immunity produced, as well as the quality of the immune response (magnitude or duration). Adjuvants can also decrease the toxicity of certain antigens; and provide solubility to some vaccine components. Almost all adjuvants used today for enhancement of the immune response against antigens are particles or form particles together with the antigen. In the book "Vaccine Design—the subunit and adjuvant approach" (Ed: Powell & Newman, Plenum Press, 1995) almost all known adjuvants are described both regarding their immunological activity and regarding their chemical characteristics. The type of adjuvants that do not form particles are a group of substances that act as immunological signal substances and that under normal conditions consist of the substances that are formed by the immune system as a consequence of the immunological activation after administration of particulate adjuvant systems.

[0241] Using particulate systems as adjuvants, the antigens are associated or mixed with or to a matrix, which has the characteristics of being slowly biodegradable. Of great importance using such matrix systems are that the matrices do not form toxic metabolites. Choosing from this point of view, the main kinds of matrices that can be used are mainly substances originating from a body. These include lactic acid polymers, poly-amino acids (proteins), carbohydrates, lipids and biocompatible polymers with low toxicity. Combinations of these groups of substances originating from a body or combinations of substances originating from a body and biocompatible polymers can also be used. Lipids are the preferred substances since they display structures that make them biodegradable as well as the fact that they are a critical element in all biological membranes.

[0242] Adjuvants for vaccines are well known in the art. Examples include, but not limited to, monoglycerides and fatty acids (e. g. a mixture of mono-olein, oleic acid, and soybean oil); mineral salts, e.g., aluminium hydroxide and aluminium or calcium phosphate gels; oil emulsions and surfactant based formulations, e.g., MF59 (microfluidised detergent stabilised oil-in-water emulsion), QS21 (purified saponin), AS02 [SBAS2] (oil-in-water emulsion + MPL + QS-21), Montanide ISA-51 and ISA-720 (stabilised water-in-oil emulsion); particulate adjuvants, e.g., virosomes (unilamellar liposomal vehicles incorporating influenza haemagglutinin), AS04 ([SBAS4] Al salt with MPL), ISCOMS (structured complex of saponins and lipids), polylactide co-glycolide (PLG); microbial derivatives (natural and

synthetic), e.g., monophosphoryl lipid A (MPL), Detox (MPL + M. Phlei cell wall skeleton), AGP [RC-529] (synthetic acylated monosaccharide), DC_Chol (lipoidal immunostimulators able to self organize into liposomes), OM-174 (lipid A derivative), CpG motifs (synthetic oligonucleotides containing immunostimulatory CpG motifs), modified LT and CT (genetically modified bacterial toxins to provide non-toxic adjuvant effects); endogenous human immunomodulators, e.g., hGM-CSF or hIL-12 (cytokines that can be administered either as protein or plasmid encoded), Immudaptin (C3d tandem array) and inert vehicles, such as gold particles. Newer adjuvants are described in U. S. Patent No. 6,890,540, U. S. Patent Application No. 20050244420, and PCT/SE97/01003, the contents of which are incorporated herein by reference in their entirety.

[0243] In some embodiments, the vaccine composition described herein further comprises pharmaceutical excipients including, but not limited to biocompatible oils, physiological saline solutions, preservatives, osmotic pressure controlling agents, carrier gases, pH-controlling agents, organic solvents, hydrophobic agents, enzyme inhibitors, water absorbing polymers, surfactants, absorption promoters and anti-oxidative agents.

[0244] In some embodiments, fusion polypeptides described herein can be solubilized in water, a solvent such as methanol, or a buffer. Suitable buffers include, but are not limited to, phosphate buffered saline Ca^{2+}/Mg^{2+} free (PBS), normal saline (150 mM NaCl in water), and Tris buffer. Fusion polypeptides described herein that are not soluble in neutral buffer can be solubilized in 10 mM acetic acid and then diluted to the desired volume with a neutral buffer such as PBS. In the case of fusion polypeptides soluble only at acid pH, acetate-PBS at acid pH may be used as a diluent after solubilization in dilute acetic acid. Glycerol can be a suitable non-aqueous buffer for use in the present invention.

[0245] If the fusion polypeptide is not soluble per se, the fusion polypeptide can be present in the formulation in a suspension or even as an aggregate. In some embodiments, hydrophobic fusion polypeptide can be solubilized in a detergent, for example a polypeptide containing a membrane-spanning domain. Furthermore, for formulations containing liposomes, an antigen in a detergent solution (e.g., a cell membrane extract) may be mixed with lipids, and liposomes then may be formed by removal of the detergent by dilution, dialysis, or column chromatography.

[0246] In one embodiment, the invention provides a composition comprising a fusion polypeptide described herein and an isolated mammalian cell. The isolated cell is preferably capable of processing and presenting target antigen fragments for display with MHC molecules. Such antigen-presenting cells can express MHC class I molecules only (so-called "non-professional" antigen presenting cells (APCs)), or MHC Class I and class II molecules (so-called "professional" APCs). Thus, in one embodiment, the mammalian cell is an antigen presenting cell, including a professional APC and/or a non-professional APC. Professional APCs include, e. g., macrophages, dendritic cells and B cells. In one embodiment, the fusion polypeptide is expressed and purified from insect cells. In one embodiment, the cells are isolated from a subject who may have been exposed to a pathogen. Such a composition is useful in screening for exposure to pathogens, such as a CMI response assay or for a mass vaccination program. CMI assays are known in the art, for example, in U. S. Patent Application 20050014205, WO/1987/005400, U. S. Patent 5,674,698 and commercially available kits such as IMMUNKNOW® CYLEX Immune cell function assay Product No. 4400.

[0247] In one embodiment, the invention provides a kit comprising a fusion polypeptide described herein and packaging materials therefor. Such a kit is useful in screening for exposure to pathogens, such as in a CMI response assay, or for a mass vaccination program. Packaging materials can include, but not limited to adjuvants, diluents, alcohols wipes for disinfecting the site of injection, disposable fix volume syringes, dosage chart and immunization schedule.

[0248] In one embodiment, a kit described herein comprises a plurality of fusion polypeptides, wherein individual members of the plurality comprise different fused antigen polypeptides. In one embodiment, individual members of the

plurality comprise different fused antigen polypeptides each comprising a fused different portion of the same target antigen polypeptide.

[0249] A CMI assay is important for assessing a subject's ability to respond to an infection by an pathogenic agent such as a microorganism, virus or parasite, to mount an autoimmune response such as in diabetes or to protect against cancers or other oncological conditions. Consequently, reference to "measuring a CMI response to a target antigen in a subject" encompasses immune diagnosis of infectious and autoimmune diseases, a marker for immunocompetence and the detection of T-cell responses to endogenous and/or exogenous antigens (including a measure of the efficacy of a vaccine) as well as a marker for inflammatory diseases and cancer. Monitoring CMI pre- and post-transplantation is necessary in the management of organ transplant patients. A CMI assay can also be used to titrate initial immunosuppression reduction and its subsequent increase in these patients.

[0250] As discussed above, any of a range of antigens can be tested such as those specific for a particular organism, pathogen, virus, auto-antigen or cancer cell. Alternatively, more general agents can be used to test generic capacity to mount a cell-mediated immune response. Examples of the latter include PPD from *M. tuberculosis* and tetanus toxoid. In general, however, any peptide, polypeptide or protein, glycoprotein, phosphoprotein, phospholipoprotein fused to an LF polypeptide, e.g., an LFn polypeptide can be used in kits or compositions as disclosed herein.

[0251] These include antigens from pathogens, particularly, but not necessarily intracellular pathogens. The pathogens include, for example, any of the viral, bacterial, fungal or parasitic pathogens described herein, among others. The antigen can also include tumor antigens and/or autoimmune antigens.

[0252] The present invention can be defined in any of the following alphabetized paragraphs:

[A] A fusion polypeptide comprising a *Bacillus anthracis* lethal factor N-terminal (LFn) polypeptide, or conservative substitution variant thereof, that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein said fusion polypeptide is N-glycosylated.

[B] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide is N-glycosylated.

[C] The fusion polypeptide of paragraph **[A]**, wherein the intact cell is a mammalian cell.

[D] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide comprises at least the 60 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[E] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide comprises at least the 104 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.

[F] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide comprises the amino acid sequence corresponding to SEQ. ID. No. 5, or a conservative substitution variant thereof.

[G] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide does not bind *B. anthracis* protective antigen protein.

[H] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide substantially lacks the amino acids 1-33 of SEQ. ID. No. 3.

[I] The fusion polypeptide of paragraph **[A]**, wherein the LFn polypeptide consists of SEQ. ID. No. 5, or a conservative substitution variant thereof.

[J] The fusion polypeptide of paragraphs **[A]-[I]**, wherein the target antigen polypeptide is an intracellular pathogen target antigen polypeptide.

[K] The fusion polypeptide of paragraph **[J]**, wherein the target antigen is an antigen from a prokaryotic pathogen.

[L] The fusion polypeptide of paragraph **[J]**, wherein the target antigen is a viral antigen from a viral pathogen, in which the virus naturally infects mammalian host cells.

[M] The fusion polypeptide of claim paragraph **[J]**, wherein the target antigen is an antigen of a parasitic pathogen.

[N] The fusion polypeptide of paragraph **[K]**, wherein the a prokaryotic pathogen is selected from the group consisting of: *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella* species, *Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Clostridium botulinum, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum, Klebsiella pneumoniae, Pseudomonas aeruginosa, Cryptosporidium parvum, Streptococcus pneumoniae, Bordetella pertussis* and *Neisseria meningitides.*

[O] The fusion polypeptide of paragraph **[L]**, wherein the viral pathogen is selected from the group consisting of: Herpes simplex virus type-1, Herpes simplex virus type-2, HBV, Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus, Human herpes virus 6, Human herpes virus 7, Human herpes virus 8, Variola virus, Vesicular stomatitis virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, poliovirus, Rhinovirus, Coronavirus, Influenza virus A, Influenza virus B. Measles virus, Polyomavirus, Human Papilomavirus, Respiratory syncytial virus, Adenovirus, Coxsackie virus, Dengue virus, Mumps virus, Poliovirus, Rabies virus, Rous sarcoma virus, Yellow fever virus, Ebola virus, Marburg virus, Lassa fever virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, St. Louis Encephalitis virus, Murray Valley fever virus, West Nile virus, Rift Valley fever virus, Rotavirus A, Rotavirus B. Rotavirus C, Sindbis virus, Rabies virus, Human T-cell Leukemia virus type-l, Hantavirus, Rubella virus and Simian Immunodeficiency virus.

[P] The fusion polypeptide of paragraph **[M]**, wherein the parasitic pathogen is selected from the group consisting of: *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella* species, *Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Bordetella pertussis, Neisseria meningitides, Leishmania donovanii, Plasmodium* species, *Pneumocystis carinii and Trypanosoma* species.

[Q] The fusion polypeptide of paragraph **[N]**, wherein the pathogen is *Myocobacterium tuberculosis.*

[R] The fusion polypeptide of paragraph **[N]**, wherein the target antigen polypeptide is a TB-specific antigen.

[S] The fusion polypeptide of paragraph **[R]**, wherein the target antigen is a TB1 (CFP-10) polypeptide comprising SEQ. ID NO: 7 or a fragment thereof.

[T] The fusion polypeptide of paragraph **[R]**, wherein the target antigen is a TB2 (ESAT-6) polypeptide comprising SEQ. ID NO: 8 or a fragment thereof.

[U] The fusion polypeptide of any of paragraphs **[A]-[T]**, wherein the target antigen polypeptide or a fragment thereof is folded in its native conformation.

[V] The fusion polypeptide of any of paragraphs **[A]-[U]**, wherein the target antigen polypeptide or a fragment thereof is part of a multi-molecular polypeptide complex.

[W] The fusion polypeptide of any of paragraphs **[A]-[V]**, wherein the target antigen polypeptide is a subunit polypeptide of a multi-molecular polypeptide target antigen.

[X] The fusion polypeptide of any of paragraphs **[A]-[W]**, wherein the fusion polypeptide is expressed and purified from a protein expression system using host cells selected from the group consisting of: mammalian cells, insect cells, yeast cells, and plant cells.

[Y] The fusion polypeptide of any of paragraphs **[A]-[W]** which is isolated from an insect cell.

[Z] The fusion polypeptide of any of paragraphs **[A]-[W]** which is produced by expression from a recombinant baculovirus vector.

[AA] An isolated polynucleotide encoding the fusion polypeptide of any of paragraphs **[A]-[Z].**

[BB] A recombinant vector comprising the polynucleotide of paragraph **[AA]**.

[CC] The recombinant vector of paragraph **[BB]**, wherein the recombinant vector is an expression vector that is compatible with a protein expression system using host cells selected from the group consisting of: mammalian cells; insect cells; yeast cells; and plant cells.

[DD] The recombinant vector of paragraph **[CC]**, wherein the vector is a viral vector.

[EE] The viral vector of paragraph **[DD]**, wherein the vector is a recombinant baculovirus vector.

[FF] A host cell comprising the expression vector of paragraph **[CC].**

[GG] The host cell of paragraph **[FF]**, wherein the host cell is an insect cell and the expression vector is a baculovirus vector.

[HH] A method of producing a N-glycosylated fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, the method comprising expressing the fusion polypeptide in a eukaryotic cell.

[II] The method of paragraph **[HH]**, wherein the method comprises infecting lepidopteran insect cells with a recombinant baculovirus vector comprising a polynucleotide encoding the fusion polypeptide, and culturing the insect cells to produce the fusion polypeptide.

[JJ] The method of paragraph **[II]**, wherein the lepidopteran insect cells are *Spodoptera frugiperda* cells.

[KK] A method of preparing a composition for increasing or detecting a cell-mediated immune response to a target antigen polypeptide, the method comprising expressing a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide in an insect cell.

[LL] The method of paragraph **[KK]**, wherein the target antigen polypeptide is an intracellular pathogen target antigen polypeptide.

[MM] The method of paragraph **[KK]**, wherein the fusion polypeptide is expressed from a recombinant baculovirus vector.

[NN] A method of introducing an intracellular pathogen target antigen polypeptide to a mammalian cell, the method comprising contacting a fusion polypeptide of any of paragraphs **[A]-[Z]** with a mammalian cell.

[OO] The method of paragraph **[NN]**, wherein the cell is a mammalian cell in vivo and the method comprises administering a fusion polypeptide to the mammal.

[PP] A method of increasing a cell-mediated immune response to a target antigen polypeptide, the method comprising administering to a mammal, a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein said fusion polypeptide is N-glycosylated.

[QQ] The method of paragraph **[PP]**, wherein the target antigen is an intracellular pathogen target antigen polypeptide or fragment thereof.

[RR] The method of paragraph **[PP]**, wherein the fusion polypeptide is isolated from an insect cell.

[SS] The method of paragraph **[PP]**, wherein the fusion polypeptide is produced by expression from a recombinant baculovirus vector.

[TT] A composition comprising a fusion polypeptide of any of paragraphs **[A]-[Z]** and an isolated mammalian cell.

[UU] The composition of paragraph **[TT]**, wherein the mammalian cell is an antigen presenting cell.

[VV] The composition of paragraph **[UU]**, wherein the antigen presenting cell expresses MHC class II molecules.

[WW] The composition of paragraph **[TT]**, wherein the fusion polypeptide is expressed and purified from insect cells.

[XX] A vaccine composition comprising a fusion polypeptide comprising a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein said fusion polypeptide is N-glycosylated and pharmaceutically acceptable carrier.

[YY] The vaccine composition of paragraph **[XX]**, wherein the fusion polypeptide is expressed and purified from insect cells.

[ZZ] The vaccine composition of paragraph **[XX]**, wherein the LFn polypeptide is N-glycosylated.

[AAA] The vaccine composition of any of paragraphs **[XX]-[ZZ]**, further comprising an adjuvant.

[BBB] A kit comprising a fusion polypeptide of any of paragraphs **[A]** to **[Z]** and packaging materials thereof.

[CCC] 55. The kit of paragraph **[BBB]** which comprises a plurality of said fusion polypeptides, wherein individual members of the plurality comprise different fused antigen polypeptides.

[DDD] 56. The kit of paragraph **[CCC]** wherein the members of said plurality each comprise a fused different portion of the same target antigen polypeptide.

[0253] The contents of all references cited throughout this application as well as the table therein are incorporated herein by reference.

CLAIMS:

1. A fusion polypeptide comprising a *Bacillus anthracis* lethal factor N-terminal (LFn) polypeptide, or conservative substitution variant thereof, that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein said fusion polypeptide is N-glycosylated.
2. The fusion polypeptide of claim 1, wherein the LFn polypeptide is N-glycosylated.
3. The fusion polypeptide of claim 1, wherein the intact cell is a mammalian cell.
4. The fusion polypeptide of claim 1, wherein the LFn polypeptide comprises at least the 60 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.
5. The fusion polypeptide of claim 1, wherein the LFn polypeptide comprises at least the 104 carboxy-terminal amino acids of SEQ. ID. No. 3, or a conservative substitution variant thereof.
6. The fusion polypeptide of claim 1, wherein the LFn polypeptide comprises the amino acid sequence corresponding to SEQ. ID. No. 5, or a conservative substitution variant thereof.
7. The fusion polypeptide of claim 1, wherein the LFn polypeptide does not bind *B. anthracis* protective antigen protein.
8. The fusion polypeptide of claim 1, wherein the LFn polypeptide substantially lacks the amino acids 1-33 of SEQ. ID. No. 3.
9. The fusion polypeptide of claim 1, wherein the LFn polypeptide consists of SEQ. ID. No. 5, or a conservative substitution variant thereof.
10. The fusion polypeptide of claim 1-9, wherein the target antigen polypeptide is an intracellular pathogen target antigen polypeptide.
11. The fusion polypeptide of claim 10, wherein said target antigen is an antigen from a prokaryotic pathogen.
12. The fusion polypeptide of claim 10, wherein said target antigen is a viral antigen from a viral pathogen, in which the virus naturally infects mammalian host cells.
13. The fusion polypeptide of claim 10, wherein said target antigen is an antigen of a parasitic pathogen.
14. The fusion polypeptide of claim 11, wherein the a prokaryotic pathogen is selected from the group consisting of: *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella species, Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Clostridium botulinum, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum, Klebsiella pneumoniae, Pseudomonas aeruginosa, Cryptosporidium parvum, Streptococcus pneumoniae, Bordetella pertussis*, and *Neisseria meningitides*.
15. The fusion polypeptide of claim 12, wherein the viral pathogen is selected from the group consisting of: Herpes simplex virus type-1, Herpes simplex virus type-2, HBV, Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus, Human herpes virus 6, Human herpes virus 7, Human herpes virus 8, Variola virus, Vesicular stomatitis virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, Hepatitis E virus, poliovirus, Rhinovirus, Coronavirus, Influenza virus A, Influenza virus B. Measles virus, Polyomavirus, Human Papilomavirus, Respiratory syncytial virus, Adenovirus, Coxsackie virus, Dengue virus, Mumps virus, Poliovirus, Rabies virus, Rous sarcoma virus, Yellow fever virus, Ebola virus, Marburg virus, Lassa fever virus, Eastern Equine Encephalitis virus, Japanese Encephalitis virus, St. Louis Encephalitis virus, Murray Valley fever virus, West Nile virus, Rift Valley fever virus, Rotavirus A, Rotavirus B. Rotavirus C, Sindbis virus, Rabies virus, Human T-cell Leukemia virus type-l, Hantavirus, Rubella virus and Simian Immunodeficiency virus.

16. The fusion polypeptide of claim 13, wherein the parasitic pathogen is selected from the group consisting of: *Myocobacterium tuberculosis, Mycobacterium leprae, Listeria monocytogenes, Salmonella typhi, Shigella dysenteriae, Yersinia pestis, Brucella species, Legionella pneumophila, Rickettsiae* species, *Chlamydia* species, *Clostridium perfringens, Staphylococcus aureus, Treponema pallidum, Haemophilus influenzae, Treponema pallidum, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Bordetella pertussis, Neisseria meningitides, Leishmania donovanii, Plasmodium species, Pneumocystis carinii,* and *Trypanosoma* species.
17. The fusion polypeptide of claim 14, wherein the pathogen is *Myocobacterium tuberculosis*.
18. The fusion polypeptide of claim 14, wherein the target antigen polypeptide is a TB-specific antigen.
19. The fusion polypeptide of claim 18, wherein the target antigen is a TB1 (CFP) polypeptide comprising SEQ ID NO: 7 or a fragment thereof.
20. The fusion polypeptide of claim 18, wherein the target antigen is a TB2 (ESAT) polypeptide comprising SEQ ID NO: 8 or a fragment thereof.
21. The fusion polypeptide of any of claims 1 to 20, wherein the target antigen polypeptide or a fragment thereof is folded in its native conformation.
22. The fusion polypeptide of any of claims 1 to 21, wherein the target antigen polypeptide or a fragment thereof is part of a multi-molecular polypeptide complex.
23. The fusion polypeptide of any of claims 1 to 22, wherein said target antigen polypeptide is a subunit polypeptide of a multi-molecular polypeptide target antigen.
24. The fusion polypeptide of any of claims 1 to 23, wherein the fusion polypeptide is expressed and purified from a protein expression system using host cells selected from the group consisting of: mammalian cells, insect cells, yeast cells, and plant cells.
25. The fusion polypeptide of any of claims 1 to 23 which is isolated from an insect cell.
26. The fusion polypeptide of any of claims 1 to 23 which is produced by expression from a recombinant baculovirus vector.
27. An isolated polynucleotide encoding the fusion polypeptide of any of claims 1 to 26.
28. A recombinant vector comprising the polynucleotide of claim 27.
29. The recombinant vector of claim 28, wherein the recombinant vector is an expression vector that is compatible with a protein expression system using host cells selected from the group consisting of: mammalian cells; insect cells; yeast cells; and plant cells.
30. The recombinant vector of claim 29, wherein the vector is a viral vector.
31. The viral vector of claim 30, wherein the vector is a recombinant baculovirus vector.
32. A host cell comprising the expression vector of claim 29.
33. The host cell of claim 32, wherein the host cell is an insect cell and the expression vector is a baculovirus vector.
34. A method of producing a N-glycosylated fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, the method comprising expressing the fusion polypeptide in a eukaryotic cell.
35. The method of claim 34, wherein the method comprises infecting Lepidopteran insect cells with a recombinant baculovirus vector comprising a polynucleotide encoding the fusion polypeptide, and culturing the insect cells to produce the fusion polypeptide.
36. The method of claim 35, wherein the Lepidopteran insect cells are *Spodoptera frugiperda* cells.

37. A method of preparing a composition for increasing or detecting a cell-mediated immune response to a target antigen polypeptide, the method comprising expressing a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide in an insect cell.
38. The method of claim 37, wherein the target antigen polypeptide is an intracellular pathogen target antigen polypeptide.
39. The method of claim 37, wherein said fusion polypeptide is expressed from a recombinant baculovirus vector.
40. A method of introducing an intracellular pathogen target antigen polypeptide to a mammalian cell, the method comprising contacting a fusion polypeptide of any of claims 1-26 with a mammalian cell.
41. The method of claim 40, wherein said cell is a mammalian cell *in vivo* and the method comprises administering a fusion polypeptide to said mammal.
42. A method of increasing a cell-mediated immune response to a target antigen polypeptide, the method comprising administering to a mammal, a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein said fusion polypeptide is N-glycosylated.
43. The method of claim 42, wherein said target antigen is an intracellular pathogen target antigen polypeptide or fragment thereof.
44. The method of claim 42, wherein said fusion polypeptide is isolated from an insect cell.
45. The method of claim 42, wherein said fusion polypeptide is produced by expression from a recombinant baculovirus vector.
46. A composition comprising a fusion polypeptide of any of claims 1 to 26 and an isolated mammalian cell.
47. The composition of claim 46, wherein the mammalian cell is an antigen presenting cell.
48. The composition of claim 47, wherein the antigen presenting cell expresses MHC class II molecules.
49. The composition of claim 46, wherein the fusion polypeptide is expressed and purified from insect cells.
50. A vaccine composition comprising a fusion polypeptide comprising a fusion polypeptide comprising a *B. anthracis* LFn polypeptide that promotes transmembrane delivery to the cytosol of an intact cell, fused to a target antigen polypeptide, wherein said fusion polypeptide is N-glycosylated and pharmaceutically acceptable carrier.
51. The vaccine composition of claim 50, wherein the fusion polypeptide is expressed and purified from insect cells.
52. The vaccine composition of claim 50, wherein the LFn polypeptide is N-glycosylated.
53. The vaccine composition of any of claims 50-52, further comprising an adjuvant.
54. A kit comprising a fusion polypeptide of any of claims 1 to 26 and packaging materials thereof.
55. The kit of claim 54 which comprises a plurality of said fusion polypeptides, wherein individual members of the plurality comprise different fused antigen polypeptides.
56. The kit of claim 55 wherein the members of said plurality each comprise a fused different portion of the same target antigen polypeptide.

1/1

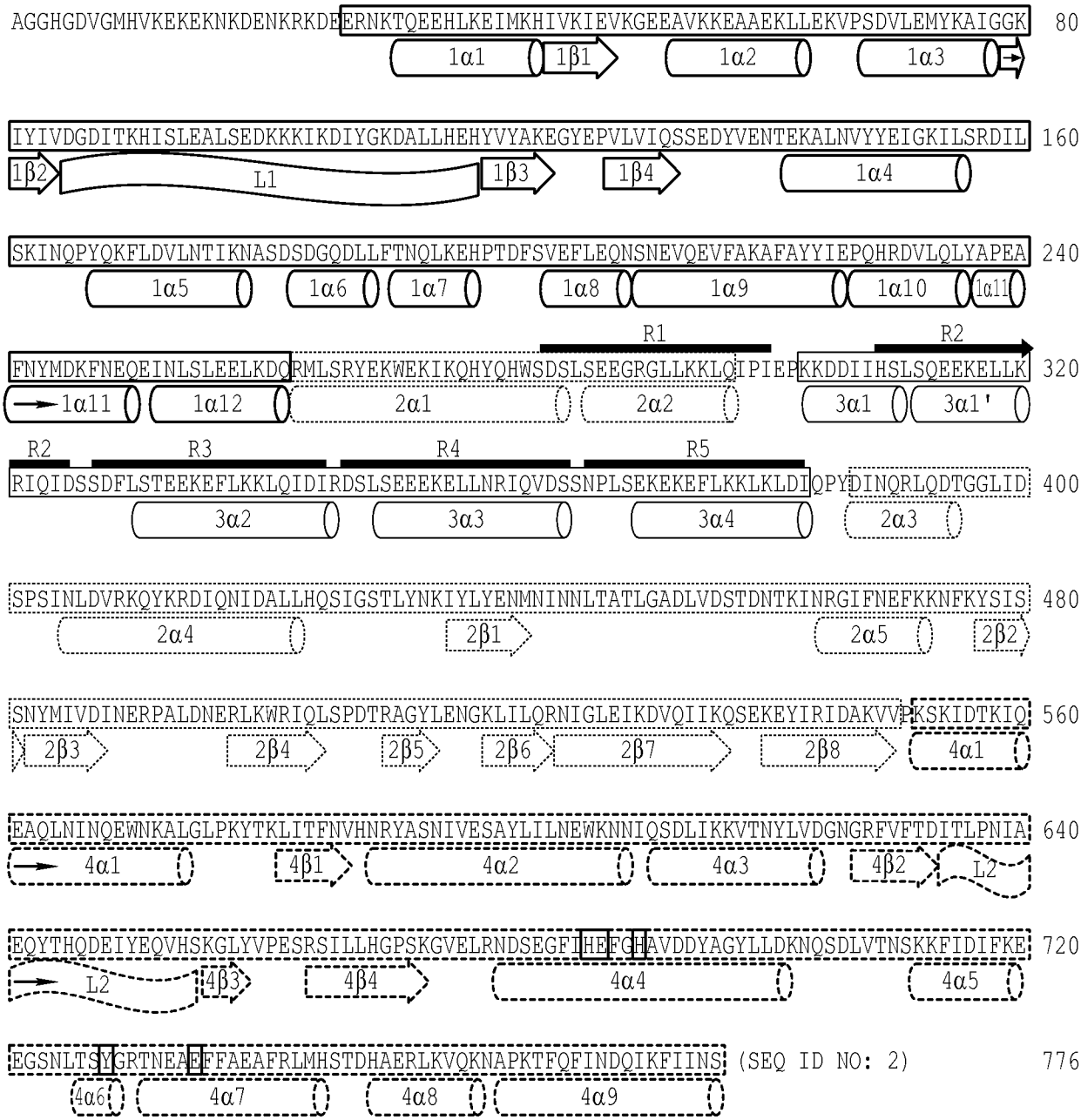
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FIG. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/038305

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/32 C12N15/62

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)
C07K C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/100340 A2 (AVANT IMMUNOTHERAPEUTICS INC [US]; THOMAS LAWRENCE J [US]; SCORPIO ANG) 19 December 2002 (2002-12-19) page 7, line 8 page 8, lines 7-10 page 10, line 19 --- -/--	1-56

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier document 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
20 September 2010	21/10/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Herrmann, Klaus

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/038305

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUSHNER N ET AL: "A fragment of anthrax lethal factor delivers proteins to the cytosol without requiring protective antigen" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES (PNAS), NATIONAL ACADEMY OF SCIENCE, US LNKD-DOI:10.1073/PNAS.1131930100, vol. 100, no. 11, 27 May 2003 (2003-05-27), pages 6652-6657, XP002354518 ISSN: 0027-8424 cited in the application abstract page 6653, left-hand column	1-56
A	WO 2007/145760 A2 (OKLAHOMA MED RES FOUND [US]; FARRIS DARISE [US]; JAMES JUDITH A [US];) 21 December 2007 (2007-12-21) page 15, line 23 page 16, line 27	1-56
A	IACONO-CONNORS LAUREN C ET AL: "Enzyme-Linked Immunosorbent Assay Using a Recombinant Baculovirus-Expressed Bacillus anthracis Protective Antigen (PA): Measurement of Human Anti-PA Antibodies" CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 1, no. 1, 1994, pages 78-82, XP002601399 ISSN: 1071-412X the whole document	1-56
A	KULAKOSKY PETER C ET AL: "N-linked glycosylation of a baculovirus-expressed recombinant glycoprotein in insect larvae and tissue culture cells" GLYCOBIOLOGY, vol. 8, no. 7, July 1998 (1998-07), pages 741-745, XP002601403 ISSN: 0959-6658 the whole document	1-56

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/038305

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02100340 A2	19-12-2002	AU 2002322059 A1	23-12-2002
		GB 2393122 A	24-03-2004
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