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(54) Title: LISTERIA-BASED ADJUVANTS

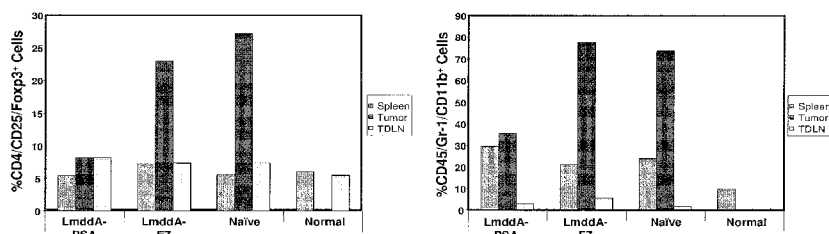


Figure 7

(57) Abstract: This invention provides methods and compositions for using *Listeria monocytogenes* as an adjuvant for enhancing immune responses in a subject.

LISTERIA-BASED ADJUVANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Provisional Patent application number 61/451,651, filed Mar. 11, 2011. This application further claims priority to United States Patent application number 13/290,783, filed November 7th, 2011, which is a Continuation-In-Part of United States application number 12/993,380, filed February 7th, 2011, which claims priority to international application number PCT/US09/44538, filed May 19th, 2009, which claims the benefit of United States provisional application number 61/071,792, filed May 19th, 2008. This application also claims priority to United States Patent application number 13/027,828 filed February 15th, 2011, which claims the benefit of United States provisional application number 61/304,701, filed February 15th, 2010. This application also claims priority to United States Patent application number 13/210,696, filed August 16th, 2011, which is a Continuation-In-Part of United States application number 12/945,386, filed November 12th, 2010, which claims the benefit of US provisional application number 61/260,277, filed November 11th, 2009. These applications are hereby incorporated in their entirety by reference herein.

FIELD OF INVENTION

[0002] This invention provides methods and compositions for using *Listeria monocytogenes* as an adjuvant for enhancing immune responses in a subject.

BACKGROUND OF THE INVENTION

[0003] Adjuvants have extensive use in immunotherapy. The majority of cellular based immunotherapies administer adjuvants prior to giving antigen specific treatment. Typically these antigens include GM-CSF, IL-1, QP-100, Keyhole Limpet Cynanin, and others. These adjuvants are typically administered systemically via IV, IM, ID or similar routes.

[0004] *Listeria monocytogenes* (*Lm*) is an intracellular pathogen that primarily infects antigen presenting cells and has adapted for life in the cytoplasm of these cells. *Listeria monocytogenes* and a protein it produces named listeriolysin O (LLO) have strong adjuvant properties, that unlike the majority of adjuvants used for cellular based immunotherapies, can be administered after providing an antigen specific treatment.

[0005] A method of rapidly elevating a subject's immune response to any antigen is needed in order to decrease disease frequency in the subject and mortality resulting thereof. The present invention provides methods of elevating an immune response in subjects such as human adults and children by taking advantage of the adjuvant properties provided by live *Lm* vaccines that secrete non-hemolytic LLO or a truncated ActA.

[0006] Further, the same method is provided to reconstitute the immune response or facilitate the recovery of an immune response to normal or approximately normal levels in subjects that have undergone cytotoxic treatment as a result of cancer.

SUMMARY OF THE INVENTION

[0007] In one embodiment the invention relates to a method of reconstituting an immune response in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to the subject.

[0008] In one embodiment the invention relates to a method of reconstituting an immune response in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to the subject, the *Listeria* strain comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide

[0009] In one embodiment, the invention relates to a method of facilitating recovery of immune responses after cytotoxic treatments in a subject, the method comprising administering a live attenuated *Listeria* vaccine strain to the subject

[00010] In one embodiment, the invention relates to a method of facilitating recovery of immune responses after cytotoxic treatments in a subject, the method comprising administering a live attenuated *Listeria* vaccine strain to the subject In another embodiment the *Listeria* strain comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide.

[00011] In one embodiment, the invention relates to a method of improving the immunogenicity of a vaccine, said method comprising the step of co-administering the vaccine and a *Listeria*-based adjuvant to a subject, wherein the *Listeria*-based adjuvant enhances the immunogenicity of said vaccine, thereby improving the immunogenicity of the vaccine.

[00012] In one embodiment, the invention relates to a method of enhancing an immune response against a disease in an antigen-independent manner in a subject, said method comprising administering a *Listeria*-based adjuvant to the subject.

[00013] Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[00014] **Figure 1** is a schematic map of *E. coli*-*Listeria* shuttle plasmids pGG55 (above) and pTV3 (below). CAT(-): *E. coli* chloramphenicol transferase; CAT(+): *Listeria* chloramphenicol transferase; Ori Lm: replication origin for *Listeria*; Ori Ec: p15 origin of replication for *E. coli*; prfA: *Listeria* pathogenicity regulating factor A; LLO: C-terminally truncated listeriolysin O, including its promoter; E7: HPV E7; p60-dal; expression cassette of p60 promoter and *Listeria* dal gene. Selected restriction sites are also depicted.

[00015] **Figure 2** shows the DNA sequences present upstream and downstream of the *inlC* region on the genome of *Listeria* strain EGD. DNA-up (red), *inlC* gene (blue) and DNA-down (black).

[00016] **Figure 3** shows the the sequence of DNA that is cloned in the temperature sensitive plasmid, pKSV7 to create *inl C* deletion mutant. The restriction enzyme sites used for cloning of these regions are indicated in caps and underlined. GAATTC- EcoRI, GGATCC-BamHI and CTGCAG-PstI. The EcoRI-PstI insert is cloned in the vector, pKSV7.

[00017] **Figure 4** shows a Schematic representation of the Lm-dd and Lm-ddD *actA* strains. The gel showing the size of PCR products using oligo's 1/2 and oligo's 3/4 obtained using e chromosomal DNA of the strains, Lm-dd and Lm-ddΔ*actA* as template.

[00018] **Figure 5** shows the DNA sequence present upstream and downstream of the *actA* gene in the *Listeria* chromosome. The region in italics contains the residual *actA* sequence element that is present in the LmddΔ*actA* strain. The underlined sequence gtcgac represent the restriction site of XhoI, which is the junction between the N-T and C-T region of *actA*.

[00019] **Figure 6** depicts tumor regression in response to administration of *Lm* vaccine strains (A). Circles represent naive mice, inverted triangles represent mice administered Lmdd-TV3, and crosses represent mice administered Lm-LLOE7.

[00020] **Figure 7** shows a decrease in MDSCs and Tregs in tumors. The number of MDSCs (right-hand panel) and Tregs (left-hand panel) following *Lm* vaccination (LmddAPSA and LmddAE7).

[00021] **Figure 8** shows suppressor assay data demonstrating that monocytic MDSCs from TPSA23 tumors are less suppressive after *Listeria* vaccination. This change in the suppressive ability of the MDSCs is not antigen specific as the same decrease in suppression is seen with PSA-antigen specific T cells and also with non-specifically stimulated T cells. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00022] **Figure 9** shows suppressor assay data demonstrating that *Listeria* has no effect on splenic monocytic MDSCs and they are only suppressive in an antigen-specific manner. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00023] **Figure 10** shows suppressor assay data demonstrating that granulocytic MDSCs from tumors have a reduced ability to suppress T cells after *Listeria* vaccination. This change in the suppressive ability of the MDSCs is not antigen specific as the same decrease in suppression is seen with PSA-antigen specific T cells and also with non-specifically stimulated T cells. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00024] **Figure 11** shows suppressor assay data demonstrating that *Listeria* has no effect on splenic granulocytic MDSCs and they are only suppressive in an antigen-specific manner. The No MDSC group shows the lack of division of the responder T cells when they are left

unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00025] **Figure 12** shows suppressor assay data demonstrating that Tregs from tumors are still suppressive. There is a slight decrease in the suppressive ability of Tregs in a non-antigen specific manner, in this tumor model. The No Treg group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no Tregs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

10 [00026] **Figure 13** shows suppressor assay data demonstrating that splenic Tregs are still suppressive. The No Treg group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no Tregs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

15 [00027] **Figure 14** shows suppressor assay data demonstrating that conventional CD4+ T cells have no effect on cell division regardless if whether they are found in the tumors or spleens of mice. Left-hand and Right-hand panels show pooled division cycles.

[00028] **Figure 15** shows suppressor assay data demonstrating that monocytic MDSCs from 4T1 tumors have decreased suppressive ability after *Listeria* vaccination. This change in the suppressive ability of the MDSCs is not antigen specific as the same decrease in suppression is seen with Her2/neu-antigen specific T cells and also with non-specifically stimulated T cells. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00029] **Figure 16** shows suppressor assay data demonstrating that there is no *Listeria*-specific effect on splenic monocytic MDSCs. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00030] **Figure 17** shows suppressor assay data demonstrating that granulocytic MDSCs from

4T1 tumors have decreased suppressive ability after *Listeria* vaccination. This change in the suppressive ability of the MDSCs is not antigen specific as the same decrease in suppression is seen with Her2/neu-antigen specific T cells and also with non-specifically stimulated T cells. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00031] **Figure 18** shows suppressor assay data demonstrating that there is no *Listeria*-specific effect on splenic granulocytic MDSCs. The No MDSC group shows the lack of division of the responder T cells when they are left unstimulated and the last group shows the division of stimulated cells with no MDSCs added to suppress division. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00032] **Figure 19** shows suppressor assay data demonstrating that decrease in the suppressive ability of Tregs from 4T1 tumors after *Listeria* vaccination. This decrease is not antigen specific, as the change in Treg suppressive ability is seen with both Her2/neu-specific and non-specific responder T cells. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00033] **Figure 20** shows suppressor assay data demonstrating that there is no *Listeria*-specific effect on splenic Tregs. The responder T cells are all capable of dividing, regardless of the whether or not they are antigen specific. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00034] **Figure 21** shows IFN- γ production is reduced in *S. mansoni* infected mice.

[00035] **Figure 22** shows IL-4 levels are increased in mice with chronic schistosomiasis.

[00036] **Figure 23** shows IL-10 production is increased in mice infected with *S. mansoni*.

[00037] **Figure 24** shows Schistosome infection does not alter the antigen-specific vaccine responses toward immunodominant CTL and helper epitopes.

DETAILED DESCRIPTION OF THE INVENTION

[00038] A novel and heretofore unexplored use is to create a live attenuated *Listeria* vaccine strain devoid of exogenous antigen

[00039] A novel and heretofore unexplored use is to create a live attenuated *Listeria* vaccine strain devoid of antigen that enables the *Listeria* to secrete only the non-hemolytic form of LLO (*Lm*-LLO) or a truncated ActA (*Lm*-ActA) as an adjuvant. The invention provided herein addresses the first live adjuvant.

[00040] In one embodiment, provided herein is a method of reconstituting an immune response in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to the subject. In another embodiment the *Listeria* strain comprises a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide.

[00041] In one embodiment, the *Listeria* over expresses said PEST-containing polypeptide. In another embodiment, the PEST-containing polypeptide is a non-hemolytic LLO protein or immunogenic fragment thereof, an ActA protein or truncated fragment thereof, or a PEST amino acid sequence.

[00042] In one embodiment, provided herein is a method of facilitating recovery of immune responses after cytotoxic treatments in a subject, the method comprising administering a live attenuated *Listeria* vaccine strain to the subject. In another embodiment, the *Listeria* strain comprises a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide.

[00043] In one embodiment, provided herein is a method of improving the immunogenicity of a vaccine, the method comprising the step of co-administering the vaccine and a *Listeria*-based adjuvant to a subject, wherein the *Listeria*-based adjuvant enhances the immunogenicity of the vaccine, thereby improving the immunogenicity of the vaccine.

[00044] In one embodiment, provided herein is a method of enhancing an immune response against a disease in an antigen-independent manner in a subject, the method comprising administering a *Listeria*-based adjuvant to the subject.

[00045] In one embodiment, provided herein is a composition and method for bioengineering a live *Lm* bacterium that infects specific cells, including; antigen processing cells (APC),

Kupffer cells, vascular endothelium, bone marrow, and others; as well as structures such as solid tumors and spleen. In another embodiment, the live *Lm* adjuvant then synthesizes and secretes a modified LLO fragment *in situ* where the adjuvant is needed and used to stimulate immune responses. In another embodiment the live *Lm* synthesizes ActA. In another
5 embodiment, unlike previous adjuvants, the instant invention administers the ability to make an adjuvant *in situ* and does not involve the systemic administration of an immune adjuvant.

[00046] In one embodiment, provided herein is a method of eliciting an adult-level enhanced immune response in neonate subjects, the method comprising the step of administering a recombinant *Listeria* vaccine strain to the subject. In another embodiment, the *Listeria* strain
10 comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a non-hemolytic listeriolysin O (LLO) or ActA, wherein the nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain.

[00047] In one embodiment, provided herein a method of facilitating recovery of immune responses after cytotoxic treatments in a subject, the method comprising administering a recombinant *Listeria* vaccine strain to the subject. In another embodiment, the *Listeria* strain comprising a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open
15 reading frame encoding a non-hemolytic listeriolysin O or ActA, wherein the nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* strain. In another embodiment, the subject is an adult human subject.

[00048] The ability of an adjuvant to increase the immune response to an antigen is typically
25 manifested by a significant increase in immune-mediated protection. For example, an increase in humoral immunity is typically manifested by a significant increase in the titer of antibodies raised to the antigen, and an increase in T-cell activity is typically manifested in increased cell proliferation, increased cytokine production and/or antigen specific cytolytic activity. An adjuvant may also alter an immune response, for example, by enabling a Th1 response against
30 a background of a persistent Th2 phenotype.

[00049] In one embodiment, this invention provides methods and compositions for preventing disease, treating disease and vaccinating a human subject.

[00050] In another embodiment, the present invention is directed to enhancing immune response of a human, a neonate, or a human that has been subjected to cytotoxic treatment as a result of cancer.

[00051] In one embodiment, a *Listeria*-based adjuvant refers to a live-attenuated *Listeria* vaccine strain. In another embodiment, the *Listeria*-based adjuvant is an *Lm*-LLO or an *Lm*-ActA. In another embodiment, *Lm*-LLO expresses a non-hemolytic LLO. In another embodiment, *Lm*-ActA expresses a truncated ActA protein. In another embodiment, *Lm*-LLO or *Lm*-ActA can be used alone, or in combination with any therapy in which an adjuvant is appropriate, and may have utility in settings where no adjuvant has been commonly used, such as chemotherapy or radiotherapy.

[00052] In another embodiment, the *Listeria* strain provided herein further comprises a third open reading frame encoding a metabolic enzyme.

[00053] In one embodiment, the metabolic enzyme is an amino acid metabolism enzyme. In another embodiment, the metabolic enzyme encoded by the second open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme. In another embodiment, the metabolic enzyme encoded by the third open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme. In another embodiment, the metabolic enzyme is encoded *dal* gene, where in another embodiment the *dal* gene is from *B. subtilis*. In another embodiment, the metabolic enzyme is encoded by the *dat* gene.

[00054] In another embodiment, the recombinant *Listeria* is an attenuated auxotrophic strain.

[00055] In one embodiment the attenuated strain is *Lmdd*. In another embodiment the attenuated strain is *Lmdda*. In another embodiment, the attenuated strain is *LmΔPrfA*. In another embodiment, the attenuated strain is *LmΔPlcB*. In another embodiment, the attenuated strain is *LmΔPlcA*. In another embodiment, the attenuated strain is *LmddAΔinlC*. In another embodiment, the *LmddAΔinlC* mutant strain is created using EGD strain of *Lm*, which is different from the background strain 10403S, the parent strain for *Lm dal dat actA (Lmdda)*. In another embodiment, this strain exerts a strong adjuvant effect which is an inherent property of *Listeria*-based vaccines. In another embodiment, this strain is constructed from the EGD *Listeria* backbone.

[00056] In another embodiment, the strain used in the invention is a *Listeria* strain that

expresses a non-hemolytic LLO. In yet another embodiment the *Listeria* strain is a *prfA* mutant, *ActA* mutant, a *plcB* deletion mutant, or a double mutant lacking both *plcA* and *plcB*. All these *Listeria* strain are contemplated for use in the methods provided herein. Each possibility represents a separate embodiment of the present invention.

- 5 [00057] In one embodiment, the *LmddAΔinlC* mutant strain is safe for use in humans and induces high levels of innate immune responses. In one embodiment, the *inlC* deletion mutant generates an enhanced level of innate immune responses that are not antigen specific.

[00058] In one embodiment, translocation of *Listeria* to adjacent cells is inhibited by two separate mechanisms, deletion of *actA* and *inlC* genes, both of which are involved in the
10 process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a vaccine backbone. In another embodiment, translocation of *Listeria* to adjacent cells is inhibited by two separate mechanisms, deletion of *actA* or *inlC* genes, both of which are involved in the process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a vaccine backbone.

- 15 [00059] Internalins are associated with increased virulence and their presence is associated with increased immunogenicity of *Listeria*, however, in the present invention, excising the *inlC* gene increases immunogenicity of the *Listeria* vaccine vector provided herein. In another embodiment, the present invention provides the novelty that the *inlC* genes are excised from a vector in which *actA* is deleted, thereby removing both, the ability to form actin flagella
20 necessary for movement through the host cell membrane and into the neighboring cell, and the ability for transmembrane passage. Therefore, the combination of these two deletions yields the surprising result of decreased virulence and increased immunogenicity of a *Listeria* vaccine vector over a wild-type *Listeria* strain or a single mutant strain.

[00060] In another embodiment, the nucleic acid molecule provided herein is integrated into
25 the *Listeria* genome. In another embodiment, the nucleic acid molecule is in a plasmid in the recombinant *Listeria* vaccine strain also provided herein. In another embodiment, the plasmid provided herein is stably maintained in the recombinant *Listeria* vaccine strain in the absence of antibiotic selection. In another embodiment, the plasmid does not confer antibiotic resistance upon said recombinant *Listeria*.

- 30 [00061] In one embodiment, the recombinant *Listeria* strain provided herein is attenuated. In another embodiment, the recombinant *Listeria* lacks the *ActA* virulence gene. In another

embodiment, the recombinant *Listeria* lacks the PrfA virulence gene.

[00062] In another embodiment, the recombinant *Listeria* vaccine strain comprises an adjuvant, wherein the adjuvant is listeriolysin O. In another embodiment, the recombinant *Listeria* vaccine strain comprises an adjuvant, wherein the adjuvant is ActA.

5 [00063] In one embodiment, the *Listeria* vaccine strain is *LmddAinlC142* strain. *LmddAinlC142* is based on a *Listeria* vaccine vector which is attenuated due to the deletion of *inlC* gene and retains the plasmid for PSA expression *in vivo* and *in vitro* by complementation of *dal* gene. In another embodiment, *LmddAinlC142* exerts strong and antigen specific anti-tumor responses with ability to break tolerance toward a heterologous antigen in a subject. In
10 another embodiment, the *LmddAinlC142* strain is highly attenuated and has a better safety profile than previous *Listeria* vaccine generation, as it is more rapidly cleared from the spleens of the immunized mice. In another embodiment, *LmddAinlC142* strain is highly immunogenic, able to break tolerance toward a heterologous antigen and prevents tumor formation in a subject.

15 [00064] In another embodiment, the methods provided herein further provide methods of overcoming or “breaking” tolerance toward a heterologous antigen that is a self-antigen. Such antigens may be aberrantly expressed by various tumors which are subject to treatment or prophylaxis under the scope of the present invention by using the methods and compositions provided herein.

20 [00065] In one embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing listeriolysin O in combination with other therapeutic modalities are useful for enhancing an immune response, and for preventing, and treating a cancer or solid tumors. In another embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing listeriolysin O alone, or in combination with other therapeutics are useful for preventing, and treating infectious
25 diseases in a subject. In another embodiment, the subject is a neonate, a child, or an adult.

[00066] In one embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing ActA in combination with other therapeutic modalities are useful for enhancing an immune response, and for preventing, and treating a cancer or solid tumors. In another embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing ActA alone, or in combination
30 with other therapeutics are useful for preventing, and treating infectious diseases in a subject. In another embodiment, the subject is a neonate, a child, or an adult.

[00067] In one embodiment, the immune response induced by the methods and compositions provided herein is a therapeutic one. In another embodiment it is a prophylactic immune response. In another embodiment, it is an enhanced immune response over methods available in the art for inducing an immune response in a subject afflicted with the conditions provided
5 herein. In another embodiment, the immune response leads to clearance of the disease provided herein that is afflicting the subject.

[00068] It is to be understood that the methods of the present invention may be used to treat any infectious disease, which in one embodiment, is bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection. In another embodiment, the
10 methods of the present invention are for inhibiting or suppressing a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a subject. In another embodiment, the present invention provides a method of eliciting a cytotoxic T-cell response against a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a subject. In another embodiment, the present invention provides a method of
15 inducing a Th1 immune response against a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a Th1 unresponsive subject. In one embodiment, the infection is viral, which in one embodiment, is HIV. In one embodiment, the infection is bacterial, which in one embodiment, is mycobacteria, which in one embodiment, is tuberculosis. In one embodiment, the infection is eukaryotic, which in one embodiment, is
20 plasmodium, which in one embodiment, is malaria.

[00069] In one embodiment, provided herein is a method of inducing a Th1 immune response in a Th1 unresponsive subject having a concomitant helminth infection, where in another embodiment, the method comprises using a *Listeria* vaccine vector.

[00070] In another embodiment, provided herein is a method of improving the
25 immunogenicity of a vaccine, the method comprising co-administering the vaccine and a *Listeria*-based adjuvant to a subject, wherein the *Listeria*-based adjuvant enhances the immunogenicity of the vaccine, thereby improving the immunogenicity of the vaccine. In one embodiment, the method enables the treatment of a disease for which said vaccine is specific against.

[00071] In one embodiment, provided herein is a method of enhancing an immune response
30 against a disease in an antigen-independent manner, the method comprising administering a *Listeria*-based adjuvant to a subject.

[00072] In another embodiment, the Listeria-based adjuvant is an LLO-expressing Listeria strain or an LLO protein or a non-hemolytic fragment thereof. In another embodiment, the Listeria-based adjuvant is an ActA-expressing Listeria strain or an ActA protein or a truncated fragment thereof. In another embodiment, Listeria-based adjuvant is used alone or is combined with an additional adjuvant. In another embodiment, the additional adjuvant is a non-nucleic acid adjuvant including aluminum adjuvant, Freund's adjuvant, MPL, emulsion, GM-CSF, QS21, SBAS2, CpG-containing oligonucleotide, a nucleotide molecule encoding an immune-stimulating cytokine, the adjuvant is or comprises a bacterial mitogen, or a bacterial toxin. In another embodiment, the LLO protein or hemolytic fragment thereof is admixed with or chemically coupled to said vaccine.

[00073] In one embodiment, the vaccine is selected from the group consisting of hepatitis B virus blood-derived vaccine, hepatitis B virus genetic engineering protein vaccines, HBV virus vector vaccine, hepatitis B virus bacterium vector vaccine, hepatitis B virus transgenic plant vaccine, rabies virus blood-derived vaccine, rabies virus genetic engineering protein vaccines, rabies virus vector vaccine, rabies virus bacterium vector vaccine, and rabies virus transgenic plant vaccine, and the DNA vaccine is selected from the group consisting of hepatitis B virus DNA vaccine and rabies DNA vaccine.

[00074] In another embodiment, the Listeria-based adjuvant is used alone or is combined with an additional adjuvant.

[00075] In another embodiment, the adjuvant of the present invention is co-administered with an additional adjuvant. In another embodiment, the additional adjuvant utilized in methods and compositions of the present invention is, in another embodiment, a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein. In another embodiment, the adjuvant comprises a GM-CSF protein. In another embodiment, the adjuvant is a nucleotide molecule encoding GM-CSF. In another embodiment, the adjuvant comprises a nucleotide molecule encoding GM-CSF. In another embodiment, the adjuvant is saponin QS21. In another embodiment, the adjuvant comprises saponin QS21. In another embodiment, the adjuvant is monophosphoryl lipid A. In another embodiment, the adjuvant comprises monophosphoryl lipid A. In another embodiment, the adjuvant is SBAS2. In another embodiment, the adjuvant comprises SBAS2. In another embodiment, the adjuvant is an unmethylated CpG-containing oligonucleotide. In another embodiment, the adjuvant comprises an unmethylated CpG-containing oligonucleotide. In another embodiment, the

adjuvant is an immune-stimulating cytokine. In another embodiment, the adjuvant comprises an immune-stimulating cytokine. In another embodiment, the adjuvant is a nucleotide molecule encoding an immune-stimulating cytokine. In another embodiment, the adjuvant comprises a nucleotide molecule encoding an immune-stimulating cytokine. In another
5 embodiment, the adjuvant is or comprises a quill glycoside. In another embodiment, the adjuvant is or comprises a bacterial mitogen. In another embodiment, the adjuvant is or comprises a bacterial toxin. In another embodiment, the adjuvant is or comprises any other adjuvant known in the art. Each possibility represents a separate embodiment of the present invention.

10 [00076] In one embodiment, provided herein is a nucleic acid molecule that encodes the adjuvant of the present invention. In another embodiment, the nucleic acid molecule is used to transform the *Listeria* in order to arrive at a recombinant *Listeria*. In another embodiment, the nucleic acid provided herein used to transform *Listeria* lacks a virulence gene. In another
15 embodiment, the nucleic acid molecule integrated into the *Listeria* genome carries a non-functional virulence gene. In another embodiment, the virulence gene is mutated in the recombinant *Listeria*. In yet another embodiment, the nucleic acid molecule is used to inactivate the endogenous gene present in the *Listeria* genome. In yet another embodiment, the virulence gene is an ActA gene, an *inlC* gene or a PrfA gene. As will be understood by a skilled artisan, the virulence gene can be any gene known in the art to be associated with
20 virulence in the recombinant *Listeria*.

[00077] In one embodiment, the metabolic gene, the virulence gene, etc. is lacking in a chromosome of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the chromosome and in any episomal genetic element of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the
25 genome of the virulence strain. In one embodiment, the virulence gene is mutated in the chromosome. In another embodiment, the virulence gene is deleted from the chromosome. Each possibility represents a separate embodiment of the present invention.

[00078] In another embodiment, the nucleic acids and plasmids provided herein do not confer antibiotic resistance upon the recombinant *Listeria*.

30 [00079] "Nucleic acid molecule" refers, in another embodiment, to a plasmid. In another embodiment, the term refers to an integration vector. In another embodiment, the term refers to a plasmid comprising an integration vector. In another embodiment, the integration vector

is a site-specific integration vector. In another embodiment, a nucleic acid molecule of methods and compositions of the present invention are composed of any type of nucleotide known in the art. Each possibility represents a separate embodiment of the present invention.

[00080] “Metabolic enzyme” refers, in another embodiment, to an enzyme involved in
5 synthesis of a nutrient required by the host bacteria. In another embodiment, the term refers to an enzyme required for synthesis of a nutrient required by the host bacteria. In another embodiment, the term refers to an enzyme involved in synthesis of a nutrient utilized by the host bacteria. In another embodiment, the term refers to an enzyme involved in synthesis of a
10 nutrient required for sustained growth of the host bacteria. In another embodiment, the enzyme is required for synthesis of the nutrient. Each possibility represents a separate embodiment of the present invention.

[00081] “Stably maintained” refers, in another embodiment, to maintenance of a nucleic acid molecule or plasmid in the absence of selection (e.g. antibiotic selection) for 10 generations, without detectable loss. In another embodiment, the period is 15 generations. In another
15 embodiment, the period is 20 generations. In another embodiment, the period is 25 generations. In another embodiment, the period is 30 generations. In another embodiment, the period is 40 generations. In another embodiment, the period is 50 generations. In another embodiment, the period is 60 generations. In another embodiment, the period is 80
20 generations. In another embodiment, the period is 100 generations. In another embodiment, the period is 150 generations. In another embodiment, the period is 200 generations. In another embodiment, the period is 300 generations. In another embodiment, the period is 500 generations. In another embodiment, the period is more than generations. In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vitro* (e.g. in culture). In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vivo*. In another embodiment, the nucleic acid molecule or plasmid is maintained stably both
25 *in vitro* and *in vivo*. Each possibility represents a separate embodiment of the present invention.

[00082] In another embodiment, the metabolic enzyme of the methods and compositions provided herein is an amino acid metabolism enzyme, where, in another embodiment, the
30 metabolic enzyme is an alanine racemase enzyme. In another embodiment, the metabolic enzyme is a D-amino acid transferase enzyme. In another embodiment, the metabolic enzyme catalyzes a formation of an amino acid used for a cell wall synthesis in the recombinant

Listeria strain, where in another embodiment the metabolic enzyme is an alanine racemase enzyme.

[00083] In another embodiment, the gene encoding the metabolic enzyme is expressed under the control of the *Listeria* p60 promoter. In another embodiment, the *inlA* (encodes internalin) promoter is used. In another embodiment, the *hly* promoter is used. In another embodiment, the *ActA* promoter is used. In another embodiment, the integrase gene is expressed under the control of any other gram positive promoter. In another embodiment, the gene encoding the metabolic enzyme is expressed under the control of any other promoter that functions in *Listeria*. The skilled artisan will appreciate that other promoters or polycistronic expression cassettes may be used to drive the expression of the gene. Each possibility represents a separate embodiment of the present invention.

[00084] The LLO utilized in the methods and compositions provided herein is, in one embodiment, a *Listeria* LLO. In one embodiment, the *Listeria* from which the LLO is derived is *Listeria monocytogenes* (Lm). In another embodiment, the *Listeria* is *Listeria ivanovii*. In another embodiment, the *Listeria* is *Listeria welshimeri*. In another embodiment, the *Listeria* is *Listeria seeligeri*.

[00085] In one embodiment, the LLO protein is encoded by the following nucleic acid sequence set forth in (SEQ ID NO: 1).

atgaaaaaataatgctagttttattacacttatattagttagttaccaattgcgcaaaaactgaagcaaaggatgcatctgcattcaataa
 agaaaattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcg
 ataagtatatcaaggattggattacaataaaaaacaatgtattagtagtaccacggagatgcagtgacaaatgtgccgccaagaaaaggta
 caaagatggaaatgaatatattgttgaggagaaaaagaagaaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttga
 gcctaacctatccaggtgctctcgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcattaacac
 tcagcattgatttccaggtatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgtaacaacgcagtaatacat
 tagtggaagatggaatgaaaaatagctcaagcttatccaaatgtaagtgcaaaattgattatgatgacgaaatggcttacagtgaatca
 caattaattgcgaaatttggtacagcatttaaagctgtaataatagcttgaaatgtaaacctcggcgcaatcagtgaaaggaaaatgcaaga
 agaagtcattagtttaacaaatttactataacgtgaatgttaatgaacctacaagacctccagattttcggcaaagctgttactaaagagc
 agttgcaagcgcttgagtgatgcagaaaatcctcctcatatatctcaagtggtggcgtatggccgtcaagtttatttgaaattatcaacta
 attcccatagtactaaagtaaaagctgctttgatgctgccgtaagcggaaaatctgtctcaggtgatgtagaactaacaataatcatcaaaa
 attcttcttcaaagccgtaatttaccggaggttcgcaaaagatgaagttcaaatcatcgacggcaacctcggagacttacgcgatatttga
 aaaaaggcgctacttttaatcgagaaacaccaggagtccattgtatatacaaaaacttcctaaaagacaatgaattagctgttataaaa

acaactcagaatatattgaacaacttcaaaagcctatacagatggaaaaattaacatcgatcactctggaggatacgttgctcaattcaaca
ttcttgggatgaagtaaattatgatctcgag (SEQ ID NO: 1).

[00086] In another embodiment, the LLO protein has the sequence SEQ ID NO: 2

5 M K K I M L V F I T L I L V S L P I A Q Q T E A K D A S A F N K E
N S I S S M A P P A S P P A S P K T P I E K K H A D E I D K Y I Q G
L D Y N K N N V L V Y H G D A V T N V P P R K G Y K D G N E Y I
V V E K K K K S I N Q N N A D I Q V V N A I S S L T Y P G A L V K
A N S E L V E N Q P D V L P V K R D S L T L S I D L P G M T N Q D
N K I V V K N A T K S N V N N A V N T L V E R W N E K Y A Q A Y
10 P N V S A K I D Y D D E M A Y S E S Q L I A K F G T A F K A V N N
S L N V N F G A I S E G K M Q E E V I S F K Q I Y Y N V N V N E P
T R P S R F F G K A V T K E Q L Q A L G V N A E N P P A Y I S S V
A Y G R Q V Y L K L S T N S H S T K V K A A F D A A V S G K S V
S G D V E L T N I I K N S S F K A V I Y G G S A K D E V Q I I D G
15 N L G D L R D I L K K G A T F N R E T P G V P I A Y T T N F L K D
N E L A V I K N N S E Y I E T T S K A Y T D G K I N I D H S G G Y
V A Q F N I S W D E V N Y D L (SEQ ID NO: 2)

The first 25 amino acids of the proprotein corresponding to this sequence are the signal
20 sequence and are cleaved from LLO when it is secreted by the bacterium. Thus, in this
embodiment, the full length active LLO protein is 504 residues long. In another embodiment,
the LLO protein has a sequence set forth in GenBank Accession No. DQ054588, DQ054589,
AY878649, U25452, or U25452. In another embodiment, the LLO protein is a variant of an
LLO protein. In another embodiment, the LLO protein is a homologue of an LLO protein.
25 Each possibility represents a separate embodiment of the present invention.

[00087] In another embodiment, “truncated LLO” or “tLLO” refers to a fragment of LLO that
comprises the PEST-like domain. In another embodiment, the terms refer to an LLO fragment
that does not contain the activation domain at the amino terminus and does not include
30 cystine 484. In another embodiment, the LLO fragment consists of a PEST sequence. In
another embodiment, the LLO fragment comprises a PEST sequence. In another embodiment,
the LLO fragment consists of about the first 400 to 441 amino acids of the 529 amino acid
full-length LLO protein. In another embodiment, the LLO fragment is a non-hemolytic form
of the LLO protein.

35 [00088] In one embodiment, the LLO fragment consists of about residues 1-25. In another
embodiment, the LLO fragment consists of about residues 1-50. In another embodiment, the
LLO fragment consists of about residues 1-75. In another embodiment, the LLO fragment
consists of about residues 1-100. In another embodiment, the LLO fragment consists of about

residues 1-125. In another embodiment, the LLO fragment consists of about residues 1-150. In another embodiment, the LLO fragment consists of about residues 1175. In another embodiment, the LLO fragment consists of about residues 1-200. In another embodiment, the LLO fragment consists of about residues 1-225. In another embodiment, the LLO fragment consists of about residues 1-250. In another embodiment, the LLO fragment consists of about residues 1-275. In another embodiment, the LLO fragment consists of about residues 1-300. In another embodiment, the LLO fragment consists of about residues 1-325. In another embodiment, the LLO fragment consists of about residues 1-350. In another embodiment, the LLO fragment consists of about residues 1-375. In another embodiment, the LLO fragment consists of about residues 1-400. In another embodiment, the LLO fragment consists of about residues 1-425. Each possibility represents a separate embodiment of the present invention.

[00089] In another embodiment, provided herein, is a vaccine comprising a recombinant form of *Listeria* of the present invention.

[00090] In another embodiment, provided herein, is a culture of a recombinant form of *Listeria* of the present invention.

[00091] In one embodiment, the live attenuated *Listeria* or recombinant *Listeria* provided herein expresses an ActA protein or a fragment thereof. In another embodiment of the methods and compositions of the present invention, a fragment of an ActA protein is fused to the heterologous antigen or a fragment thereof also provided herein. In another embodiment, the fragment of an ActA protein has the sequence:

[00092] MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKTEEQPSEVNTGPRYETAREVSSRDIKELEKSNKVRNTNKADLIAMLKEKAKEGPNINNNNSEQTENAAI NEEASGADRP AIQVERRHPGLPSDSA AEIKRRRKAIASSDSELESLTYPDKPTKVNKK KVAKESVADASESDL DSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKID ENPEVKKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEPFPPPTDEELRLALPETPMLLGFNAPATSEPSSFEPFPPPTDEELEIIRETASSLDSSFTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGGRP (SEQ ID No: 3). In another embodiment, an ActA AA sequence of methods and compositions of the present invention comprises the sequence set forth in SEQ ID No: 3. In another embodiment, the ActA AA sequence is a homologue of SEQ ID No: 3. In another embodiment, the ActA AA sequence is a variant of SEQ ID No: 3. In another embodiment, the ActA AA sequence is a

fragment of SEQ ID No: 3. In another embodiment, the ActA AA sequence is an isoform of SEQ ID No: 3. Each possibility represents a separate embodiment of the present invention.

[00093] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide comprising the sequence:

ATGCGTGCGATGATGGTGGTTTTTCATTACTGCCAATTGCATTACGATTAACCCCGA
 CATAATATTTGCAGCGACAGATAGCGAAGATTCTAGTCTAAACACAGATGAATGG
 5 GAAGAAGAAAAAACAGAAGAGCAACCAAGCGAGGTAAATACGGGACCAAGATA
 CGAAACTGCACGTGAAGTAAGTTCACGTGATATTAAAGAACTAGAAAAATCGAA
 TAAAGTGAGAAATACGAACAAAGCAGACCTAATAGCAATGTTGAAAGAAAAAGC
 AGAAAAAGGTCCAAATATCAATAATAACAACAGTGAACAAACTGAGAATGCGGC
 TATAAATGAAGAGGCTTCAGGAGCCGACCGACCAGCTATACAAGTGGAGCGTCG
 10 TCATCCAGGATTGCCATCGGATAGCGCAGCGGAAATTAAAAAAGAAGGAAAGC
 CATAGCATCATCGGATAGTGAGCTTGAAAGCCTTACTTATCCGGATAAACCAACA
 AAAGTAAATAAGAAAAAAGTGGCGAAAGAGTCAGTTGCGGATGCTTCTGAAAGT
 GACTTAGATTCTAGCATGCAGTCAGCAGATGAGTCTTCACCACAACCTTTAAAG
 CAAACCAACAACCATTTTTCCCTAAAGTATTTAAAAAATAAAAGATGCGGGGA
 15 AATGGGTACGTGATAAAATCGACGAAAATCCTGAAGTAAAGAAAGCGATTGTTG
 ATAAAAGTGCAGGGTTAATTGACCAATTATTAACCAAAAAGAAAAGTGAAGAGG
 TAAATGCTTCGGACTTCCCGCCACCACCTACGGATGAAGAGTTAAGACTTGCTTT
 GCCAGAGACACCAATGCTTCTTGTTTTAATGCTCCTGCTACATCAGAACCGAGC
 TCATTGGAATTTCCACCACCACCTACGGATGAAGAGTTAAGACTTGCTTTGCCAG
 20 AGACGCCAATGCTTCTTGTTTTAATGCTCCTGCTACATCGGAACCGAGCTCGTTC
 GAATTTCCACCGCCTCCAACAGAAGATGAACTAGAAATCATCCGGGAAACAGCA
 TCCTCGCTAGATTCTAGTTTTACAAGAGGGGATTTAGCTAGTTTGAGAAATGCTAT
 TAATCGCCATAGTCAAAATTTCTCTGATTTCCACCAATCCCAACAGAAGAAGAG
 TTGAA CGGGAGAGGCGGTAGACCA (SEQ ID NO: 4). In another embodiment, the
 25 recombinant nucleotide has the sequence set forth in SEQ ID NO: 4. In another embodiment,
 an ActA-encoding nucleotide of methods and compositions of the present invention comprises
 the sequence set forth in SEQ ID No: 4. In another embodiment, the ActA-encoding
 nucleotide is a homologue of SEQ ID No: 4. In another embodiment, the ActA-encoding
 nucleotide is a variant of SEQ ID No: 4. In another embodiment, the ActA-encoding
 30 nucleotide is a fragment of SEQ ID No: 4. In another embodiment, the ActA-encoding
 nucleotide is an isoform of SEQ ID No: 4. Each possibility represents a separate embodiment
 of the present invention.

[00094] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide comprising the sequence:

[00095] Tttatcacgtacccatttccccgcacatcttttatttttttaataacttttagggaaaa
 atggtttttgatttgcttttaaggttggtgtagactcgtctgctgactgcatgctagaa
 5 tctaagtcactttcagaagcatccacaactgactctttcgccacttttctcttatttgcttt
 tgttggtttatctggataagtaaggctttcaagctcactatccgacgacgctatggcttttc
 ttctttttttaatttccgctgcgctatccgatgacagacctggatgacgacgctccacttgc
 agagttggctcggctcactcctgaagcctcttcatttatagccacatttctgtttgctcacc
 gttgttattattgttattcggacctttctctgcttttgctttcaacattgctattaggtctg
 10 ctttggttcgtatttttctactttattcgtattttctagttcctcaatatcacgtgaacttact
 tcacgtgcagtttcgtatcttggtcccgtatttacctcgcttggtgctcttctgttttttc
 ttcttcccatcatctgtgttttagactggaatcttcgctatctgtcgctgcaaataattatgt
 cgggggttaatcgtaatgcagttggcagtaatgaaaactaccatcatcgcacgcat (SEQ ID

NO: 5). In another embodiment, the recombinant nucleotide has the sequence set forth in
 15 SEQ ID NO: 5. In another embodiment, an ActA-encoding nucleotide of methods and
 compositions of the present invention comprises the sequence set forth in SEQ ID No: 5. In
 another embodiment, the ActA-encoding nucleotide is a homologue of SEQ ID No: 5. In
 another embodiment, the ActA-encoding nucleotide is a variant of SEQ ID No: 5. In another
 embodiment, the ActA-encoding nucleotide is a fragment of SEQ ID No: 5. In another
 20 embodiment, the ActA-encoding nucleotide is an isoform of SEQ ID No: 5. Each possibility
 represents a separate embodiment of the present invention.

[00096] In another embodiment of methods and compositions of the present invention, a
 fragment of an ActA protein is fused to a heterologous antigen or fragment thereof. In another
 embodiment, the fragment of an ActA protein has the sequence as set forth in Genbank
 25 Accession No. AAF04762. In another embodiment, an ActA AA sequence of methods and
 compositions of the present invention comprises the sequence set forth in Genbank Accession
 No. AAF04762. In another embodiment, the ActA AA sequence is a homologue of Genbank
 Accession No. AAF04762. In another embodiment, the ActA AA sequence is a variant of
 Genbank Accession No. AAF04762. In another embodiment, the ActA AA sequence is a
 30 fragment of Genbank Accession No. AAF04762. In another embodiment, the ActA AA
 sequence is an isoform of Genbank Accession No. AAF04762. Each possibility represents a
 separate embodiment of the present invention.

[00097] An N-terminal fragment of an ActA protein utilized in methods and compositions of the present invention has, in another embodiment, the sequence set forth in SEQ ID NO: 6:

MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKTEEQPSEVNTGPRYETA
REVSSRDIKELEKSNKVRNTNKADLIAMLEKEAEKGPNNNNNSEQTENAAINEEAS
5 GADRPAlQVERRHPGLPSDSAAEIKRRRKAIASSDSELESLTYPDKPTKVNKKKVAKE
SVADASESDLDSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKIDENPEV
KKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSEP
SSFEFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTDELEIIRETASSLDSS
FTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGRG. In another embodiment, the

10 ActA fragment comprises the sequence set forth in SEQ ID NO: 6. In another embodiment, the ActA fragment is any other ActA fragment known in the art. Each possibility represents a separate embodiment of the present invention.

[00098] In another embodiment, the recombinant nucleotide encoding a fragment of an ActA protein comprises the sequence set forth in SEQ ID NO: 7

15 Atgcgtgcgatgatggtggttttcattactgccaatgcattacgattaaccccgacataatattgcagcgacagatagcgaagattcta
gtctaaacacagatgaatgggaagaagaaaaacagaagagcaaccaagcgaggtaaatacgggaccaagatacgaaactgcacg
tgaagtaagttcacgtgatattaagaactagaaaaatcgaataaagtgaataacgaacaagcagacctaataagcaatgttgaaag
aaaaagcagaaaaaggtccaaatatcaataataacaacagtgaacaaactgagaatgcggctataatgaagaggcttcaggagccg
accgaccagctatacaagtgaggcgtcgtcatccaggattgccatcgatagcgagcggaattaaaaaagaaggaaagccatag
20 catcatcgatagtgagcttgaaagccttacttatccggataaaccaacaaaagtaataagaaaaagtgccgaaagagtcagttgcg
gatgcttctgaaagtacttagattctagcatgcagtcagcagatgagtcctaccacaacctttaaagcaaaccaacaaccattttccc
taaagtatttaaaaaataaaagatgcggggaaatgggtacgtgataaatcgacgaaaatcctgaagtaagaaagcgattgttgata
aaagtgcagggttaattgaccaattattaacaaaaagaaaagtgaagaggtaaatgcttcggactcccgccaccacctacggatgaa
gagttaagacttgcttgccagagacaccaatgcttcttggttttaatgctcctgctacatcagaaccgagctcattcgaatttcaccacca
25 cctacggatgaagagttaagacttgcttgccagagacccaatgcttcttggttttaatgctcctgctacatcggaaccgagctcgttcga
atttcaccgcctccaacagaagatgaactagaaatcatccgggaacagcatcctcgctagattctagttttacaagaggggatttagct
agtttgagaaatgctattaatgcccatagtaaaatttctctgattcccaccaatcccaacagaagaagagttgaacgggagagggcgt
agacca. In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ
ID NO: 7. In another embodiment, the recombinant nucleotide comprises any other sequence
30 that encodes a fragment of an ActA protein. Each possibility represents a separate
embodiment of the present invention.

[00099] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide comprising the sequence as set forth in Genbank Accession No. AF103807. In another embodiment, the recombinant nucleotide has the sequence set forth in Genbank Accession No. AF103807. In another embodiment, an ActA-encoding nucleotide of methods and compositions of the present invention comprises the sequence set forth in Genbank Accession No. AF103807. In another embodiment, the ActA-encoding nucleotide is a homologue of Genbank Accession No. AF103807. In another embodiment, the ActA-encoding nucleotide is a variant of Genbank Accession No. AF103807. In another embodiment, the ActA-encoding nucleotide is a fragment of Genbank Accession No. AF103807. In another embodiment, the ActA-encoding nucleotide is an isoform of Genbank Accession No. AF103807. Each possibility represents a separate embodiment of the present invention.

[000100] In another embodiment, the ActA fragment is any other ActA fragment known in the art. In another embodiment, a recombinant nucleotide of the present invention comprises any other sequence that encodes a fragment of an ActA protein. In another embodiment, the recombinant nucleotide comprises any other sequence that encodes an entire ActA protein. Each possibility represents a separate embodiment of the present invention.

[000101] In one embodiment, the live attenuated *Listeria* or recombinant *Listeria* provided herein expresses a PEST sequence peptide. In another embodiment of methods and compositions of the present invention, a PEST AA sequence is fused to the heterologous antigen or fragment. In another embodiment, the PEST AA sequence is KENSISSMAPPASPPASPKTPIEKKHADEIDK (SEQ ID NO: 8). In another embodiment, the PEST sequence is KENSISSMAPPASPPASPK (SEQ ID No: 9).

[000102] In another embodiment, the PEST AA sequence is a PEST sequence from a *Listeria* ActA protein. In another embodiment, the PEST sequence is KTEEQPSEVNTGPR (SEQ ID NO: 10), KASVTDTSEGLDSSMQSADESTPQPLK (SEQ ID NO: 11), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 12), or RGGIPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 13). In another embodiment, the PEST-like sequence is a variant of the PEST sequence described hereinabove, which in one embodiment, is KESVYDASESDLDSSMQSADESTPQPLK (SEQ ID NO: 14, KSEEVNASDFPPPTDEELR (SEQ ID NO: 15), or RGGRPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 16), as would be understood by a skilled artisan. In another embodiment, the PEST-like sequence is from

Listeria seeligeri cytolysin, encoded by the lso gene. In another embodiment, the PEST sequence is RSEVTISPAETPESPPATP (SEQ ID NO: 17). In another embodiment, the PEST sequence is from Streptolysin O protein of *Streptococcus sp.* In another embodiment, the PEST sequence is from *Streptococcus pyogenes* Streptolysin O, e.g. KQNTASTETTTTNEQPK (SEQ ID NO: 18) at AA 35-51. In another embodiment, the PEST-like sequence is from *Streptococcus equisimilis* Streptolysin O, e.g. KQNTANTETTTTNEQPK (SEQ ID NO: 19) at AA 38-54. In another embodiment, the PEST-like sequence has a sequence selected from SEQ ID NO: 8-16. In another embodiment, the PEST-like sequence has a sequence selected from SEQ ID NO: 8-19. In another embodiment, the PEST sequence is another PEST AA sequence derived from a prokaryotic organism.

[000103] Identification of PEST sequences is well known in the art, and is described, for example in Rogers S et al (Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science 1986; 234(4774):364-8) and Rechsteiner M et al (PEST sequences and regulation by proteolysis. Trends Biochem Sci 1996; 21(7):267-71). "PEST sequence" refers, in another embodiment, to a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues. In another embodiment, the PEST sequence is flanked by one or more clusters containing several positively charged amino acids. In another embodiment, the PEST sequence mediates rapid intracellular degradation of proteins containing it. In another embodiment, the PEST sequence fits an algorithm disclosed in Rogers et al. In another embodiment, the PEST sequence fits an algorithm disclosed in Rechsteiner et al. In another embodiment, the PEST sequence contains one or more internal phosphorylation sites, and phosphorylation at these sites precedes protein degradation.

[000104] In one embodiment, PEST sequences of prokaryotic organisms are identified in accordance with methods such as described by, for example Rechsteiner and Rogers (1996, Trends Biochem. Sci. 21:267-271) for *Lm* and in Rogers S et al (Science 1986; 234(4774):364-8). Alternatively, PEST AA sequences from other prokaryotic organisms can also be identified based on this method. Other prokaryotic organisms wherein PEST AA sequences would be expected to include, but are not limited to, other *Listeria* species. In one embodiment, the PEST sequence fits an algorithm disclosed in Rogers et al. In another embodiment, the PEST sequence fits an algorithm disclosed in Rechsteiner et al. In another embodiment, the PEST sequence is identified using the PEST-find program.

[000105] In another embodiment, identification of PEST motifs is achieved by an initial scan for positively charged AA R, H, and K within the specified protein sequence. All AA between the positively charged flanks are counted and only those motifs are considered further, which contain a number of AA equal to or higher than the window-size parameter. In
5 another embodiment, a PEST-like sequence must contain at least 1 P, 1 D or E, and at least 1 S or T.

[000106] In another embodiment, the quality of a PEST motif is refined by means of a scoring parameter based on the local enrichment of critical AA as well as the motif's hydrophobicity. Enrichment of D, E, P, S and T is expressed in mass percent (w/w) and corrected for 1
10 equivalent of D or E, 1 of P and 1 of S or T. In another embodiment, calculation of hydrophobicity follows in principle the method of J. Kyte and R.F. Doolittle (Kyte, J and Doolittle, RF. J. Mol. Biol. 157, 105 (1982).

[000107] In another embodiment, a potential PEST motif's hydrophobicity is calculated as the sum over the products of mole percent and hydrophobicity index for each AA species. The
15 desired PEST score is obtained as combination of local enrichment term and hydrophobicity term as expressed by the following equation:

[000108] $\text{PEST score} = 0.55 * \text{DEPST} - 0.5 * \text{hydrophobicity index}.$

[000109] In another embodiment, "PEST sequence", "PEST-like sequence" or "PEST-like sequence peptide" refers to a peptide having a score of at least +5, using the above algorithm.

20 In another embodiment, the term refers to a peptide having a score of at least 6. In another embodiment, the peptide has a score of at least 7. In another embodiment, the score is at least 8. In another embodiment, the score is at least 9. In another embodiment, the score is at least 10. In another embodiment, the score is at least 11. In another embodiment, the score is at least 12. In another embodiment, the score is at least 13. In another embodiment, the score is
25 at least 14. In another embodiment, the score is at least 15. In another embodiment, the score is at least 16. In another embodiment, the score is at least 17. In another embodiment, the score is at least 18. In another embodiment, the score is at least 19. In another embodiment, the score is at least 20. In another embodiment, the score is at least 21. In another embodiment, the score is at least 22. In another embodiment, the score is at least 22. In
30 another embodiment, the score is at least 24. In another embodiment, the score is at least 24. In another embodiment, the score is at least 25. In another embodiment, the score is at least 26. In another embodiment, the score is at least 27. In another embodiment, the score is at

least 28. In another embodiment, the score is at least 29. In another embodiment, the score is at least 30. In another embodiment, the score is at least 32. In another embodiment, the score is at least 35. In another embodiment, the score is at least 38. In another embodiment, the score is at least 40. In another embodiment, the score is at least 45. Each possibility

5 represents a separate embodiment of the present invention.

[000110] In another embodiment, the PEST sequence is identified using any other method or algorithm known in the art, e.g. the CaSPredictor (Garay-Malpartida HM, Occhiucci JM, Alves J, Belizario JE. *Bioinformatics*. 2005 Jun;21 Suppl 1:i169-76). In another embodiment, the following method is used:

10 [000111] A PEST index is calculated for each stretch of appropriate length (e.g. a 30-35 AA stretch) by assigning a value of 1 to the AA Ser, Thr, Pro, Glu, Asp, Asn, or Gln. The coefficient value (CV) for each of the PEST residue is 1 and for each of the other AA (non-PEST) is 0.

[000112] Each method for identifying a PEST-like sequence represents a separate
15 embodiment of the present invention.

[000113] In another embodiment, the PEST sequence is any other PEST sequence known in the art. Each PEST sequence and type thereof represents a separate embodiment of the present invention.

[000114] "Fusion to a PEST sequence" refers, in another embodiment, to fusion to a protein
20 fragment comprising a PEST sequence. In another embodiment, the term includes cases wherein the protein fragment comprises surrounding sequence other than the PEST sequence. In another embodiment, the protein fragment consists of the PEST sequence. Thus, in another embodiment, "fusion" refers to two peptides or protein fragments either linked together at their respective ends or embedded one within the other. Each possibility represents a separate
25 embodiment of the present invention.

[000115] In another embodiment, provided herein is a vaccine comprising a recombinant form of *Listeria* of the present invention.

[000116] In another embodiment, provided herein, is a culture of a recombinant form of *Listeria* of the present invention.

[000117] In another embodiment, the *Listeria* of methods and compositions of the present invention is *Listeria monocytogenes*. In another embodiment, the *Listeria* is *Listeria ivanovii*. In another embodiment, the *Listeria* is *Listeria welshimeri*. In another embodiment, the *Listeria* is *Listeria seeligeri*. Each type of *Listeria* represents a separate embodiment of the present invention.

[000118] In one embodiment, attenuated *Listeria* strains, such as *Lm* delta-*actA* mutant (Brundage et al, 1993, Proc. Natl. Acad. Sci., USA, 90:11890-11894), *L. monocytogenes* delta-*plcA* (Camilli et al, 1991, J. Exp. Med., 173:751-754), or delta-*ActA*, delta *INL-b* (Brockstedt et al, 2004, PNAS, 101:13832-13837) are used in the present invention. In another embodiment, attenuated *Listeria* strains are constructed by introducing one or more attenuating mutations, as will be understood by one of average skill in the art when equipped with the disclosure herein. Examples of such strains include, but are not limited to *Listeria* strains auxotrophic for aromatic amino acids (Alexander et al, 1993, Infection and Immunity 61:2245-2248) and mutant for the formation of lipoteichoic acids (Abachin et al, 2002, Mol. Microbiol. 43:1-14) and those attenuated by a lack of a virulence gene (see examples herein).

[000119] In another embodiment, the nucleic acid molecule of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, the first open reading frame of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, the second open reading frame of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, each of the open reading frames are operably linked to a promoter/regulatory sequence. Each possibility represents a separate embodiment of the present invention.

[000120] The skilled artisan, when equipped with the present disclosure and the methods provided herein, will readily understand that different transcriptional promoters, terminators, carrier vectors or specific gene sequences (e.g. those in commercially available cloning vectors) can be used successfully in methods and compositions of the present invention. As is contemplated in the present invention, these functionalities are provided in, for example, the commercially available vectors known as the pUC series. In another embodiment, non-essential DNA sequences (e.g. antibiotic resistance genes) are removed. Each possibility represents a separate embodiment of the present invention. In another embodiment, a

commercially available plasmid is used in the present invention. Such plasmids are available from a variety of sources, for example, Invitrogen (La Jolla, CA), Stratagene (La Jolla, CA), Clontech (Palo Alto, CA), or can be constructed using methods well known in the art.

[000121] Another embodiment is a plasmid such as pCR2.1 (Invitrogen, La Jolla, CA), which
5 is a prokaryotic expression vector with a prokaryotic origin of replication and promoter/regulatory elements to facilitate expression in a prokaryotic organism. In another embodiment, extraneous nucleotide sequences are removed to decrease the size of the plasmid and increase the size of the cassette that can be placed therein.

[000122] Such methods are well known in the art, and are described in, for example, Sambrook
10 et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubei et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York).

[000123] Antibiotic resistance genes are used in the conventional selection and cloning
15 processes commonly employed in molecular biology and vaccine preparation. Antibiotic resistance genes contemplated in the present invention include, but are not limited to, gene products that confer resistance to ampicillin, penicillin, methicillin, streptomycin, erythromycin, kanamycin, tetracycline, chloramphenicol (CAT), neomycin, hygromycin, gentamicin and others well known in the art. Each gene represents a separate embodiment of the present invention.

[000124] Methods for transforming bacteria are well known in the art, and include calcium-
20 chloride competent cell-based methods, electroporation methods, bacteriophage-mediated transduction, chemical, and physical transformation techniques (de Boer et al, 1989, Cell 56:641-649; Miller et al, 1995, FASEB J., 9:190-199; Sambrook et al. 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York; Ausubel et al.,
25 1997, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Gerhardt et al., eds., 1994, Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, DC; Miller, 1992, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) In another embodiment, the *Listeria* vaccine strain of the present invention is transformed by electroporation. Each
30 method represents a separate embodiment of the present invention.

[000125] In another embodiment, conjugation is used to introduce genetic material and/or plasmids into bacteria. Methods for conjugation are well known in the art, and are described, for example, in Nikodinovic J et al. (A second generation snp-derived *Escherichia coli*-*Streptomyces* shuttle expression vector that is generally transferable by conjugation. Plasmid. 2006 Nov;56(3):223-7) and Auchtung JM et al (Regulation of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA damage response. Proc Natl Acad Sci U S A. 2005 Aug 30;102 (35):12554-9). Each method represents a separate embodiment of the present invention.

[000126] "Transforming," in one embodiment, is used identically with the term "transfecting," and refers to engineering a bacterial cell to take up a plasmid or other heterologous DNA molecule. In another embodiment, "transforming" refers to engineering a bacterial cell to express a gene of a plasmid or other heterologous DNA molecule. Each possibility represents a separate embodiment of the present invention.

[000127] Plasmids and other expression vectors useful in the present invention are described elsewhere herein, and can include such features as a promoter/regulatory sequence, an origin of replication for gram negative and gram positive bacteria, an isolated nucleic acid encoding a fusion protein and an isolated nucleic acid encoding an amino acid metabolism gene. Further, an isolated nucleic acid encoding a fusion protein and an amino acid metabolism gene will have a promoter suitable for driving expression of such an isolated nucleic acid. Promoters useful for driving expression in a bacterial system are well known in the art, and include bacteriophage lambda, the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325. Further examples of prokaryotic promoters include the major right and left promoters of bacteriophage lambda (PL and PR), the trp, recA, lacZ, lad, and gal promoters of *E. coli*, the alpha-amylase (Ulmanen et al, 1985. J. Bacteriol. 162:176-182) and the S28-specific promoters of *B. subtilis* (Gilman et al, 1984 Gene 32:11- 20), the promoters of the bacteriophages of *Bacillus* (Gryczan, 1982, In: The Molecular Biology of the Bacilli, Academic Press, Inc., New York), and *Streptomyces* promoters (Ward et al, 1986, Mol. Gen. Genet. 203:468-478). Additional prokaryotic promoters contemplated in the present invention are reviewed in, for example, Glick (1987, J. Ind. Microbiol. 1:277-282); Cenatiempo, (1986, Biochimie, 68:505-516); and Gottesman, (1984, Ann. Rev. Genet. 18:415-442). Further examples of promoter/regulatory elements contemplated in the present invention include, but are not limited to the *Listerial* prfA promoter, the *Listerial* hly promoter, the *Listerial* p60

promoter and the *Listerial* ActA promoter (GenBank Acc. No. NC_003210) or fragments thereof.

[000128] In one embodiment, DNA encoding the recombinant non-hemolytic LLO is produced using DNA amplification methods, for example polymerase chain reaction (PCR). First, the segments of the native DNA on either side of the new terminus are amplified separately. The 5' end of the one amplified sequence encodes the peptide linker, while the 3' end of the other amplified sequence also encodes the peptide linker. Since the 5' end of the first fragment is complementary to the 3' end of the second fragment, the two fragments (after partial purification, e.g. on LMP agarose) can be used as an overlapping template in a third PCR reaction. The amplified sequence will contain codons, the segment on the carboxy side of the opening site (now forming the amino sequence), the linker, and the sequence on the amino side of the opening site (now forming the carboxyl sequence). The antigen is ligated into a plasmid. Each method represents a separate embodiment of the present invention.

[000129] In another embodiment, the present invention further comprises a phage based chromosomal integration system for clinical applications. A host strain that is auxotrophic for essential enzymes, including, but not limited to, d-alanine racemase will be used, for example Lmdal(-)dat(-). In another embodiment, in order to avoid a "phage curing step," a phage integration system based on PSA is used (Lauer, et al., 2002 J Bacteriol, 184:4177-4186). This requires, in another embodiment, continuous selection by antibiotics to maintain the integrated gene. Thus, in another embodiment, the current invention enables the establishment of a phage based chromosomal integration system that does not require selection with antibiotics. Instead, an auxotrophic host strain will be complemented.

[000130] The recombinant proteins of the present invention are synthesized, in another embodiment, using recombinant DNA methodology. This involves, in one embodiment, creating a DNA sequence, placing the DNA in an expression cassette, such as the plasmid of the present invention, under the control of a particular promoter/regulatory element, and expressing the protein. DNA encoding the protein (e.g. non-hemolytic LLO) of the present invention is prepared, in another embodiment, by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang et al. (1979, Meth. Enzymol. 68: 90-99); the phosphodiester method of Brown et al. (1979, Meth. Enzymol 68: 109-151); the

diethylphosphoramidite method of Beaucage et al. (1981, Tetra. Lett., 22: 15 1859-1862); and the solid support method of U.S. Pat. No. 4,458,066.

[000131] In another embodiment, chemical synthesis is used to produce a single stranded oligonucleotide. This single stranded oligonucleotide is converted, in various embodiments,
5 into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill in the art would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences can be obtained by the ligation of shorter sequences. In another embodiment, subsequences are cloned and the appropriate subsequences cleaved
10 using appropriate restriction enzymes. The fragments are then be ligated to produce the desired DNA sequence.

[000132] In another embodiment, DNA encoding the recombinant protein of the present invention is cloned using DNA amplification methods such as polymerase chain reaction (PCR). Thus, the gene for non-hemolytic LLO is PCR amplified, using a sense primer
15 comprising a suitable restriction site and an antisense primer comprising another restriction site, e.g. a non-identical restriction site to facilitate cloning.

[000133] In another embodiment, the recombinant fusion protein gene is operably linked to appropriate expression control sequences for each host. Promoter/ regulatory sequences are described in detail elsewhere herein. In another embodiment, the plasmid further comprises
20 additional promoter regulatory elements, as well as a ribosome binding site and a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and an enhancer derived from e.g. immunoglobulin genes, SV40, cytomegalovirus, etc., and a polyadenylation sequence. In another embodiment, the sequences include splice donor and acceptor sequences.

25 [000134] In one embodiment, the term “operably linked” refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

30 [000135] In another embodiment, in order to select for an auxotrophic bacteria comprising the plasmid, transformed auxotrophic bacteria are grown on a media that will select for

expression of the amino acid metabolism gene. In another embodiment, a bacteria auxotrophic for D-glutamic acid synthesis is transformed with a plasmid comprising a gene for D-glutamic acid synthesis, and the auxotrophic bacteria will grow in the absence of D-glutamic acid, whereas auxotrophic bacteria that have not been transformed with the plasmid, or are not expressing the plasmid encoding a protein for D-glutamic acid synthesis, will not grow. In another embodiment, a bacterium auxotrophic for D-alanine synthesis will grow in the absence of D-alanine when transformed and expressing the plasmid of the present invention if the plasmid comprises an isolated nucleic acid encoding an amino acid metabolism enzyme for D-alanine synthesis. Such methods for making appropriate media comprising or lacking necessary growth factors, supplements, amino acids, vitamins, antibiotics, and the like are well known in the art, and are available commercially (Becton-Dickinson, Franklin Lakes, NJ). Each method represents a separate embodiment of the present invention.

[000136] In another embodiment, once the auxotrophic bacteria comprising the plasmid of the present invention have been selected on appropriate media, the bacteria are propagated in the presence of a selective pressure. Such propagation comprises growing the bacteria in media without the auxotrophic factor. The presence of the plasmid expressing an amino acid metabolism enzyme in the auxotrophic bacteria ensures that the plasmid will replicate along with the bacteria, thus continually selecting for bacteria harboring the plasmid. The skilled artisan, when equipped with the present disclosure and methods herein will be readily able to scale-up the production of the *Listeria* vaccine vector by adjusting the volume of the media in which the auxotrophic bacteria comprising the plasmid are growing.

[000137] The skilled artisan will appreciate that, in another embodiment, other auxotroph strains and complementation systems are adopted for the use with this invention.

[000138] In one embodiment, provided herein is a method of administering the composition of the present invention. In another embodiment, provided herein is a method of administering the vaccine of the present invention. In another embodiment, provided herein is a method of administering the recombinant polypeptide or recombinant nucleotide of the present invention. In another embodiment, the step of administering the composition, vaccine, recombinant polypeptide or recombinant nucleotide of the present invention is performed with an attenuated recombinant form of *Listeria* comprising the composition, vaccine, recombinant nucleotide or expressing the recombinant polypeptide, each in its own discrete

embodiment. In another embodiment, the administering is performed with a different attenuated bacterial vector. In another embodiment, the administering is performed with a DNA vaccine (e.g. a naked DNA vaccine). In another embodiment, administration of a recombinant polypeptide of the present invention is performed by producing the recombinant protein, then administering the recombinant protein to a subject. Each possibility represents a separate embodiment of the present invention.

[000139] In one embodiment, the vaccine for use in the methods of the present invention comprises a recombinant *Listeria monocytogenes*, in any form or embodiment as described herein. In one embodiment, the vaccine for use in the present invention consists of a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In another embodiment, the vaccine for use in the methods of the present invention consists essentially of a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In one embodiment, the term “comprise” refers to the inclusion of a recombinant *Listeria monocytogenes* in the vaccine, as well as inclusion of other vaccines or treatments that may be known in the art. In another embodiment, the term “consisting essentially of” refers to a vaccine, whose functional component is the recombinant *Listeria monocytogenes*, however, other components of the vaccine may be included that are not involved directly in the therapeutic effect of the vaccine and may, for example, refer to components which facilitate the effect of the recombinant *Listeria monocytogenes* (e.g. stabilizing, preserving, etc.). In another embodiment, the term “consisting” refers to a vaccine, which contains the recombinant *Listeria monocytogenes*.

[000140] In another embodiment, the methods of the present invention comprise the step of administering a recombinant *Listeria monocytogenes*, in any form or embodiment as described herein. In one embodiment, the methods of the present invention consist of the step of administering a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In another embodiment, the methods of the present invention consist essentially of the step of administering a recombinant *Listeria monocytogenes* of the present invention, in any form or embodiment as described herein. In one embodiment, the term “comprise” refers to the inclusion of the step of administering a recombinant *Listeria monocytogenes* in the methods, as well as inclusion of other methods or treatments that may be known in the art. In another embodiment, the term “consisting essentially of” refers to a method, whose functional component is the administration of recombinant *Listeria monocytogenes*, however, other steps of the methods may be included

that are not involved directly in the therapeutic effect of the methods and may, for example, refer to steps which facilitate the effect of the administration of recombinant *Listeria monocytogenes*. In one embodiment, the term “consisting” refers to a method of administering recombinant *Listeria monocytogenes* with no additional steps.

- 5 [000141] In another embodiment, the immune response elicited by methods and compositions of the present invention comprises a CD8⁺ T cell-mediated response. In another embodiment, the immune response consists primarily of a CD8⁺ T cell-mediated response. In another embodiment, the only detectable component of the immune response is a CD8⁺ T cell-mediated response (see Examples 7-11).
- 10 [000142] In another embodiment, the immune response elicited by methods and compositions provided herein comprises a CD4⁺ T cell-mediated response. In another embodiment, the immune response consists primarily of a CD4⁺ T cell-mediated response. In another embodiment, the only detectable component of the immune response is a CD4⁺ T cell-mediated response. In another embodiment, the CD4⁺ T cell-mediated response is
- 15 accompanied by a measurable antibody response against the antigen. In another embodiment, the CD4⁺ T cell-mediated response is not accompanied by a measurable antibody response against the antigen (see Examples 7-11).

- [000143] In another embodiment, the immune response elicited by methods and compositions provided herein comprises an innate immune response wherein M1 macrophages and
- 20 dendritic cells (DCs) are activated.

[000144] In one embodiment, provided herein is a method of increasing intratumoral ratio of CD8⁺/T regulatory cells, whereby and in another embodiment, the method comprising the step of administering to the subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present invention (see Examples 7-11).

- 25 [000145] In another embodiment, provided herein is a method of increasing intratumoral ratio of CD8⁺/T regulatory cells, whereby and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present invention (see Examples 7-11).

- [000146] In one embodiment, provided herein is a method of increasing intratumoral ratio of
- 30 CD8⁺/ myeloid-derived suppressor cells (MDSC), whereby and in another embodiment, the

method comprises the step of administering to the subject a composition comprising the recombinant *Listeria*, or recombinant vector of the present invention.

[000147] In another embodiment, provided herein is a method of increasing the ratio of CD8+ myeloid-derived suppressor cells (MDSC) at sites of disease, whereby and in another
5 embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria*, or recombinant vector of the present invention.

[000148] Common plasma markers in human MDSCs include CD33, CD11b, CD15, CD14 negative, MHC class II negative, HLA DR^{low} or -. Common intracellular markers include arginase, and iNOS. Further, human MDSCs' suppressive activity or mechanism includes use
10 of nitric oxide (NO), arginase, or nitrotyrosine. In mice, myeloid-derived suppressor cells (MDSC) are CD11b and Gr-1 double positive and have also have been described as F4/80^{int}, CD11c^{low}, MHCII-/^{low}, Ly6C+. CD11b+/Gr-1+ cells that have immunosuppressive ability have been described to produce IFN-g. MDSCs can be monocytic and/or granulocytic as well.

[000149] In one embodiment, MDSCs at disease sites can unexpectedly inhibit both, the
15 function of antigen-specific and non-specific T cell function, while spleen MDSCs can only inhibit the function of antigen-specific T cells. As demonstrated in the Examples below (see Examples 21-24), the live attenuated *Listeria* provided herein reduces the amount or quantity of suppressor cells in a disease thereby allowing CD8 T cell replication and infiltration at the disease site, for example, a tumor site.

20 [000150] *Lm* or sublytic doses of LLO in human epithelial Caco-2 cells induce the expression of IL-6 that reduces bacterial intracellular growth and causes over expression of inducible nitric oxide synthase (NOS). Nitric oxide appears to be an essential component of the innate immune response to *Lm*, having an important role in listericidal activity of neutrophils and macrophages, with a deficiency of inducible NO synthase (iNOS) causing susceptibility to *Lm*
25 infection.

[000151] *Lm* infection also results in the generation of robust MHC Class 2 restricted CD4⁺ T cell responses, and shifts the phenotype of CD4⁺ T cells to Th-1. Further, CD4⁺ T cell help is required for the generation and maintenance of functional CD8⁺ T cell memory against *Lm*. Moreover, it has been reported infection of mice intraperitoneally with *Lm* caused a local
30 induction of CD4⁺ T_{γδ} cells associated with IL-17 secretion in the peritoneal cavity however no changes were observed in the splenic or lymph node T cell populations after these

injections. In addition, *Listeria* infection also involves other systems not essentially a part of the immune system but which support immune function to affect a therapeutic outcome, such as myelopoiesis and vascular endothelial cell function.

[000152] *Lm* infected macrophages produce TNF- α , IL-18 and IL-12, all of which are
5 important in inducing the production of IFN- γ and subsequent killing and degradation of *Lm* in the phagosome. IL-12 deficiency results in an increased susceptibility to listeriosis, which can be reversed through administration of IFN- γ . NK cells are the major source of IFN- γ in early infection. Upon reinfection memory CD8⁺ T cells have the ability to produce IFN- γ in response to IL-12 and IL-18 in the absence of the cognate antigen. CD8⁺ T cells co-localize
10 with the macrophages and *Lm* in the T cell area of the spleen where they produce IFN- γ independent of antigen. IFN- γ production by CD8⁺ T cells depends partially on the expression of LLO.

[000153] IFN- γ plays an important role in anti-tumor responses obtained by *Lm*-based vaccines. Although produced initially by NK cells, IFN- γ levels are subsequently maintained
15 by CD4⁺ T-helper cells for a longer period. *Lm* vaccines require IFN- γ for effective tumor regression, and IFN- γ is specifically required for tumor infiltration of lymphocytes. IFN- γ also inhibits angiogenesis at the tumor site in the early effector phase following vaccination.

[000154] A poorly described property of LLO, is its ability to induce epigenetic modifications affecting control of DNA expression. Extracellular LLO induces a dephosphorylation of the
20 histone protein H3 and a similar deacetylation of the histone H4 in early phases of *Listeria* infection. This epigenetic effect results in reduced transcription of certain genes involved in immune function, thus providing a mechanism by which LLO may regulate the expression of gene products required for immune responses. Another genomic effect of LLO is its ability to increase NF- κ B translocation in association with the expression of ICAM and E-selectin, and
25 the secretion of IL-8 and MCP-1. Another signaling cascade affected by LLO is the Mitogen Activated Protein Kinase (MAPK) pathway, resulting in increase of Ca²⁺ influx across the cell membrane, which facilitates the entry of *Listeria* into endothelial cells and their subsequent infection.

[000155] LLO is also a potent inducer of inflammatory cytokines such as IL-6, IL-8, IL-12,
30 IL-18, TNF- α , and IFN- γ , GM-CSF as well as NO, chemokines, and costimulatory molecules that are important for innate and adaptive immune responses. The proinflammatory cytokine-inducing property of LLO is thought to be a consequence of the activation of the TLR4 signal

pathway. One evidence of the high Th1 cytokine-inducing activity of LLO is in that protective immunity to *Lm* can be induced with killed or avirulent *Lm* when administered together with LLO, whereas the protection is not generated in the absence of LLO. Macrophages in the presence of LLO release IL-1 α , TNF- α , IL-12 and IL-18, which in turn
5 activate NK cells to release IFN- γ resulting in enhanced macrophage activation.

[000156] IL-18 is also critical to resistance to *Lm*, even in the absence of IFN- γ , and is required for TNF- α and NO production by infected macrophages. A deficiency of caspase-1 impairs the ability of macrophages to clear *Lm* and causes a significant reduction in IFN- γ production and listericidal activity that can be reversed by IL-18. Recombinant IFN- γ
10 injection restores innate resistance to listeriosis in caspase-1^{-/-} mice. Caspase-1 activation precedes the cell death of macrophages infected with *Lm*, and LLO deficient mutants that cannot escape the phagolysosome have an impaired ability to activate caspase-1.

[000157] LLO secreted by cytosolic *Lm* causes specific gene upregulation in macrophages resulting in significant IFN- γ transcription and secretion. Cytosolic LLO activates a potent
15 type I interferon response to invasive *Lm* independent of Toll-like receptors (TLR) without detectable activation of NF-KB and MAPK. One of the IFN I-specific apoptotic genes, TNF- α related apoptosis-inducing ligand (TRAIL), is up-regulated during *Lm* infection in the spleen. Mice lacking TRAIL are also more resistant to primary listeriosis coincident with lymphoid and myeloid cell death in the spleen.

20 [000158] *Lm* also secretes P60 which acts directly on naïve DCs to stimulate their maturation in a manner that permits activation of NK cells. Both activated DCs and IFN- γ that is produced by NK cells can boost cellular (Th1-type) immune responses. ActA stimulate toll receptors, for example TLR-5, which plays a fundamental role in pathogen recognition and activation of innate immune response.

25 [000159] In one embodiment, the *Lm* vaccines provided herein reduce the number of Tregs and MDSCs in a disease further provided herein. In another embodiment, *Lm* vaccines provided herein are useful to improve immune responses by reducing the number of Tregs and MDSCs at a specific site in a subject. Such a site can be an inflammation site due to allergies, trauma, infection, disease or the site can be a tumor site.

30 [000160] In another embodiment, both monocytic and granulocytic MDSCs purified from the tumors of *Listeria*-treated mice are less able to suppress the division of CD8+ T cells than

MDSCs purified from the tumors of untreated mice, whereas monocytic and granulocytic MDSCs purified from the spleens of these same tumor-bearing mice show no change in their function after vaccination with *Listeria* (see Examples 7-11 herein). In one embodiment, this effect is seen because splenic MDSCs are only suppressive in an antigen-specific manner.

5 Hence, treatment with *Listeria* has the distinct advantage that it allows for tumor-specific inhibition of tumor suppressive cells such as Tregs and MDSCs (see Examples 7-11 herein). Another unexpected advantage provided by the live attenuated *Listeria* of the methods and compositions provided herein is that there are lower amount of Tregs in the tumor, and the ones that persist lose the ability to suppress T cell replication (see Examples 7-11 herein).

10 [000161] In one embodiment, provided herein is a method of reducing the percentage of suppressor cells in a disease site in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to the subject.

[000162] In another embodiment, provided herein is a method of reducing suppressor cells' ability to suppress T cell replication in a disease site in a subject, the method comprising the
15 step of administering a live attenuated *Listeria* vaccine strain to said subject.

[000163] In one embodiment, reducing the number of the suppressor cells at a disease site effectively treats the disease. In another embodiment, reducing the number of the suppressor cells at the disease site enhances an anti-disease immune response in the subject having the disease at the disease site. In another embodiment, the immune response is a cell-mediated
20 immune response. In another embodiment, the immune response is a tumor infiltrating T-lymphocytes (TILs) immune response.

[000164] In one embodiment, provided herein is a method of reducing the percentage of suppressor cells in a disease in a subject and enhancing a therapeutic response against the disease in the subject, the method comprising the step of administering a live attenuated
25 *Listeria* vaccine strain to the subject, thereby reducing the percentage of suppressor cells in the disease and enhancing a therapeutic response against the disease in the subject.

[000165] In another embodiment, provided herein is a method of reducing suppressor cells' ability to suppress replication of T cells in a disease in a subject and enhancing a therapeutic response against the disease in the subject, the method comprising the step of administering a
30 live attenuated *Listeria* vaccine strain to the subject.

[000166] In one embodiment, the term “percentage” is representative of the amount, quantity, or numbers, etc., of either Tregs, MDSCs, or CD8/CD4 T cells measures in an assay or in an immune response. In another embodiment, it refers to the amount, quantity, percentage, etc. of any composition, cell, protein, bacteria or *Listeria* cell provided herein.

5 [000167] In one embodiment, provided herein is a method of attenuating a recombinant *Listeria* vaccine strain, wherein the method comprises deleting the genomic *prfA*, *inlC* and *actA* genes, where in another embodiment, the attenuation is relative to the wild-type strain or a mutant strain having a mutant *prfA*, *inlC*, or *actA*, or any virulence gene thereof. In another embodiment, provided herein is a method of further enhancing the immunogenicity of a
10 recombinant *Listeria* vaccine strain also provided herein, wherein the method comprises deleting the genomic *prfA*, *inlC* and *actA* genes. In one embodiment, provided herein is a method of attenuating a recombinant *Listeria* vaccine strain, wherein the method comprises deleting the genomic *prfA*, *inlC* or *actA* genes, where in another embodiment, the attenuation is relative to the wild-type strain or a mutant strain having a mutant *prfA*, *inlC*, or *actA*, or any
15 virulence gene thereof. In another embodiment, provided herein is a method of further enhancing the immunogenicity of a recombinant *Listeria* vaccine strain also provided herein, wherein the method comprises deleting the genomic *prfA*, *inlC* or *actA* genes.

[000168] In another embodiment, provided herein is a method of eliciting an enhanced immune response in a subject recovering from cytotoxic treatment to a tumor or a cancer, the
20 method comprising administering to said subject a composition comprising the recombinant *Listeria* strain provided herein. In another embodiment, the recombinant *Listeria* strain comprises a mutation or deletion of the *inlC* gene, an *actA* gene, a *prfA* gene, a *PlcA* gene, a *PLcB* gene, a *dal* gene or a *dal/dat* gene. In another embodiment, the recombinant *Listeria* strain comprises an *inlC* and *actA* mutation or deletion. In another embodiment, the
25 recombinant *Listeria* strain comprises an *inlC* or *actA* mutation or deletion. In another embodiment, the recombinant *Listeria* strain consists of an *inlC* or *actA* mutation or deletion.

[000169] In one embodiment, the immune response elicited by the compositions and methods provided herein is not antigen specific.

[000170] In another embodiment, the present invention provides a method of reducing an
30 incidence of cancer or infectious disease, comprising administering a composition of the present invention. In another embodiment, the present invention provides a method of ameliorating cancer or infectious disease, comprising administering a composition of the

present invention. Each possibility represents a separate embodiment of the present invention.

[000171] In one embodiment, the cancer treated by a method of the present invention is breast cancer. In another embodiment, the cancer is a cervix cancer. In another embodiment, the cancer is an Her2 containing cancer. In another embodiment, the cancer is a melanoma. In another embodiment, the cancer is pancreatic cancer. In another embodiment, the cancer is ovarian cancer. In another embodiment, the cancer is gastric cancer. In another embodiment, the cancer is a carcinomatous lesion of the pancreas. In another embodiment, the cancer is pulmonary adenocarcinoma. In another embodiment, it is a glioblastoma multiforme. In another embodiment, it is a hypoxic solid tumor. In another embodiment, the cancer is colorectal adenocarcinoma. In another embodiment, the cancer is pulmonary squamous adenocarcinoma. In another embodiment, the cancer is gastric adenocarcinoma. In another embodiment, the cancer is an ovarian surface epithelial neoplasm (e.g. a benign, proliferative or malignant variety thereof). In another embodiment, the cancer is an oral squamous cell carcinoma. In another embodiment, the cancer is non small-cell lung carcinoma. In another embodiment, the cancer is an endometrial carcinoma. In another embodiment, the cancer is a bladder cancer. In another embodiment, the cancer is a head and neck cancer. In another embodiment, the cancer is a prostate carcinoma. Each possibility represents a separate embodiment of the present invention.

[000172] It is to be understood that the methods of the present invention may be used to treat any infectious disease, which in one embodiment, is bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection. In another embodiment, the methods of the present invention are for inhibiting or suppressing a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a subject. In another embodiment, the present invention provides a method of eliciting a cytotoxic T-cell response against a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a subject. In another embodiment, the present invention provides a method of inducing an immune response against a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a subject. In one embodiment, the infection is viral, which in one embodiment, is HIV. In one embodiment, the infection is bacterial, which in one embodiment, is mycobacterial, which in one embodiment, is tuberculosis. In one embodiment, the infection is eukaryotic, which in one embodiment, is plasmodium, which in one embodiment, is malaria.

[000173] In one embodiment, the present invention provides a method of inducing an immune response in a subject having a concomitant helminth infection, where in another embodiment, the method comprises using a *Listeria* vaccine vector.

5 [000174] In another embodiment, the present invention provides a method of inducing an immune response in a subject having concomitant infectious disease and helminth infections, the method comprising administering to the subject a therapeutically effective dose of a *Listeria* vaccine vector, wherein the *Listeria* vaccine vector expresses and secretes an antigen of the infectious disease.

10 [000175] In another embodiment, the present invention provides a method of inducing an immune response in a subject having concomitant infectious disease and helminth infections, the method comprising administering to the subject a therapeutically effective dose of a *Listeria* vaccine vector, wherein the *Listeria* vaccine vector expresses and secretes an antigen of the infectious disease fused to an additional immunogenic polypeptide.

15 [000176] In another embodiment, the present invention provides a method of enhancing an innate immune response against an infectious disease in a subject, the method comprising the step of administering to the subject a therapeutically effective dose of the composition comprising the *Listeria* vaccine vector provided herein.

20 [000177] In one embodiment, the present invention provides a method of eliciting an enhanced immune response to an infectious disease in a subject, the method comprising administering to the subject a therapeutically effective dose of the composition comprising the *Listeria* vaccine vector provided herein. In another embodiment, the immune response is not antigen specific.

25 [000178] In another embodiment, the present invention provides a method of preventing the onset of an infectious disease in a subject, the method comprising the step of administering to the subject a therapeutically effective dose of the composition comprising the *Listeria* vaccine vector provided herein. In another embodiment, the immune response is not antigen specific.

[000179] In one embodiment, the present invention provides a method of treating an infectious disease in a subject, the method comprising the step of administering to the subject a therapeutically effective dose of the composition comprising the *Listeria* vaccine vector provided herein. In another embodiment, the immune response is not antigen specific.

30 [000180] In one embodiment, the infectious disease is one caused by, but not limited to, any

one of the following pathogens: BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio

5 Vaccines, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arena viruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filo

10 viruses (Ebola , Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic *E.coli*, Pathogenic Vibrios, Shigella species, Salmonella

15 BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tick borne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tick borne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human

20 immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis,

25 Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Trichomonas vaginalis, or any other infectious disease known in the art that is not listed herein.

[000181] In another embodiment, the infectious disease is a livestock infectious disease. In another embodiment, livestock diseases can be transmitted to man and are called “zoonotic

30 diseases.” In another embodiment, these diseases include, but are not limited to, Foot and mouth disease, West Nile Virus, rabies, canine parvovirus, feline leukemia virus, equine influenza virus, infectious bovine rhinotracheitis (IBR), pseudorabies, classical swine fever (CSF), IBR, caused by bovine herpesvirus type 1 (BHV-1) infection of cattle, and pseudorabies (Aujeszky's disease) in pigs, toxoplasmosis, anthrax, vesicular stomatitis virus,

rhodococcus equi, Tularemia, Plague (*Yersinia pestis*), trichomonas.

[000182] In another embodiment of the methods of the present invention, the subject mounts an immune response against an antigen-expressing tumor or target antigen, thereby mediating anti-tumor effects.

5 [000183] In one embodiment, the recombinant *Listeria monocytogenes* for use in the present invention secretes a heterologous peptide. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present invention expresses a heterologous peptide. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present invention expresses and secretes a non-hemolytic LLO, as described herein.

10 [000184] In one embodiment, a treatment protocol of the present invention is therapeutic. In another embodiment, the protocol is prophylactic. In another embodiment, the vaccines of the present invention are used to protect people at risk for cancer such as breast cancer or other types of tumors because of familial genetics or other circumstances that predispose them to these types of ailments as will be understood by a skilled artisan. Similarly, in another
15 embodiment, the vaccines of the present invention are used to protect people at risk for infectious disease; such as tuberculosis, malaria, influenza, and leishmaniasis. In another embodiment, the vaccines are used as a cancer immunotherapy in early stage disease, or after debulking of tumor growth by surgery, conventional chemotherapy or radiation treatment. Following such treatments, the vaccines of the present invention are administered so that the
20 CTL response to the tumor antigen of the vaccine destroys remaining metastases and prolongs remission from the cancer. In another embodiment, vaccines of the present invention are used to effect the growth of previously established tumors and to kill existing tumor cells. Each possibility represents a separate embodiment of the present invention.

[000185] In another embodiment, the vaccines and immunogenic compositions utilized in any
25 of the methods described above have any of the characteristics of vaccines and immunogenic compositions of the present invention. Each characteristic represents a separate embodiment of the present invention.

[000186] Various embodiments of dosage ranges are contemplated by this invention. In one embodiment, in the case of vaccine vectors, the dosage is in the range of 0.4 LD₅₀/dose. In
30 another embodiment, the dosage is from about 0.4-4.9 LD₅₀/dose. In another embodiment the dosage is from about 0.5-0.59 LD₅₀/dose. In another embodiment the dosage is from about

0.6-0.69 LD₅₀/dose. In another embodiment the dosage is from about 0.7-0.79 LD₅₀/dose. In another embodiment the dosage is about 0.8 LD₅₀/dose. In another embodiment, the dosage is 0.4 LD₅₀/dose to 0.8 of the LD₅₀/dose.

[000187] In another embodiment, the dosage is 10⁷ bacteria/dose. In another embodiment, the dosage is 1.5 x 10⁷ bacteria/dose. In another embodiment, the dosage is 2 x 10⁷ bacteria/dose. In another embodiment, the dosage is 3 x 10⁷ bacteria/dose. In another embodiment, the dosage is 4 x 10⁷ bacteria/dose. In another embodiment, the dosage is 6 x 10⁷ bacteria/dose. In another embodiment, the dosage is 8 x 10⁷ bacteria/dose. In another embodiment, the dosage is 1 x 10⁸ bacteria/dose. In another embodiment, the dosage is 1.5 x 10⁸ bacteria/dose. In another embodiment, the dosage is 2 x 10⁸ bacteria/dose. In another embodiment, the dosage is 3 x 10⁸ bacteria/dose. In another embodiment, the dosage is 4 x 10⁸ bacteria/dose. In another embodiment, the dosage is 6 x 10⁸ bacteria/dose. In another embodiment, the dosage is 8 x 10⁸ bacteria/dose. In another embodiment, the dosage is 1 x 10⁹ bacteria/dose. In another embodiment, the dosage is 1.5 x 10⁹ bacteria/dose. In another embodiment, the dosage is 2 x 10⁹ bacteria/dose. In another embodiment, the dosage is 3 x 10⁹ bacteria/dose. In another embodiment, the dosage is 5 x 10⁹ bacteria/dose. In another embodiment, the dosage is 6 x 10⁹ bacteria/dose. In another embodiment, the dosage is 8 x 10⁹ bacteria/dose. In another embodiment, the dosage is 1 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 1.5 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 2 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 3 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 5 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 6 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 8 x 10¹⁰ bacteria/dose. In another embodiment, the dosage is 8 x 10⁹ bacteria/dose. In another embodiment, the dosage is 1 x 10¹¹ bacteria/dose. In another embodiment, the dosage is 1.5 x 10¹¹ bacteria/dose. In another embodiment, the dosage is 2 x 10¹¹ bacteria/dose. In another embodiment, the dosage is 3 x 10¹¹ bacteria/dose. In another embodiment, the dosage is 5 x 10¹¹ bacteria/dose. In another embodiment, the dosage is 6 x 10¹¹ bacteria/dose. In another embodiment, the dosage is 8 x 10¹¹ bacteria/dose. Each possibility represents a separate embodiment of the present invention.

[000188] In one embodiment, the adjuvant vaccine of the present invention comprise a vaccine given in conjunction. In another embodiment, the adjuvant vaccine of the present invention is administered following administration of a vaccine regimen, wherein the vaccine regimen is a viral, bacteria, nucleic acid, or recombinant polypeptide vaccine formulation.

[000189] “Adjuvant” typically refers, in another embodiment, to compounds that, when administered to an individual or tested *in vitro*, increase the immune response to an antigen in the individual or test system to which the antigen is administered. In another embodiment, an immune adjuvant enhances an immune response to an antigen that is weakly immunogenic when administered alone, i.e., inducing no or weak antibody titers or cell-mediated immune response. In another embodiment, the adjuvant increases antibody titers to the antigen. In another embodiment, the adjuvant lowers the dose of the antigen effective to achieve an immune response in the individual. However, in one embodiment, in the present invention, the adjuvant enhances an immune response in an antigen-unspecific manner in order to enable a heightened state of an immune response, as it applies to neonates, or in order to enable the recovery of the immune response following cytotoxic treatment, as it applies to older children and adults and also as further provided herein.

[000190] In another embodiment, the methods of the present invention further comprise the step of administering to the subject a booster vaccination. In one embodiment, the booster vaccination follows a single priming vaccination. In another embodiment, a single booster vaccination is administered after the priming vaccinations. In another embodiment, two booster vaccinations are administered after the priming vaccinations. In another embodiment, three booster vaccinations are administered after the priming vaccinations. In one embodiment, the period between a prime and a boost vaccine is experimentally determined by the skilled artisan. In another embodiment, the period between a prime and a boost vaccine is 1 week, in another embodiment it is 2 weeks, in another embodiment, it is 3 weeks, in another embodiment, it is 4 weeks, in another embodiment, it is 5 weeks, in another embodiment it is 6-8 weeks, in yet another embodiment, the boost vaccine is administered 8-10 weeks after the prime vaccine.

[000191] In one embodiment, a vaccine or immunogenic composition of the present invention is administered alone to a subject. In another embodiment, the vaccine or immunogenic composition is administered together with another cancer therapy. In another embodiment, the cancer therapy is chemotherapy, immuno therapy, radiation, surgery or any other type of therapy available in the art as will be understood by a skilled artisan. Each possibility represents a separate embodiment of the present invention.

[000192] In one embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using homologous recombination. Techniques for homologous

recombination are well known in the art, and are described, for example, in Baloglu S, Boyle SM, et al. (Immune responses of mice to vaccinia virus recombinants expressing either *Listeria monocytogenes* partial listeriolysin or Brucella abortus ribosomal L7/L12 protein. Vet Microbiol 2005, 109(1-2): 11-7); and Jiang LL, Song HH, et al., (Characterization of a mutant *Listeria monocytogenes* strain expressing green fluorescent protein. Acta Biochim Biophys Sin (Shanghai) 2005, 37(1): 19-24). In another embodiment, homologous recombination is performed as described in United States Patent No. 6,855,320. In this case, a recombinant *Lm* strain that expresses E7 was made by chromosomal integration of the E7 gene under the control of the hly promoter and with the inclusion of the hly signal sequence to ensure secretion of the gene product, yielding the recombinant referred to as Lm-AZ/E7. In another embodiment, a temperature sensitive plasmid is used to select the recombinants. Each technique represents a separate embodiment of the present invention.

[000193] In another embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using transposon insertion. Techniques for transposon insertion are well known in the art, and are described, *inter alia*, by Sun et al. (Infection and Immunity 1990, 58: 3770-3778) in the construction of DP-L967. Transposon mutagenesis has the advantage, in another embodiment, that a stable genomic insertion mutant can be formed but the disadvantage that the position in the genome where the foreign gene has been inserted is unknown.

[000194] In another embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using phage integration sites (Lauer P, Chow MY et al, Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J Bacteriol 2002; 184(15): 4177-86). In certain embodiments of this method, an integrase gene and attachment site of a bacteriophage (e.g. U153 or PSA listeriophage) is used to insert the heterologous gene into the corresponding attachment site, which may be any appropriate site in the genome (e.g. comK or the 3' end of the arg tRNA gene). In another embodiment, endogenous prophages are cured from the attachment site utilized prior to integration of the construct or heterologous gene. In another embodiment, this method results in single-copy integrants. Each possibility represents a separate embodiment of the present invention.

[000195] In another embodiment, one of various promoters is used to express protein containing same. In one embodiment, an *Lm* promoter is used, e.g. promoters for the genes

hly, *actA*, *plcA*, *plcB* and *mpl*, which encode the *Listerial* proteins hemolysin, ActA, phosphatidylinositol-specific phospholipase, phospholipase C, and metalloprotease, respectively. Each possibility represents a separate embodiment of the present invention.

[000196] In another embodiment, the construct or nucleic acid molecule is expressed from an episomal vector, with an endogenous nucleic acid sequence encoding an LLO, PEST or ActA sequence or functional fragments thereof. In another embodiment, the construct or nucleic acid molecule comprises a first and at least a second open reading frame each encoding a first and at least a second polypeptide, wherein the first and the at least second polypeptide each comprise a heterologous antigen or a functional fragment thereof fused to an endogenous PEST-containing polypeptide. Such compositions are described in US Patent application serial no. 13/290,783, incorporated by reference herein in its entirety.

[000197] In another embodiment, the PEST-containing polypeptide is a truncated non-hemolytic LLO, an N-terminal ActA, or a PEST sequence.

[000198] In another embodiment, provided herein is a recombinant *Listeria* strain comprising an episomal recombinant nucleic acid molecule, the nucleic acid molecule comprising a first and at least a second open reading frame each encoding a first and at least a second polypeptide, wherein the first and the at least second polypeptide each comprise a heterologous antigen or a functional fragment thereof fused to an endogenous PEST-containing polypeptide, wherein the nucleic acid further comprises an open reading frame encoding a plasmid replication control region. Such compositions are described in US Patent application serial no. 13/290,783, incorporated by reference herein in its entirety.

[000199] In another embodiment, methods and compositions of the present invention utilize a homologue of a heterologous antigen or LLO sequence of the present invention. The terms "homology," "homologous," etc, when in reference to any protein or peptide, refer in one embodiment, to a percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Methods and computer programs for the alignment are well known in the art.

[000200] In another embodiment, the term "homology," when in reference to any nucleic acid sequence similarly indicates a percentage of nucleotides in a candidate sequence that are

identical with the nucleotides of a corresponding native nucleic acid sequence.

[000201] Homology is, in one embodiment, determined by computer algorithm for sequence alignment, by methods well described in the art. For example, computer algorithm analysis of nucleic acid sequence homology may include the utilization of any number of software packages available, such as, for example, the BLAST, DOMAIN, BEAUTY (BLAST Enhanced Alignment Utility), GENPEPT and TREMBL packages.

[000202] In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-41 of greater than about 70%. In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-41 of greater than about 70%. In another embodiment, the identity is greater than about 75%. In another embodiment, the identity is greater than about 78%. In another embodiment, the identity is greater than about 80%. In another embodiment, the identity is greater than about 82%. In another embodiment, the identity is greater than about 83%. In another embodiment, the identity is greater than about 85%. In another embodiment, the identity is greater than about 87%. In another embodiment, the identity is greater than about 88%. In another embodiment, the identity is greater than about 90%. In another embodiment, the identity is greater than about 92%. In another embodiment, the identity is greater than about 93%. In another embodiment, the identity is greater than about 95%. In another embodiment, the identity is greater than about 96%. In another embodiment, the identity is greater than about 97%. In another embodiment, the identity is greater than 98%. In another embodiment, the identity is greater than about 99%. In another embodiment, the identity is 100%. Each possibility represents a separate embodiment of the present invention.

[000203] In another embodiment, homology is determined via determination of candidate sequence hybridization, methods of which are well described in the art (See, for example, "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., Eds. (1985); Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y). For example methods of hybridization may be carried out under moderate to stringent conditions, to the complement of a DNA encoding a native caspase peptide. Hybridization conditions being, for example, overnight incubation at 42 °C in a solution comprising: 10-20 % formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 X Denhardt's solution, 10 % dextran sulfate, and 20

µg/ml denatured, sheared salmon sperm DNA.

[000204] In one embodiment of the present invention, “nucleic acids” refers to a string of at least two base-sugar-phosphate combinations. The term includes, in one embodiment, DNA and RNA. “Nucleotides” refers, in one embodiment, to the monomeric units of nucleic acid polymers. RNA may be, in one embodiment, in the form of a tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, small inhibitory RNA (siRNA), micro RNA (miRNA) and ribozymes. The use of siRNA and miRNA has been described (Caudy AA et al, *Genes & Devel* 16: 2491-96 and references cited therein). DNA may be in form of plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition, these forms of DNA and RNA may be single, double, triple, or quadruple stranded. The term also includes, in another embodiment, artificial nucleic acids that may contain other types of backbones but the same bases. In one embodiment, the artificial nucleic acid is a PNA (peptide nucleic acid). PNA contain peptide backbones and nucleotide bases and are able to bind, in one embodiment, to both DNA and RNA molecules. In another embodiment, the nucleotide is oxetane modified. In another embodiment, the nucleotide is modified by replacement of one or more phosphodiester bonds with a phosphorothioate bond. In another embodiment, the artificial nucleic acid contains any other variant of the phosphate backbone of native nucleic acids known in the art. The use of phosphothiorate nucleic acids and PNA are known to those skilled in the art, and are described in, for example, Neilsen PE, *Curr Opin Struct Biol* 9:353-57; and Raz NK et al *Biochem Biophys Res Commun.* 297:1075-84. The production and use of nucleic acids is known to those skilled in art and is described, for example, in *Molecular Cloning*, (2001), Sambrook and Russell, eds. and *Methods in Enzymology: Methods for molecular cloning in eukaryotic cells* (2003) Purchio and G. C. Fareed. Each nucleic acid derivative represents a separate embodiment of the present invention.

[000205] Protein and/or peptide homology for any amino acid sequence listed herein is determined, in one embodiment, by methods well described in the art, including immunoblot analysis, or via computer algorithm analysis of amino acid sequences, utilizing any of a number of software packages available, via established methods. Some of these packages may include the FASTA, BLAST, MPsrch or Scanps packages, and may employ the use of the Smith and Waterman algorithms, and/or global/local or BLOCKS alignments for analysis, for example. Each method of determining homology represents a separate embodiment of the present invention.

[000206] In another embodiment, the present invention provides a kit comprising a reagent utilized in performing a method of the present invention. In another embodiment, the present invention provides a kit comprising a composition, tool, or instrument of the present invention.

5 [000207] The terms “contacting” or “administering,” in one embodiment, refer to directly contacting the cancer cell or tumor with a composition of the present invention. In another embodiment, the terms refer to indirectly contacting the cancer cell or tumor with a composition of the present invention. In another embodiment, methods of the present invention include methods in which the subject is contacted with a composition of the present
10 invention after which the composition is brought in contact with the cancer cell or tumor by diffusion or any other active transport or passive transport process known in the art by which compounds circulate within the body. Each possibility represents a separate embodiment of the present invention.

[000208] In another embodiment, the terms “gene” and “recombinant gene” refer to nucleic
15 acid molecules comprising an open reading frame encoding a polypeptide of the invention. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of a given gene. Alternative alleles can be identified by sequencing the gene of interest in a number of different individuals or organisms. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individuals or
20 organisms. Any and all such nucleotide variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity are intended to be within the scope of the invention.

Pharmaceutical Compositions

[000209] The pharmaceutical compositions containing vaccines and compositions of the
25 present invention are, in another embodiment, administered to a subject by any method known to a person skilled in the art, such as parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intra-dermally, subcutaneously, intra-peritoneally, intra-ventricularly, intra-cranially, intra-vaginally or intra-tumorally.

[000210] In another embodiment of the methods and compositions provided herein, the
30 vaccines or compositions are administered orally, and are thus formulated in a form suitable for oral administration, i.e. as a solid or a liquid preparation. Suitable solid oral formulations

include tablets, capsules, pills, granules, pellets and the like. Suitable liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In another embodiment of the present invention, the active ingredient is formulated in a capsule. In accordance with this embodiment, the compositions of the present invention comprise, in
5 addition to the active compound and the inert carrier or diluent, a gelatin capsule.

[000211] In another embodiment, the vaccines or compositions are administered by intravenous, intra-arterial, or intra-muscular injection of a liquid preparation. Suitable liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are administered intravenously and are thus
10 formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions are administered intra-arterially and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions are administered intra-muscularly and are thus formulated in a form suitable for intra-muscular administration.

15 [000212] In one embodiment, the term "treating" refers to curing a disease. In another embodiment, "treating" refers to preventing a disease. In another embodiment, "treating" refers to reducing the incidence of a disease. In another embodiment, "treating" refers to ameliorating symptoms of a disease. In another embodiment, "treating" refers to inducing remission. In another embodiment, "treating" refers to slowing the progression of a disease.
20 The terms "reducing", "suppressing" and "inhibiting" refer in another embodiment to lessening or decreasing. Each possibility represents a separate embodiment of the present invention.

[000213] The term "therapeutically effective dose" or "therapeutic effective amount" means a dose that produces the desired effect for which it is administered. The exact dose will be
25 ascertainable by one skilled in the art using known techniques.

[000214] The term "about" as used herein means in quantitative terms plus or minus 5%, or in another embodiment plus or minus 10%, or in another embodiment plus or minus 15%, or in another embodiment plus or minus 20%.

[000215] The term "subject" refers in one embodiment to a mammal including a human in need
30 of therapy for, or susceptible to, a condition or its sequelae. The subject may include dogs,

cats, pigs, cows, sheep, goats, horses, rats, and mice and humans. The term "subject" does not exclude an individual that is normal in all respects.

[000216] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the
5 broad scope of the invention.

EXAMPLES

MATERIALS AND EXPERIMENTAL METHODS

Bacterial strains, transformation and selection

[000217] *E. coli* strain MB2159 was used for transformations, using standard protocols.

10 Bacterial cells were prepared for electroporation by washing with H₂O.

[000218] *E. coli* strain MB2159 (Strych U et al, FEMS Microbiol Lett. 2001 Mar 15;196(2):93-8) is an *alr* (-)/*dadX* (-) deficient mutant that is not able to synthesize D-alanine racemase. *Listeria* strain *Lm dal*(-)/*dat*(-) (*Lmdd*) similarly is not able to synthesize D-alanine racemase due to partial deletions of the *dal* and the *dat* genes.

15 ***Plasmid Constructions***

[000219] Using the published sequence of the *plcA* gene (Mengaud et al., Infect. Immun. 1989 57, 3695-3701), PCR was used to amplify the gene from chromosomal DNA. The amplified product was then ligated into pAM401 using *Sal*I- and *Xba*I-generated DNA ends to generate pDP1462.

20 [000220] Plasmid pDP1500, containing *prfA* alone, was constructed by deleting the *plcA* gene, bases 429 to 1349 (Mengaud et al., supra), from pDP1462 after restriction with *Xba*I and *Pst*I, treatment of the DNA ends with T4 DNA polymerase to make them blunt, and intramolecular ligation.

[000221] Plasmid pDP1499, containing the *plcA* promoter and a portion of the 3' end of *plcA*,
25 was constructed by deleting a *plcA* internal fragment, bases 428 to 882 (Mengaud et al., Infect. Immun. 1989 57, 3695-3701), from pDP1339 after restriction with *Pst*I and *Nsi*I and intramolecular ligation.

[000222] pDP1526 (pKSV7:: Δ plcA) was constructed by a single three-part ligation of pKSV7 restricted with BAMHI and XbaI, the 468 bp XbaI and NsiI-generated fragment from pAM401::plcA containing the 5' end of plcA (bases 882 to 1351; Mengaud et al., supra) and, the 501 bp PstI- and BamHI-generated fragment from pAM401::plcA prfA containing the 3' end of plcA (bases 77 to 429; Mengaud et al., supra).

[000223] The prfA promoter, bases 1-429 (Mengaud et al., supra), was isolated by EcoRI and PstI double digestion of pDP1462 and the fragment was subsequently ligated into EcoRI- and PstI-restricted pKSV7 to generate pDP1498. Two random HindIII-generated 10403S chromosomal DNA fragments, approximately 3kb in length, were ligated into HindIII-restricted pKSV7, to generate the random integration control plasmids pDP1519 and pDP1521.

Construction of L. Monocytogenes Mutant Strains

[000224] L. monocytogenes strain DP-L1387 was isolated as a mutant with reduced lecithinase (PC-PLC) from a Tn917-LTV3 bank of SLCC 5764, constructed as previously described (Camilli et al., J. Bacteriol. 1990, 172,3738-3744). The site of Tn917-LTV3 insertion was determined by sequencing one transposon-chromosomal DNA junction as previously described (Sun et al., Infect. Immun. 1990 58, 3770-3778). L. monocytogenes was transformed with plasmid DNA as previously described (Camilli et al., supra). Selective pressure for maintenance of pAM401, pKSV7, and their derivatives in L. monocytogenes was exerted in the presence of 10 .mu.g of chloramphenicol per ml of media. In addition, maintenance of pKSV7 derivatives required growth at 30°C., a permissive temperature for plasmid replication in Gram-positive bacteria.

[000225] Integration of pKSV7 derivatives into the L. monocytogenes chromosome occurred by homologous recombination between L. monocytogenes DNA sequences on the plasmids and their corresponding chromosomal alleles. Integration mutants were enriched by growth for approximately 30 generations at 40°C., a non-permissive temperature for pKSV7 replication, in Brain Heart Infusion (BHI) broth containing 10 .mu.g chloramphenicol per ml of media. Each integration strain was subsequently colony purified on BHI agar containing 10 .mu.g chloramphenicol per ml of media and incubated at 40°C. Southern blot analyses of chromosomal DNA isolated from each integration strain confirmed the presence of the integrated plasmid.

[000226] Construction of DP-L1552 is achieved by integration of the pKSV7 derivative, pDP1526, to generate a merodiploid intermediate was done as described above. Spontaneous excision of the integrated plasmid, through intramolecular homologous recombination, occurred at a low frequency. Bacteria in which the plasmid had excised from the chromosome were enriched by growth at 30°C. in BHI broth for approximately 50 generations. The nature of the selective pressure during this step was not known but may be due to a slight growth defect of strains containing integrated temperature-sensitive plasmids. Approximately 50% of excision events, i.e., those resulting from homologous recombination between sequences 3' of the deletion, resulted in allelic exchange of Δ plcA for the wild-type allele on the chromosome.

10 [000227] The excised plasmids were cured by growing the bacteria at 40°C in BHI for approximately 30 generations. Bacteria cured of the plasmid retaining the Δ plcA allele on the chromosome were identified by their failure to produce a zone of turbidity surrounding colonies after growth on BHI agar plates containing a 5 ml overlay of BHI agar/2.5% egg yolk/2.5% phosphate-buffered saline (PBS) (BHI/egg yolk agar). The turbid zones resulted from PI-PLC hydrolysis of PI in the egg yolk, giving an insoluble diacylglycerol precipitate. 15 The correct plcA deletion on the *L. monocytogenes* chromosome was confirmed by amplifying the deleted allele using PCR and sequencing across the deletion.

[000228] Thus, PI-PLC negative mutants (plcA deletion mutants) may be used according to the present invention to generate attenuated *L. monocytogenes* vaccines. Other mutants were made using the same method, namely, an actA deletion mutant, a plcB deletion mutant, and a double mutant lacking both plcA and plcB, all of which may also be used according to the present disclosure to generate attenuated *L. monocytogenes* vaccines. Given the present disclosure, one skilled in the art would be able to create other attenuated mutants in addition to those mentioned above.

25 *Construction of Lmdd*

[000229] The dal gene was initially inactivated by means of a double-allelic exchange between the chromosomal gene and the temperature-sensitive shuttle plasmid pKSV7 (Smith K et al, Biochimie. 1992 Jul-Aug;74(7-8):705-11) carrying an erythromycin resistance gene between a 450-bp fragment from the 5' end of the original 850-bp dal gene PCR product and a 450-bp fragment from the 3' end of the dal gene PCR product. Subsequently, a dal deletion mutant covering 82% of the gene was constructed by a similar exchange reaction with pKSV7 carrying homology regions from the 5' and 3' ends of the intact gene (including sequences 30

upstream and downstream of the gene) surrounding the desired deletion. PCR analysis was used to confirm the structure of this chromosomal deletion.

[000230] The chromosomal *dat* gene was inactivated by a similar allelic exchange reaction. pKSV7 was modified to carry 450-bp fragments derived by PCR from both the 5' and 3' ends of the intact *dat* gene (including sequences upstream and downstream of the gene). These two fragments were ligated by appropriate PCR. Exchange of this construct into the chromosome resulted in the deletion of 30% of the central bases of the *dat* gene, which was confirmed by PCR analysis.

Bacterial culture and in vivo passaging of Listeria

10 [000231] *E. coli* were cultured following standard methods. *Listeria* were grown at 37° C, 250 rpm shaking in LB media (Difco, Detroit, MI). + 50 µg/ml streptomycin, and harvested during exponential growth phase. For Lm-LLOE7, 37 µg/ml chloramphenicol was added to the media. For growth kinetics determinations, bacteria were grown for 16 hours in 10 ml of LB + antibiotics. The OD_{600nm} was measured and culture densities were normalized between the strains. The culture was diluted 1:50 into LB + suitable antibiotics and D-alanine if applicable.

Passaging of Lm in mice

[000232] 1 x 10⁸ CFU were injected intraperitoneally (ip.) into C57BL/6 mice. On day three, spleens were isolated and homogenized in PBS. An aliquot of the spleen suspension was 20 plated on LB plates with antibiotics as applicable. Several colonies were expanded and mixed to establish an injection stock.

Construction of antibiotic resistance factor free plasmid pTV3

[000233] **Construction of p60-dal cassette.** The first step in the construction of the antibiotic resistance gene-free vector was construction of a fusion of a truncated p60 promoter to the dal gene. The *Lm* alanine racemase (*dal*) gene (forward primer: 5'-CCA TGG TGA CAG GCT GGC ATC-3'; SEQ ID NO: 20) (reverse primer: 5'-GCT AGC CTA ATG GAT GTA TTT TCT AGG-3'; SEQ ID NO: 21) and a minimal p60 promoter sequence (forward primer: 5'-TTA ATT AAC AAA TAG TTG GTA TAG TCC-3'; SEQ ID No: 22) (reverse primer: 5'-GAC GAT GCC AGC CTG TCA CCA TGG AAA ACT CCT CTC-3'; SEQ ID No: 23) were 25 isolated by PCR amplification from the genome of *Lm* strain 10403S. The primers introduced a *PacI* site upstream of the p60 sequence, an *NheI* site downstream of the *dal* sequence (restriction sites in bold type), and an overlapping *dal* sequence (the first 18 bp) downstream 30

of the p60 promoter for subsequent fusion of p60 and dal by splice overlap extension (SOE)-PCR. The sequence of the truncated p60 promoter was:
 CAAATAGTTGGTATAGTCCTCTTTAGCCTTTGGAGTATTATCTCATCATTTGTTTT
 TAGGTGAAAACCTGGGTAACTTAGTATTATCAATATAAAATTAATTCTCAAATAC
 5 TTAATTACGTACTGGGATTTTCTGAAAAAAGAGAGGAGTTTTCC (SEQ ID NO: 24
 Kohler et al, J Bacteriol 173: 4668-74, 1991). Using SOE-PCR, the p60 and dal PCR
 products were fused and cloned into cloning vector pCR2.1 (Invitrogen, La Jolla, CA) .

[000234] **Removal of antibiotic resistance genes from pGG55.** The subsequent cloning
 strategy for removing the Chloramphenicol acetyltransferase (CAT) genes from pGG55 and
 10 introducing the p60-dal cassette also intermittently resulted in the removal of the gram-
 positive replication region (oriRep; Brantl et al, Nucleic Acid Res 18: 4783-4790, 1990). In
 order to re-introduce the gram-positive oriRep, the oriRep was PCR-amplified from pGG55,
 using a 5'-primer that added a NarI/EheI site upstream of the sequence
 (GGCGCCACTAACTCAACGCTAGTAG, SEQ ID NO: 25) and a 3'-primer that added a
 15 NheI site downstream of the sequence (GCTAGCCAGCAAAGAAAAACAAACACG, SEQ
 ID NO: 26). The PCR product was cloned into cloning vector pCR2.1 and sequence verified.

[000235] In order to incorporate the p60-dal sequence into the pGG55 vector, the p60-dal
 expression cassette was excised from pCR-p60dal by PacI/NheI double digestion. The
 replication region for gram-positive bacteria in pGG55 was amplified from pCR-oriRep by
 20 PCR (primer 1, 5'-GTC GAC GGT CAC CGG CGC CAC TAA CTC AAC GCT AGT AG-3';
 SEQ ID No: 27); (primer 2, 5'-TTA ATT AAG CTA GCC AGC AAA GAA AAA CAA ACA
 CG-3'; SEQ ID No: 28) to introduce additional restriction sites for EheI and NheI. The PCR
 product was ligated into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.), and the sequence was
 verified. The replication region was excised by EheI/NheI digestion, and vector pGG55 was
 25 double digested with EheI and NheI, removing both CAT genes from the plasmid
 simultaneously. The two inserts, p60-dal and oriRep, and the pGG55 fragment were ligated
 together, yielding pTV3 (Figure 1). pTV3 also contains a prfA (pathogenicity regulating
 factor A) gene. This gene is not necessary for the function of pTV3, but can be used in
 situations wherein an additional selected marker is required or desired.

30 **Preparation of DNA for real-time PCR**

[000236] Total *Listeria* DNA was prepared using the Masterpure® Total DNA kit (Epicentre,
 Madison, WI). *Listeria* were cultured for 24 hours at 37° C and shaken at 250 rpm in 25 ml of

Luria-Bertoni broth (LB). Bacterial cells were pelleted by centrifugation, resuspended in PBS supplemented with 5 mg/ml of lysozyme and incubated for 20 minutes at 37° C, after which DNA was isolated.

[000237] In order to obtain standard target DNA for real-time PCR, the LLO-E7 gene was PCR amplified from pGG55 (5'-ATGAAAAAATAATGCTAGTTTTTATTAC-3' (SEQ ID NO: 29); 5'-GCGGCCGCTTAATGATGATGATGATGATGTGGTTTCTG AGAACAGATG-3' (SEQ ID NO: 30)) and cloned into vector pETblue1 (Novagen, San Diego, CA). Similarly, the plcA amplicon was cloned into pCR2.1. *E. coli* were transformed with pET-LLOE7 and pCR-plcA, respectively, and purified plasmid DNA was prepared for use in real-time PCR.

10 **Real-time PCR**

[000238] Taqman primer-probe sets (Applied Biosystems, Foster City, CA) were designed using the ABI PrimerExpress software (Applied Biosystems) with E7 as a plasmid target, using the following primers: 5'-GCAAGTGTGACTCTACGCTTCG-3' (SEQ ID NO: 31); 5'-TGCCCATTAACAGGTCTTCCA-3' (SEQ ID NO: 32); 5'-FAM-TGCGTAAAGCACACACGTAGACATTCGTAC-TAMRA-3' (SEQ ID NO: 33) and the one-copy gene plcA (TGACATCGTTTGTGTTTGAGCTAG -3' (SEQ ID NO: 34, 5'-GCAGCGCTCTCTATACCAGGTAC-3' (SEQ ID NO: 35); 5'-TET-TTAATGTCCATGTATGTCTCCGTTATAGCTCATCGTA-TAMRA-3'; SEQ ID NO: 36) as a *Listeria* genome target.

[000239] 0.4 µM primer and 0.05 mM probe were mixed with PuRE Taq RTG PCR beads (Amersham, Piscataway, NJ) as recommended by the manufacturer. Standard curves were prepared for each target with purified plasmid DNA, pET-LLOE7 and pCR-plcA (internal standard) and used to calculate gene copy numbers in unknown samples. Mean ratios of E7 copies / plcA copies were calculated based on the standard curves and calibrated by dividing the results for Lmdd-TV3 and Lm-LLOE7 with the results from Lm-E7, a *Listeria* strain with a single copy of the E7 gene integrated into the genome. All samples were run in triplicate in each qPCR assay which was repeated three times. Variation between samples was analyzed by Two-Way ANOVA using the KyPlot software. Results were deemed statistically significant if $p < 0.05$.

30 **Growth measurements**

[000240] Bacteria were grown at 37°C, 250 rpm shaking in Luria Bertani (LB) Medium +/- 100 micrograms (µg)/ml D-alanine and/or 37 µg/ml chloramphenicol. The starting inoculum was adjusted based on OD₆₀₀ nm measurements to be the same for all strains.

Hemolytic Lysis Assay

5 [000241] 4 x 10⁹ CFU of *Listeria* were thawed, pelleted by centrifugation (1 minute, 14000 rpm) and resuspended in 100 µl PBS, pH 5.5 with 1 M cysteine. Bacteria were serially diluted 1:2 and incubated for 45 minutes at 37° C in order to activate secreted LLO. Defibrinated total sheep blood (Cedarlane, Hornby, Ontario, Canada) was washed twice with 5 volumes of PBS and three to four times with 6 volumes of PBS-Cysteine until the supernatant remained
10 clear, pelleting cells at 3000 x g for 8 minutes between wash steps, then resuspended to a final concentration of 10 % (v/v) in PBS-Cysteine. 100 µl of 10% washed blood cells were mixed with 100 µl of *Listeria* suspension and incubated for additional 45 minutes at 37° C. Un-lysed blood cells were then pelleted by centrifugation (10 minutes, 1000 x g). 100 µl of supernatant was transferred into a new plate and the OD_{530nm} was determined and plotted against the sample
15 dilution.

Therapeutic efficacy of Lmdd-Tv3

[000242] 10⁵ TC-1 (ATCC, Manassas, VA) were implanted subcutaneously in C57BL/6 mice (n=8) and allowed to grow for about 7 days, after which tumors were palpable. TC-1 is a C57BL/6 epithelial cell line that was immortalized with HPV E6 and E7 and transformed with
20 activated ras, which forms tumors upon subcutaneous implantation. Mice were immunized with 0.1 LD₅₀ of the appropriate *Listeria* strain on days 7 and 14 following implantation of tumor cells. A non-immunized control group (naïve) was also included. Tumor growth was measured with electronic calipers.

Construction of LmddAinlC

25 [000243] The deletions in the *Listeria* chromosome are introduced by homologous recombination between a target gene and homologous sequences present on the plasmid, which is temperature sensitive for DNA replication. After transformation of plasmid into the host, the integration of the plasmid into the chromosome by single crossover event is selected during growth at non-permissive temperature (42°C) while maintaining selective pressure.
30 Subsequent growth of co-integrates at permissive temperatures (30°C) leads to second recombination event, resulting in their resolution.

[000244] To create deletion mutant, DNA fragments that are present upstream and downstream of *inl C* region (indicated in the figure is amplified by PCR (indicated in Fig. 2 and 3 and respective SEQ ID NO: 37 and SEQ ID NO: 38).

[000245] *atggcgcgggatggtatactatacaagcgatatggttcaaaaagatactttgaattaagaagtacaataaagttaactca*

5 *ttagacaaaaagaaaaacaaggaagaatagtagatagttataaatacttggagagtgagggtgaatatggggcagctgatttt*
ggggtttcataatgtagtttcaagattagccattgttgcggcagtagtttacttctatacttattgagaaaaattgcaataaatagaa
aaaaagccttgtcaaacgaggctttttatgcaaaaaatacgcgaatgaagccatgtgagacaatttgaatagcagacaaca
aggaaggtagaacatgtttgaaaaatttactgattttcgattattattaacgcttgtaatttaaacatctcttattttgctaacataag
tatacaaaggacataaaaaaggtaacagcggttgtaaataggaagtatatgaaaatcctcttttggtttctaaattttttaagga
10 *gtggagaatgttgaaaaaaaataattggttacaaaatgcagtaatagcaatgctagtgtaattgtaggtctgtgcattaatatg*
ggttctggaacaaaagtacaagctgagagtattcaacgaccaacgcctattaaccaagttttccagatcccggcctagegaat
gcagtgaacaaaatttagggaagcaaagtgtacagacctgtatcacaaaaggaactatctggagtacaaaatttcaatgg
agataatagcaacattcaatctcttgcgggaatgcaatttttactaatttaaaagaacttcattctatccataatcaataagt
acctagtcctttaaaggatctaactaagttagaagagctatctgtgaatagaaacagactgaaaaatttaacggaattccaa
15 *gtgcttgtttatctcgcttgttttagataacaacgaactcagagatactgactcgcttattcatttgaaaaatctagaaatcttct*
attcgtaataataagttaaaaagtattgtgatgcttgggtttttatcaaaactagaggtattagatttgcattggaatgaaataaca
aatacaggtggactaactagattgaagaaagttaactggatagatttaactggtcagaaatgtgtgaatgaaccagtaaaata
ccaaccagaattgtatataacaaatactgtcaaagaccagatggaagatggatatctccatattacatcagtaatgggtgggag
ttatgtagatgggtgtgtcctgtgggaattgccagtttatacagatgaagtaagctataagtttagcgaatatataaacgttgggg
20 *agactgaggctatattttagtggaacagttacacaacctatcaagaattaggacttgtgcacacctgtatactttgagctctctgataat*
cacgagagctttttaatatgtaatgttaattatctcttgacaaaaagaacgtttattctgataaggttaccagagatgaagaaactttta
ttacaattcaccttgacacaaaaactccatgatagtaataaggttattaaacaagaaagaagaagcaaccgcttctgcctcgt
taacacgaacgtttcaggcaaaaaattcaactttctgcgcgtagcttacgcgattttgaatgtcgggattgctgaaaagcagcccgtt
tttatggcctccgaacgaatgagtagcaggccgcagattgaacagctattttctatcttgttgtaacaaaattaaagtgaggtggctcac
25 *cattagcaaaagacatgttgtaaacgatgggattcgtgcacgtgaagtaagattgatcgaccaagacggtgaacaattaggcgtgaaga*
gtaaaatcgatgcgttcaaattgctgaaaaggctaattctgatctagtgcttgtgtccaacagcgaaaccgccagtagctcgta
 (SEQ ID NO: 37).

[000246] GAATTC*atggcgcgggatggtatactatacaagcgatatggttcaaaaagatactttgaattaagaagtacaataaa*

30 *gtaacttcattagacaaaaagaaaaacaaggaagaatagtagatagttataaatacttggagagtgagggtgaatatgggggc*
agctgatttttggggtttcataatgtagtttcaagattagccattgttgcggcagtagtttacttctatacttattgagaaaaattgcaa
taaatagaaaaaaaagccttgtcaaacgaggctttttatgcaaaaaatacgcgaatgaagccatgtgagacaatttgaatagc
agacaacaaggaaggtagaacatgtttgaaaaatttactgattttcgattattattaacgcttgtaatttaaacatctcttattttgcta
acataaagtatacaaaaggacataaaaaaggtaacagcggttgtaaataggaagtatatgaaaatcctcttttggtttctaaattta

ttttaaggagtggagaGGATCCggacttgtgcacacctgtatactttgagctctcgtataatcacgagagctttttaaataatgtaagtc
 ttaattatctcttgacaaaaagaacgtttatcgtataaggttaccagagatgaagaaactattttattacaattcaccttgacacaaaaac
 tccatatgatataagtaataaggttattaacaagaagaagaacacccgcttctgcctcgttaacacgaacgttttcaggcaaaaaa
 ttcaaacttcgtcgcgtagcttacgcgattttgaatgtcgggattgctgaaaagcagcccgttttttatggcctccgaacgaatgagtta
 5 gcaggccgcagattgaacagctattttctatcttgttgaacaaaattaagtggaggtggctcaccattagcaaagacatgttggtaaacg
 atgggatcgtgcacgtgaagtaagattgatcgaccaagacgggtgaacaattaggcgtgaagagtaaaatcgatgcgttcaaattgctg
 aaaaggctaattcttgatctagtgttgcctccaacagcgaaaccgccagtagctcgtaCTGCAG (SEQ ID NO: 38).

[000247] The *inl C* gene codes for 296 amino acid protein and the entire gene for this protein is
 deleted. The DNA fragments, DNA-up and DNA-down are amplified by PCR and cloned
 10 sequentially in the plasmid, pNEB193 using restriction enzyme sites EcoRI/BamHI and
 BamHI/PstI, respectively as indicated in Fig 3. The DNA cassette up-down (EcoRI and PstI
 fragment) is excised and further cloned in the temperature sensitive shuttle vector, pKSV7.
 After cloning, the plasmid, pKSV7/up-down is transformed in the strain *Lm dal dat actA* and
 the resulting colonies are tested for the presence of plasmid using colony PCR.

15 [000248] For homologous recombination, the bacteria is cultured repeatedly for 5 days under
 chloramphenicol (Cm) selection at 30°C, conditions permissive for plasmid replication and
 during which time random DNA crossover events occur. This incubation step allowed for the
 integration of the shuttle plasmid into the genome, thus initially transferring Cm resistance.
 Bacteria containing a chromosomally integrated plasmid copy are selected by growth under
 20 Cm selective pressure during a temperature shift to 42°C, conditions not permissive for
 plasmid replication. The colonies are verified for the first recombination using PCR and the
 growth temperature are again shifted to 30°C to allow for a second DNA cross over occurring
 at homologous sites, thus excising unwanted plasmid sequences and leaving only the
 recombinant gene copy behind in the *Lm* chromosome. By employing an additional
 25 temperature shift to 42°C, the excised plasmid is prohibited from replicating, so that it is
 diluted out during expansion of the bacterial culture. Furthermore, subsequent replica plating
 is used for selecting the Cm sensitive bacteria. The Cm sensitive colonies are analyzed for the
 deletion of *inl C* gene using colony PCR.

Generation of an ActA deletion mutant

30 [000249] The strain *Lm dal dat* (Lmdd) was attenuated by the irreversible deletion of the
 virulence factor, ActA. An in frame deletion of *actA* in the *Lmdal dat* (Lmdd) background was
 constructed to avoid any polar effects on the expression of downstream genes. The *Lm dal dat*

$\Delta actA$ contains the first 19 amino acids at the N-terminal and 28 amino acid residues of the C-terminal with a deletion of 591 amino acids of ActA. The deletion of the gene into the chromosomal spot was verified using primers that anneal external to the *actA* deletion region. These are primers 3 (Adv 305-tgggatggccaagaaattc) (SEQ ID NO: 39) and 4 (Adv304-ctaccatgtcttcggttgcttg) (SEQ ID NO: 40) as shown in the Fig. 4. The PCR analysis was performed on the chromosomal DNA isolated from Lmdd and Lm-dd $\Delta actA$. The sizes of the DNA fragments after amplification with two different set of primer pairs 1, 2 and 3, 4 in Lm-dd chromosomal DNA was expected to be 3.0 Kb and 3.4 Kb. However, for the Lm-dd $\Delta actA$ the expected sizes of PCR using the primer pairs 1, 2 and 3, 4 was 1.2 Kb and 1.6 Kb. Thus, PCR analysis in Fig. 3 confirms that 1.8 kb region of *actA* was deleted in the strain, Lm-dd $\Delta actA$. DNA sequencing was also performed on PCR products to confirm the deletion of *actA* containing region in the strain, Lm-dd $\Delta actA$ (Figure 5).

[000250] gcgccaaatcattggtgattggtgaggatgtctgtgtgcgtgggtcgcgagatgggcgaataagaagcattaaagatcctg
 acaataataatcaagcggtcatatgaaagattacgaatcgttccactcacagaggaaggcgactggggcggagttcattataatagt
 15 gtatcccgaataaagcagcctataatactatcactaaacttggaagaaaaaacagacagctttatttcgcgcctaaagtactatttaa
 cgaaaaatcccagttaccgatgcgaaaaagcgctcaacaagcagcgaaagatttatgtgaagatgcttctaaaaagttgctg
 aagcttgggaagcagttggggtaactgattaacaaatgtagagaaaaattaattctccaagtatattctaaaataattcatgaatattttt
 cttatattagctaattaagaagataactaactgctaattccaatttttaacggaacaaattagtgaatgaaggccgaatttccttgttctaaa
 aaggttgattagcgtatcacgaggaggaggtataagtgggattaaacagatttatgcgtgcgatgatgggtgtttcattactgccaatt
 20 gcattacgattaaccccgacgacgacccatacgcaggttaattcttgcaatgtagctattggcgtgttctttagggcggttatcaaaa
 ttattcaattaagaaaaataattaaaaacacagaaacgaaagaaaaagtgaggtgaatgatatgaaattcaaaaaggtggttctaggtat
 tgtgcttgatcgcaagtgttctagtctttccggaacgataaaaagcaaatgcctgtgtgatgaatacttacaacacccgcagctccgat
 gatattgacagcaaaattaccacataaacttagttggtccgcgataacccgacaaatactgacgtaataacgcactattggcttttaaacaa
 agcggaaaaatactagctaagatgtaaatcatatgcgagctaatttaataatgaacttaaaaattcgataacaaatagctcaagga
 25 atatatgatcggatcataaaaatccatattatgatactagtagctttttatctcattttataatcctgatagagataatactatttgcgggtttt
 gctaattgcgaaaataacaggagcaagattttcaatcaatcggtgactgattaccgagaaggga (SEQ ID NO: 41).

Production of Inflammatory cytokines:

[000251] Macrophages such as RAW 264.7 are infected with different *Listeria* backbones such as *Lm dal dat*, *Lm dal dat actA*, *Lm dal dat actA Δ inlC* and *Lm dal dat Δ inlC* and supernatant is harvested at different time points to quantify the level of various cytokines using different ELISA based kits. The cytokines that are quantified include IFN-γ, TNF-α and IL-6.

In vivo cytokine production:

[000252] To measure the *in vivo* cytokine production and recruitment of neutrophils, C57BL/6 mice are injected intraperitoneally with different 10⁸ CFU of *inlC* mutant, *Listeria* control or an equivalent volume of saline. After 12 h mice are killed and peritoneal cavities are washed with 2 mL of PBS. The peritoneal washes are examined for bacterial load after plating on growth medium and analysis of proinflammatory cytokines such as MIP-1α, KC, MCP etc. Using flow cytometry the number of neutrophils and macrophages is determine after staining with markers such as Gr-1, CD11b and F4/80 and further these populations are quantified using CellQuest software.

Transwell migration assay:

[000253] This assay is done to determine if there is an increase in the migration of neutrophils following infection of bone marrow derived macrophages or dendritic cells with the *inlC* deletion strain. Bone marrow-derived macrophages or dendritic cells are isolated from mice such as C57BL/6 and are infected with the *inlC* deletion mutants or control *Listeria*. Using infected cells the transwell assay is set up using corning costar Transwell plates. The assay is initially standardize using 3, 5, or 8 micron pore transwell plates. To test neutrophil migration, plate the infected APCs in the bottom of the plate and the neutrophils in the top of the well in the chamber. At different time points the cells are counted to determine the number of neutrophils that have migrated to the bottom.

Therapeutic efficacy of the *Lm dal dat actA Δ inlC* mutant:

[000254] To determine the therapeutic efficacy of *inlC* mutant, human Prostate specific antigen (PSA) is used as tumor antigen as proof of concept. The backbone *Lm dal dat actA inlC* are transformed with the plasmid, pAdv142 that contains expression cassette for human PSA resulting in LmddA*inlC*142. The strain LmddA*inlC*142 is characterized for the

expression and secretion of fusion protein, tLLO-PSA. Further the strain *LmddAin/C142* are passaged twice in vivo in mice and the colonies obtained after two in vivo passages are examined for the expression and secretion of fusion protein, tLLO-PSA. The vaccine working stock are prepared from the colonies obtained after second in vivo passage and this are used
 5 for the assessment of therapeutic effects and immunogenicity. -

Impact on tumor microenvironment:

[000255] The ability of *LmddAin/C142*, *LmddA142* and other control strains to cause infiltration of immune cells in the tumor microenvironment are determined. In this study mice are inoculated with 1×10^6 TPSA23 tumor cells on day 0 and are vaccinated on day 7, 14 and
 10 21 with 10^8 CFU of *LmddAin/C142*, *LmddA142* and other control strains. Tumors are harvested on day 28 and processed for further staining with different cell surface markers such as Gr-1, CD11b, CD3, CD4, CD8, CD25, Foxp3, NK1.1 and CD62L. Using these markers different cell populations that are examined include macrophages (CD11b⁺), NK cells (NK1.1⁺), neutrophils (Gr-1⁺ CD11b⁺), myeloid derived suppressor cells (MDSCs) (Gr-1⁺
 15 CD11b⁺), regulatory T cells (CD4⁺ CD25⁺ Foxp3⁺) and effector T cells (CD8⁺ CD3⁺ CD62L^{low}). Further effector T cells are characterized for their functional ability to produce effector cytokines such as IFN- γ , TNF- α and IL-2. The intratumoral regulatory T cells and MDSCs are tested for their ability to cause suppression of T cell proliferation.

Listeria immunization and *S. mansoni* infection

[000256] Female (6–8 weeks old) BALB/c mice were maintained as naïve (un-infected) or infected with *S. mansoni*. For infection, mice were injected i.p. with 50 cercariae. Eight weeks later, both infected and un-infected mice were immunized i.p. (100 μ g/injection) with 0.1 LD50 Lm-gag, 0.2 LD50 Lm-gag, or 1 LD50 Lm-gag, or orally with 10 LD50 Lm-gag or 100 LD50 Lm-gag. Two weeks later, some groups of mice were boosted i.p. with 0.1 LD50 Lm-gag or 0.2 LD50 Lm-gag or orally with 10 LD50 Lm-gag or 100 LD50 Lm-gag in a similar
 25 manner. Lm-E7 was used as a negative control. Two weeks after the final immunization, the T-cell immune response was analyzed as described below. Infection was confirmed at the time of sacrifice by examining the mice for the presence of worms, liver eggs and hepatosplenomegally.

MDSC and Treg Function

[000257] Tumors were implanted in mice on the flank or a physiological site depending on the tumor model. After 7 days, mice were then vaccinated, the initial vaccination day depends on the tumor model being used. The mice were then administered a booster vaccine one week after the vaccine was given.

- 5 [000258] Mice were then sacrificed and tumors and spleen were harvested 1 week after the boost or, in the case of an aggressive tumor model, 3-4 days after the boost. Five days before harvesting the tumor, non-tumor bearing mice were vaccinated to use for responder T cells. Splenocytes were prepared using standard methodology.

[000259] Briefly, single cell suspensions of both the tumors and the spleens were prepared.

- 10 Splens were crushed manually and red blood cells were lysed. Tumors were minced and incubated with collagenase/DNase. Alternatively, the GENTLEMACS™ dissociator was used with the tumor dissociation kit.

[000260] MDSCs were purified from tumors and spleens using a Miltenyi kit and columns or the autoMACs separator. Cells were then counted.

- 15 [000261] Single cell suspension was prepared and the red blood cells were lysed. Responder T cells were then labeled with CFSE.

[000262] Cells were plated together at a 2:1 ratio of responder T cells (from all division cycle stages) to MDSCs at a density of 1×10^5 T cells per well in 96 well plates. Responder T cells were then stimulated with either the appropriate peptide (PSA OR CA9) or non-specifically with PMA/ionomycin. Cells were incubated in the dark for 2 days at 37°C with 5% CO₂. Two days later, the cells were stained for FACS and analyzed on a FACS machine.

20

Analysis of T-cell responses

[000263] For cytokine analysis by ELISA, splenocytes were harvested and plated at 1.5 million cells per well in 48-well plates in the presence of media, SEA or conA (as a positive control).

- 25 After incubation for 72 hours, supernatants were harvested and analyzed for cytokine level by ELISA (BD). For antigen-specific IFN- γ ELISpot, splenocytes were harvested and plated at 300K and 150K cells per well in IFN- γ ELISpot plates in the presence of media, specific CTL peptide, irrelevant peptide, specific helper peptide or conA (as a positive control). After incubation for 20 hours, ELISpots (BD) were performed and spots counted by the
- 30 Immunospot analyzer (C.T.L.). Number of spots per million splenocytes were graphed.

[000264] Splenocytes were counted using a Coulter Counter, Z1. The frequency of IFN- γ producing CD8⁺ T cells after re-stimulation with gag-CTL, gag-helper, medium, an irrelevant antigen, and con A (positive control) was determined using a standard IFN- γ -based ELISPOT assay.

- 5 [000265] Briefly, IFN- γ was detected using the mAb R46-A2 at 5 mg/ml and polyclonal rabbit anti- IFN- γ used at an optimal dilution (kindly provided by Dr. Phillip Scott, University of Pennsylvania, Philadelphia, PA). The levels of IFN- γ were calculated by comparison with a standard curve using murine rIFN- γ (Life Technologies, Gaithersburg, MD). Plates were developed using a peroxidase-conjugated goat anti-rabbit IgG Ab (IFN- γ). Plates were then
10 read at 405 nm. The lower limit of detection for the assays was 30 pg/ml.

RESULTS

EXAMPLE 1: A PLASMID CONTAINING AN AMINO ACID METABOLISM ENZYME INSTEAD OF AN ANTIBIOTIC RESISTANCE GENE IS RETAINED IN

E. COLI AND *Lm* BOTH IN VITRO AND IN VIVO

- [000266] An auxotroph complementation system based on D-alanine racemase was utilized to mediate plasmid retention in *Lm* without the use of an antibiotic resistance gene. *E. coli* strain MB2159 is an *alr* (-)/*dadX* (-) deficient mutant that is not able to synthesize D-alanine racemase. *Listeria* strain *Lm* *dal*(-)/*dat*(-) (*Lmdd*) similarly is not able to synthesize D-alanine
20 racemase due to partial deletions of the *dal* and the *dat* genes. Plasmid pGG55, which is based on *E. coli*-*Listeria* shuttle vector pAM401, was modified by removing both CAT genes and replacing them with a p60-*dal* expression cassette under control of the *Listeria* p60 promoter to generate pTV3 (Figure 1). DNA was purified from several colonies.

EXAMPLE 2: PLASMIDS CONTAINING A METABOLIC ENZYME DO NOT 25 INCREASE THE VIRULENCE OF BACTERIA

- [000267] As virulence is linked to LLO function, the hemolytic lysis activity between *Lmdd*-TV3 and *Lm*-LLOE7 was compared. This assay tests LLO function by lysis of red blood cells and can be performed with culture supernatant, purified LLO or bacterial cells. *Lmdd*-TV3 displayed higher hemolytic lysis activity than *Lm*-LLOE7.

[000268] *In vivo* virulence was also measured by determining LD₅₀ values, a more direct, and therefore accurate, means of measuring virulence. The LD₅₀ of Lmdd-TV3 (0.75×10^9) was very close to that of Lm-LLOE7 (1×10^9), showing that plasmids containing a metabolic enzyme do not increase the virulence of bacteria.

5 **EXAMPLE 3: INDUCTION OF ANTI-TUMOR IMMUNITY BY PLASMIDS**
 CONTAINING A METABOLIC ENZYME

[000269] Efficacy of the metabolic enzyme-containing plasmid as a cancer vaccine was determined in a tumor regression model. The TC-1 cell line model, which is well characterized for HPV vaccine development and which allowed for a controlled comparison of
10 the regression of established tumors of similar size after immunization with Lmdd-TV3 or Lm-LLOE7, was used. In two separate experiments, immunization of mice with Lmdd-TV3 and Lm-LLOE7 resulted in similar tumor regression (Figure 6) with no statistically significant difference ($p < 0.05$) between vaccinated groups. All immunized mice were still alive after 63
15 days, whereas non-immunized mice had to be sacrificed when their tumors reached 20 mm diameter. Cured mice remained tumor-free until the termination of the experiment.

[000270] Thus, metabolic enzyme-containing plasmids are efficacious as a therapeutic cancer vaccine. Because immune responses required for a therapeutic cancer vaccine are stronger than those required for a prophylactic cancer vaccine, these results demonstrate utility as well for a prophylactic cancer vaccine.

20 **EXAMPLE 4: *inlC*-DELETION MUTANT GENERATE SIGNIFICANTLY HIGH**
 LEVELS OF THE CHEMOKINES AND CYTOKINES.

[000271] *inlC* deletion mutant generates significantly high levels of the chemokines such as MIP-1 α , KC (mouse homolog of IL-8), MCP resulting in infiltration of neutrophils and leukocytes towards the site of infection. Thus when different *Listeria* strains are administered
25 intraperitoneally, the *inlC* mutant demonstrate an increase production of these cytokines and chemokines, which attract neutrophils and macrophages in the peritoneal fluid obtained 12 h after injection. Further, *inlC* deletion mutant generate significantly high levels of the inflammatory cytokines when compared to control strains.

**EXAMPLE 5: *inlC*-DELETION MUTANTS INDUCE NEUTROPHIL
MIGRATION**

[000272] The macrophages infected with *inlC* deletion mutant show significant increase in the migration of neutrophils at different time points when compared to other control strains. The results of this experiment strongly support the ability of this strain to attract immune cells such as neutrophils during infection.

**EXAMPLE 6: *inlC*-DELETION MUTANTS EFFECT A THERAPEUTIC ANTI-
TUMOR RESPONSE**

[000273] The results of anti-tumor studies using both LmddA142 and LmddA*inlC*142 are very comparable to each other and therapeutic regression of tumors is observed. Further, two doses of LmddA*inlC*142 are comparable to three doses of the strain LmddA142 because of its ability to generate high levels of innate responses and increased secretion of proinflammatory cytokines.

[000274] At day 0 tumors were implanted in mice. At day 7 mice were vaccinated with Lmdda-E7 or LmddA-PSA. At day 14 tumors were harvested and MDSCs and Treg percentages and numbers were measured for vaccinated and naïve groups. It was found that there is a decrease in the percentages of both MDSC and Tregs in the tumors of Listeria-treated mice, whereas the same effect is not observed in the spleens or the draining lymph nodes (TLDN) (Fig. 7).

[000275] Isolated splenocytes and tumor-infiltrating lymphocytes (TILs) extracted from tumor bearing mice in the above experiment were pooled and stained for CD3, and CD8 to elucidate the effect of immunization with Lm-LLO-E7, *Lm*-LLO-PSA and *Lm*-LLO-CA9, Lm-LLO-Her2 (Fig. 8-20) on the presence of MDSCs and Tregs (both splenic and tumoral MDSCs and Tregs) in the tumor. Each column represents the % of T cell population at a particular cell division stage and is subgrouped under a particular treatment group (naïve, peptide –CA9 or PSA- treated, no MDSC/Treg, and no MDSC + PMA/ionomycin) (see Figs 8-20).

Analysis of cells in the blood of tumor-bearing mice

[000276] Blood from tumor-bearing mice was analyzed for the percentages of Tregs and MDSCs present. There is a decrease in both MDSC and Tregs in the blood of mice after *Lm* vaccination.

EXAMPLE 7: SUPPRESSOR CELL FUNCTION AFTER *LISTERIA* VACCINE TREATMENT

[000277] At day 0 tumors were implanted in mice. At day 7 mice were vaccinated with Lmdda-
 5 E7 or Lmdda-PSA. At day 14 tumors were harvested and MDSCs and Treg percentages and numbers were measured for vaccinated and naïve groups. It was found that there is a decrease in the percentages of both MDSC and Tregs in the tumors of *Listeria*-treated mice, whereas the same effect is not observed in the spleens or the draining lymph nodes (TLDN) (Fig. 7).

[000278] Isolated splenocytes and tumor-infiltrating lymphocytes (TILs) extracted from tumor
 10 bearing mice in the above experiment were pooled and stained for CD3, and CD8 to elucidate the effect of immunization with Lm-LLO-E7, *Lm*-LLO-PSA and *Lm*-LLO-CA9, Lm-LLO-Her2 (Fig. 8-20) on the presence of MDSCs and Tregs (both splenic and tumoral MDSCs and Tregs) in the tumor. Each column represents the % of T cell population at a particular cell division stage and is subgrouped under a particular treatment group (naïve, peptide -CA9 or
 15 PSA- treated, no MDSC/Treg, and no MDSC + PMA/ionomycin) (see Figs 8-20).

Analysis of cells in the blood of tumor-bearing mice

[000279] Blood from tumor-bearing mice was analyzed for the percentages of Tregs and MDSCs present. There is a decrease in both MDSC and Tregs in the blood of mice after *Lm* vaccination.

20 EXAMPLE 8: MDSCs FROM TPSA23 TUMORS BUT NOT SPLEENS ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[000280] Suppressor assays were carried out using monocytic and granulocytic MDSCs isolated from TPSA23 tumors with non-specifically activated naïve murine cells, and specifically activated cells (PSA, CA9, PMA/ionomycin). Results demonstrated that the
 25 MDSCs isolated from tumors from the *Lm* vaccinated groups have a diminished capacity to suppress the division of activated T cells as compared to MDSC from the tumors of naïve mice. (see Lm-LLO-PSA and Lm-LLO-treated Groups in Figs. 8 & 10, right-hand panel in figures represents pooled cell division data from left-hand panel). In addition, T responder cells from untreated mice where no MDSCs were present and where the cells were
 30 unstimulated/activated, remained in their parental (resting) state (Fig. 8 & 10), whereas T cells stimulated with PMA or ionomycin were observed to replicate (Fig. 8 & 10). Further, it was observed that both, the Gr⁺Ly6G⁺ and the Gr^{dim}Ly6G⁻ MDSCs are less suppressive after

treatment with *Listeria* vaccines. This applies to their decreased abilities to suppress both the division of activated PSA-specific T cells and non-specific (PMA/Ionomycin stimulated) T cells.

[000281] Moreover, suppressor assays carried out using MDSCs isolated from TPSA23 tumors with non-specifically activated naïve murine cells demonstrated that the MDSCs isolated from tumors from the *Lm* vaccinated groups have a diminished capacity to suppress the division of activated T cells as compared to MDSC from the tumors of naïve mice (see Figs. 8 & 10).

[000282] In addition, the observations discussed immediately above relating to Figures 8 and 10 were not observed when using splenic MDSCs. In the latter, splenocytes/ T cells from the naïve group, the *Listeria*-treated group (PSA, CA9), and the PMA/ionomycin stimulated group (positive control) all demonstrated the same level of replication (Fig. 9 & 11). Hence, these results show that *Listeria*-mediated inhibition of suppressor cells in tumors worked in an antigen-specific and non-specific manner, whereas *Listeria* has no effect on splenic granulocytic MDSCs as they are only suppressive in an antigen-specific manner.

EXAMPLE 9: TUMOR T REGULATORY CELLS' REDUCED SUPPRESSION BUT NOT THOSE FROM SPLEENS

[000283] Suppressor assays were carried out using Tregs isolated from TPSA23 tumors after *Listeria* treatment. It was observed that after treatment with *Listeria* there is a reduction of the suppressive ability of Tregs from tumors (Fig. 12), however, it was found that splenic Tregs are still suppressive (Fig. 13).

[000284] As a control conventional CD4+ T cells were used in place of MDSCs or Tregs and were found not to have an effect on cell division (Fig. 14).

EXAMPLE 10: MDSCs AND TREGS FROM 4T1 TUMORS BUT NOT SPLEENS ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[000285] As in the above, the same experiments were carried out using 4T1 tumors and the same observations were made, namely, that MDSCs are less suppressive after *Listeria* vaccination (Figs. 15 & 17), that *Listeria* has no specific effect on splenic monocytic MDSCs (Figs. 16 & 18), that there is a decrease in the suppressive ability of Tregs from 4T1 tumors after *Listeria* vaccination (Fig. 19), and that *Listeria* has no effect on the suppressive ability of splenic Tregs (Fig. 20).

[000286] Finally, it was observed that *Listeria* has no effect on the suppressive ability of splenic Tregs

[000287] The preceding examples are presented in order to more fully illustrate the embodiments of the invention. They should in no way be construed, however, as limiting the

5 broad scope of the invention.

EXAMPLE 11: LISTERIA VECTORS ARE CAPABLE OF DRIVING A Th1 T-CELL IMMUNE RESPONSE DESPITE HELMINTH INFECTION-MEDIATED SUPPRESSION OF Th1 T-CELL IMMUNE RESPONSE

[000288] Despite systemic biasing toward Th2, as evidenced by a reduced IFN- γ response

10 (Figure 21) and an increase in IL-4 and IL-10 production (Figure 22 and 23, respectively), antigen-specific production of IFN- γ remains unchanged (Figure 24), indicating this vaccine can produce a functional cell-mediated immune response in the presence of a Th2 environment. This observation suggests that *Listeria* vector vaccines are capable of driving vaccine-specific immune responses in helminth infected populations. Further, *Listeria* vectors
15 should be considered in the development of new generation HIV-1, malaria or TB vaccines to be administered to populations in sub-Saharan Africa where helminth infection is highly prevalent.

[000289] The preceding examples are presented in order to more fully illustrate the embodiments of the invention. They should in no way be construed, however, as limiting the

20 broad scope of the invention.

[000290]

What is claimed is:

1. A method of reconstituting an immune response in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to said subject.
2. The method of claim 1, wherein said *Listeria* strain comprising a nucleic acid molecule, wherein said nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide.
3. The method of claim 1, wherein said *Listeria* over expresses said PEST-containing polypeptide.
4. The method of claim 3, wherein said PEST-containing polypeptide is a non-hemolytic LLO protein or immunogenic fragment thereof, an ActA protein or truncated fragment thereof, or a PEST amino acid sequence.
5. The method of claim 1, wherein said recombinant *Listeria* comprises a mutation or a deletion of a genomic *internalin C (inlC)* gene, an *ActA* gene, a *PlcA* gene, *PrfA* gene or a *PlcB* gene.
6. The method of claim 2, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* strain.
7. The method of claim 6, wherein said *Listeria* further comprises a third open reading frame encoding a metabolic enzyme.
8. The method of claim 6, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme.

9. The method of claim 7, wherein said metabolic enzyme encoded by said third open reading frame is a D-amino acid transferase enzyme.
10. The method of claim 2, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
11. The method of claim 2, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
12. The method of claim 1, wherein said recombinant *Listeria* strain has been passaged through an animal host.
13. The method of claim 1, wherein said subject is an adult human or a child.
14. A method of facilitating recovery of immune responses after cytotoxic treatments in a subject, the method comprising administering a live attenuated *Listeria* vaccine strain to said subject
15. The method of claim 14, wherein said *Listeria* strain comprises a nucleic acid molecule, wherein said nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide.
16. The method of claim 14, wherein said *Listeria* over expresses said PEST-containing polypeptide.
17. The method of claim 16, wherein said PEST-containing polypeptide is a non-hemolytic LLO protein or immunogenic fragment thereof, an ActA protein or truncated fragment thereof, or a PEST amino acid sequence.
18. The method of claim 14, wherein said recombinant *Listeria* comprises a mutation or a

deletion of a genomic *internalin C (inlC)* gene, an *ActA* gene, a *PlcA* gene, *PrfA* gene or a *PlcB* gene.

19. The method of claim 15,, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* strain.
20. The method of claim 19,, wherein said *Listeria* further comprises a third open reading frame encoding a metabolic enzyme.
21. The method of claim 19, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme.
22. The method of claim 20, wherein said metabolic enzyme encoded by said third open reading frame is a D-amino acid transferase enzyme.
23. The method of claim 14, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
24. The method of claim 14, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
25. The method of claim 14, wherein said recombinant *Listeria* strain has been passaged through an animal host.
26. The method of claim 14, wherein said subject is an adult human or a child.
27. A method of improving the immunogenicity of a vaccine, said method comprising the step of co-administering the vaccine and a *Listeria*-based adjuvant to a subject,

wherein the *Listeria*-based adjuvant enhances the immunogenicity of said vaccine, thereby improving the immunogenicity of said vaccine.

28. The method of claim 27, wherein the *Listeria*-based adjuvant is a *Listeria* strain comprising a nucleic acid molecule comprising a first open reading frame expressing a PEST-containing polypeptide or a fragment thereof.
29. The method of claim 28, wherein said *Listeria* over expresses said PEST-containing polypeptide or fragment thereof.
30. The method of claim 29, wherein said PEST-containing polypeptide is a non-hemolytic LLO protein or immunogenic fragment thereof, an ActA protein or truncated fragment thereof, or a PEST amino acid sequence.
31. The method of claim 27, wherein said *Listeria* strain comprises a mutation or a deletion of a genomic *internalin C (inlC)* gene, an *ActA* gene, a *PlcA* gene, *PrfA* gene or a *PlcB* gene.
32. The method of claim 28, wherein said nucleic acid molecule comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* strain.
33. The method of claim 32, wherein said *Listeria* further comprises a third open reading frame encoding a metabolic enzyme.
34. The method of claim 32, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme.
35. The method of claim 33, wherein said metabolic enzyme encoded by said third open reading frame is a D-amino acid transferase enzyme.

36. The method of claim 28, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
37. The method of claim 28, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
38. The method of claim 27, wherein said recombinant *Listeria* strain has been passaged through an animal host.
39. The method of claim 27, wherein said subject is an adult human or a child.
40. The method of claim 27, wherein the *Listeria*-based adjuvant is used alone or is combined with an additional adjuvant.
41. The method of claim 40, wherein said additional adjuvant is a non-nucleic acid adjuvant including aluminum adjuvant, Freund's adjuvant, MPL, emulsion, GM-CSF, QS21, SBAS2, CpG-containing oligonucleotide, a nucleotide molecule encoding an immune-stimulating cytokine, the adjuvant is or comprises a bacterial mitogen, or a bacterial toxin.
42. The method of claim 27, wherein said method enables the treatment of said disease.
43. A method of enhancing an immune response against a disease in an antigen-independent manner in a subject, said method comprising administering a *Listeria*-based adjuvant to said subject.
44. The method of claim 43, wherein the *Listeria*-based adjuvant is a *Listeria* strain comprising a nucleic acid molecule comprising a first open reading frame expressing a PEST-containing polypeptide or a fragment thereof.

45. The method of claim 44, wherein said *Listeria* over expresses said PEST-containing polypeptide or fragment thereof.
46. The method of claim 45, wherein said PEST-containing polypeptide is a non-hemolytic LLO protein or immunogenic fragment thereof, an ActA protein or truncated fragment thereof, or a PEST amino acid sequence.
47. The method of claim 44, wherein said *Listeria* strain comprises a mutation or a deletion of a genomic *internalin C (inlC)* gene, an *ActA* gene, a *PlcA* gene, *PrfA* gene or a *PlcB* gene.
48. The method of claim 44, wherein said nucleic acid molecule comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements an endogenous gene that is lacking in the chromosome of said recombinant *Listeria* strain.
49. The method of claim 48, wherein said *Listeria* further comprises a third open reading frame encoding a metabolic enzyme.
50. The method of claim 48, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme.
51. The method of claim 49, wherein said metabolic enzyme encoded by said third open reading frame is a D-amino acid transferase enzyme.
52. The method of claim 44, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
53. The method of claim 44, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.

54. The method of claim 44, wherein said recombinant *Listeria* strain has been passaged through an animal host.
55. The method of claim 43, wherein said subject is an adult human or a child.
56. The method of claim 43, wherein the *Listeria*-based adjuvant is used alone or is combined with an additional adjuvant.
57. The method of claim 56, wherein said additional adjuvant is a non-nucleic acid adjuvant including aluminum adjuvant, Freund's adjuvant, MPL, emulsion, GM-CSF, QS21, SBAS2, CpG-containing oligonucleotide, a nucleotide molecule encoding an immune-stimulating cytokine, the adjuvant is or comprises a bacterial mitogen, or a bacterial toxin.
58. The method of claim 43, wherein said method enables the treatment of said disease.

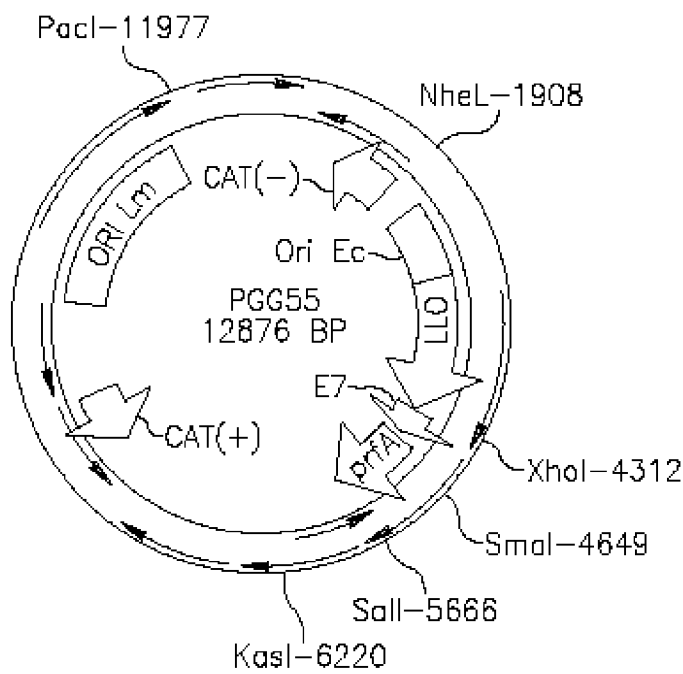


FIG.1A

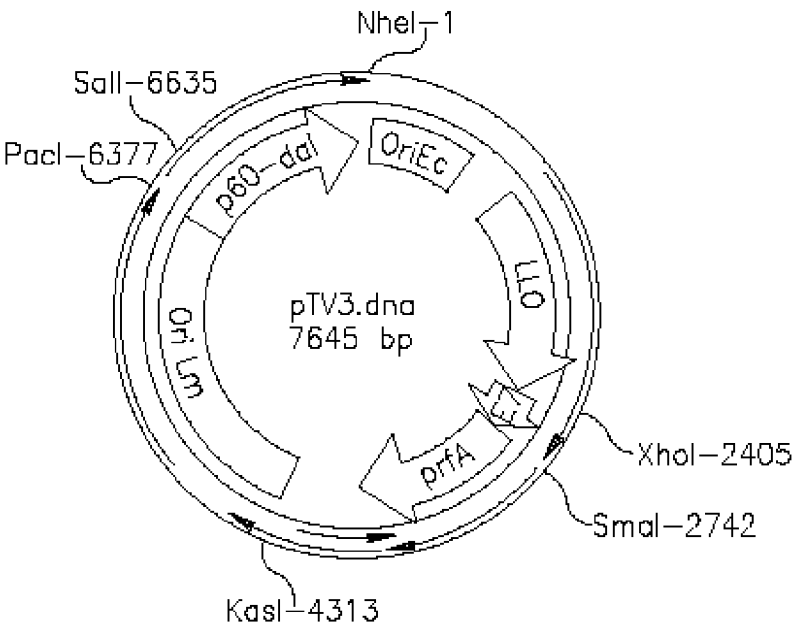


FIG.1B

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Figure 2

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GAATTCatggcgcgggatggtatactatacaagcgtatgggttcaaaaagatactttgaattaa
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CTGCAG (SEQ ID NO: 38)

Figure 3

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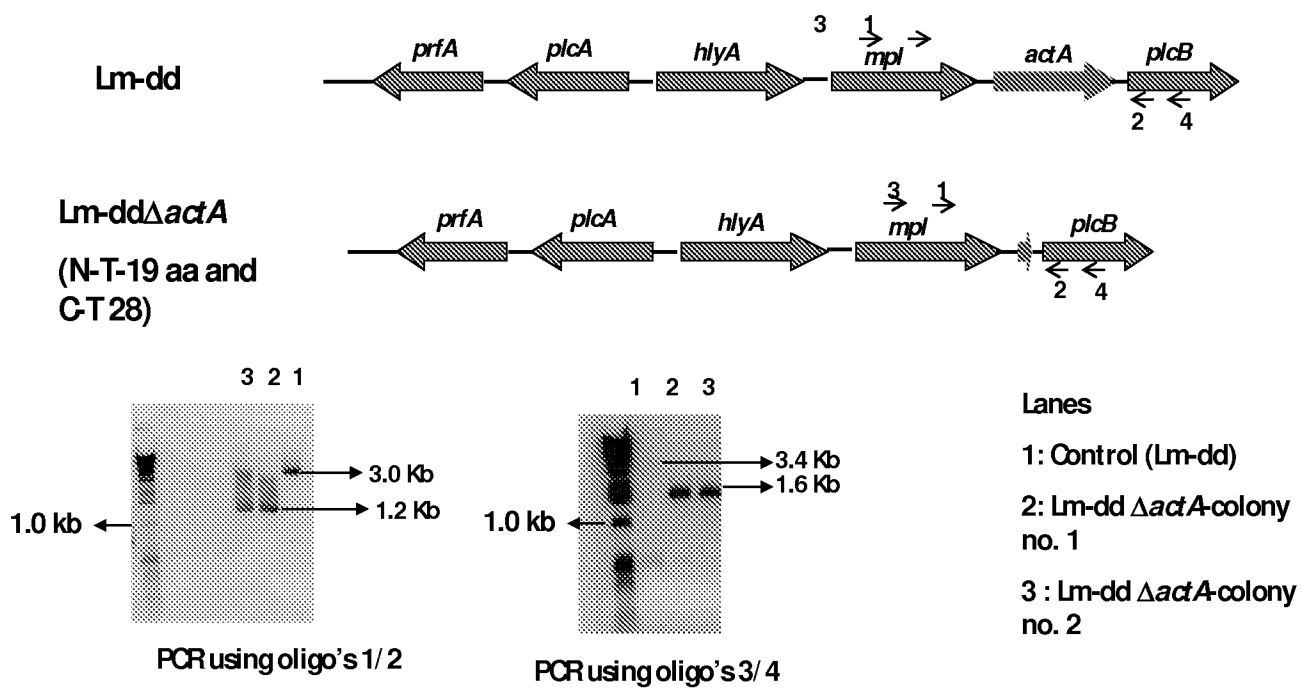


Figure 4

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gcgccaaatcattggttgattggtgaggatgtctgtgtgcgtgggtcgcgagatgggcgaataagaagcattaagatcctgacaaatat
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Figure 5

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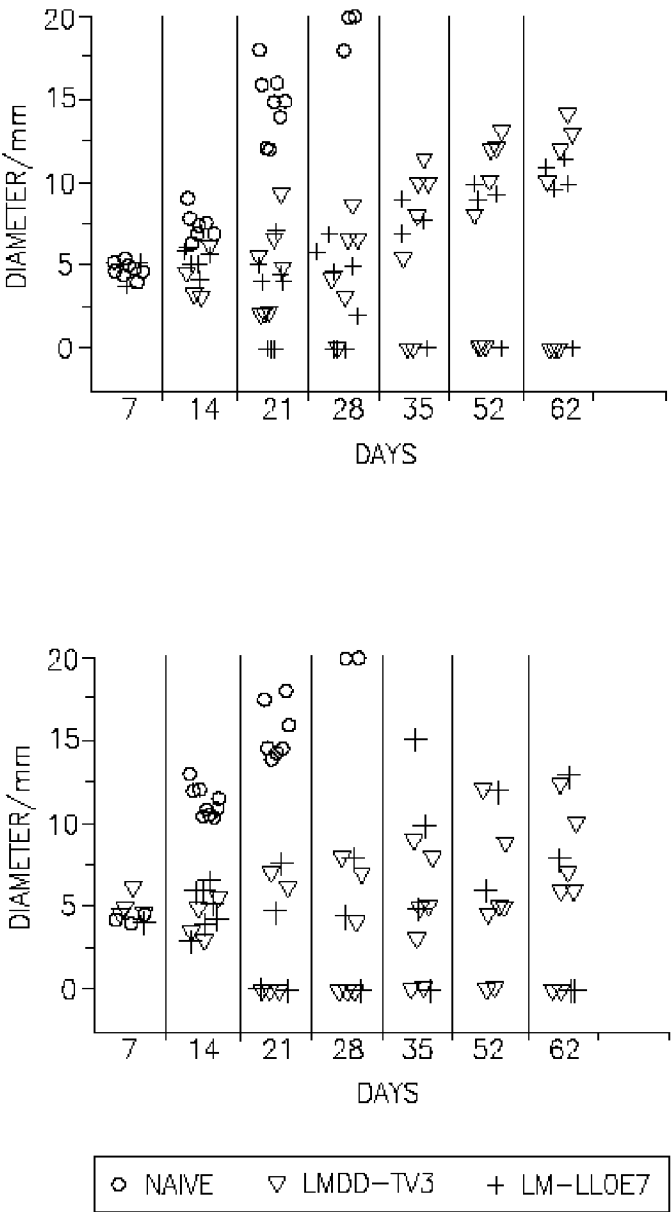


Figure 6

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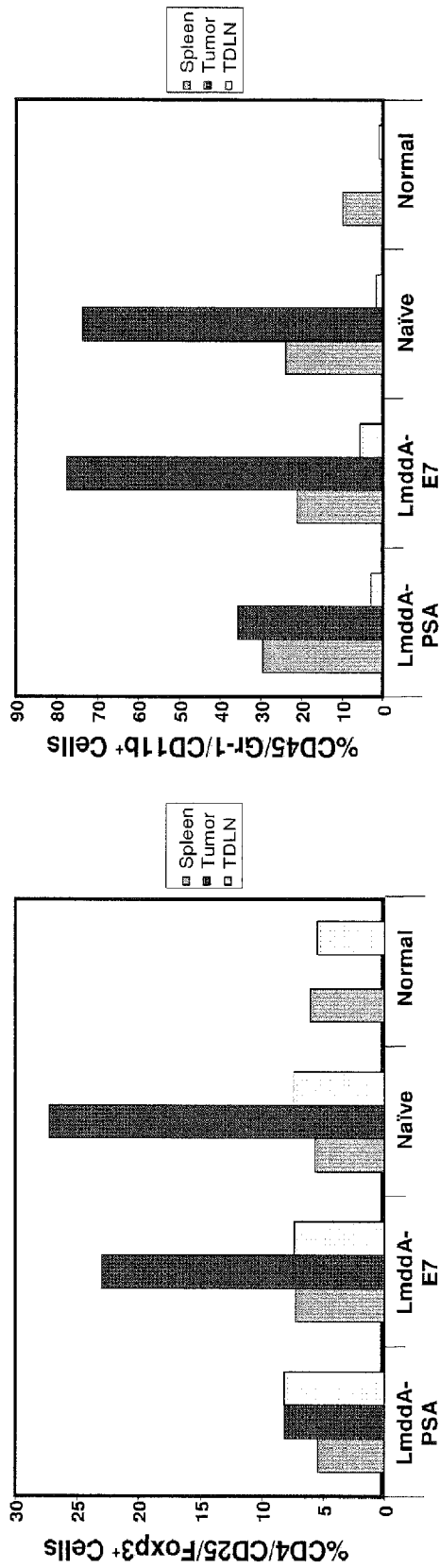


Figure 7

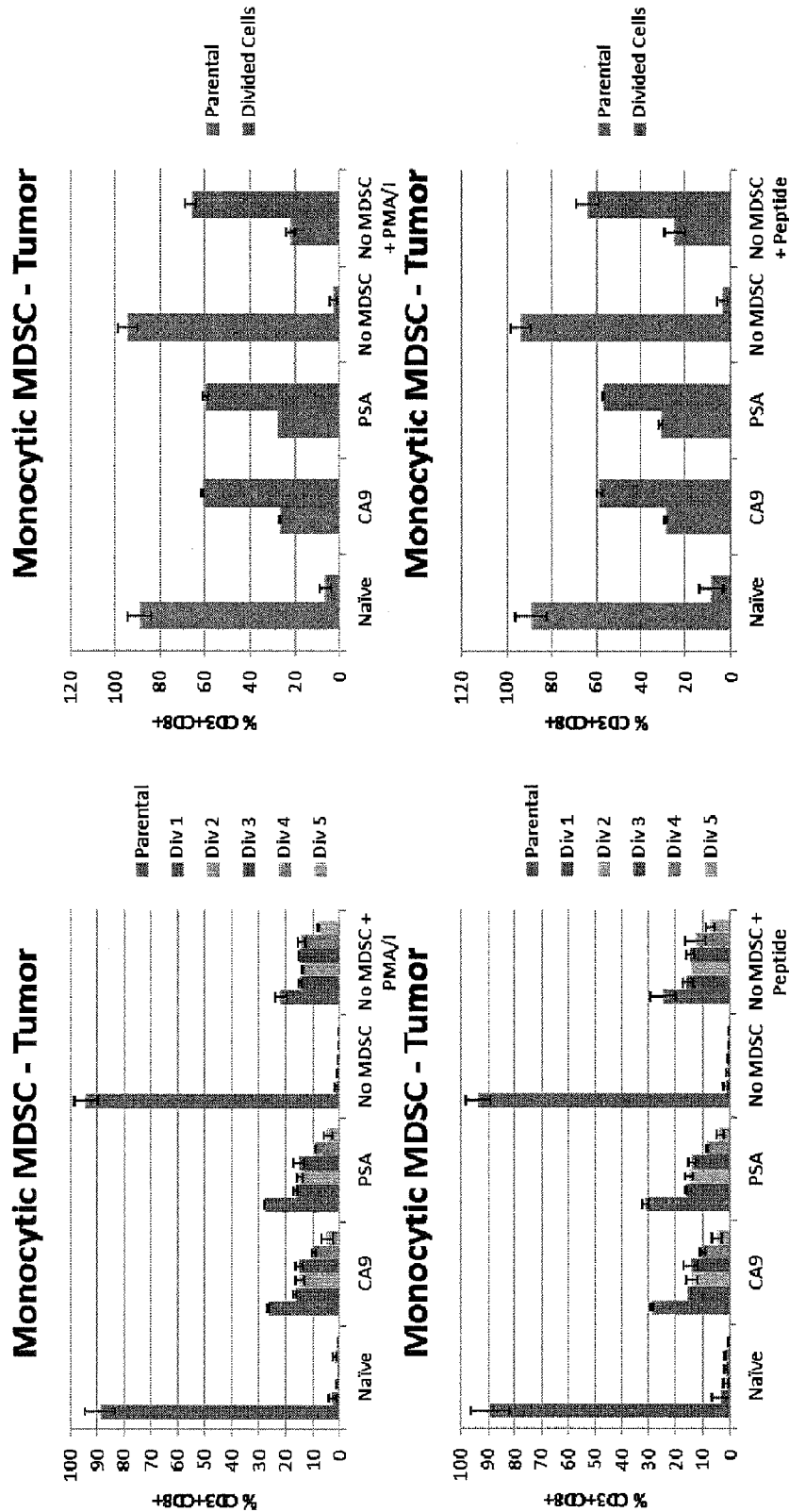


Figure 8

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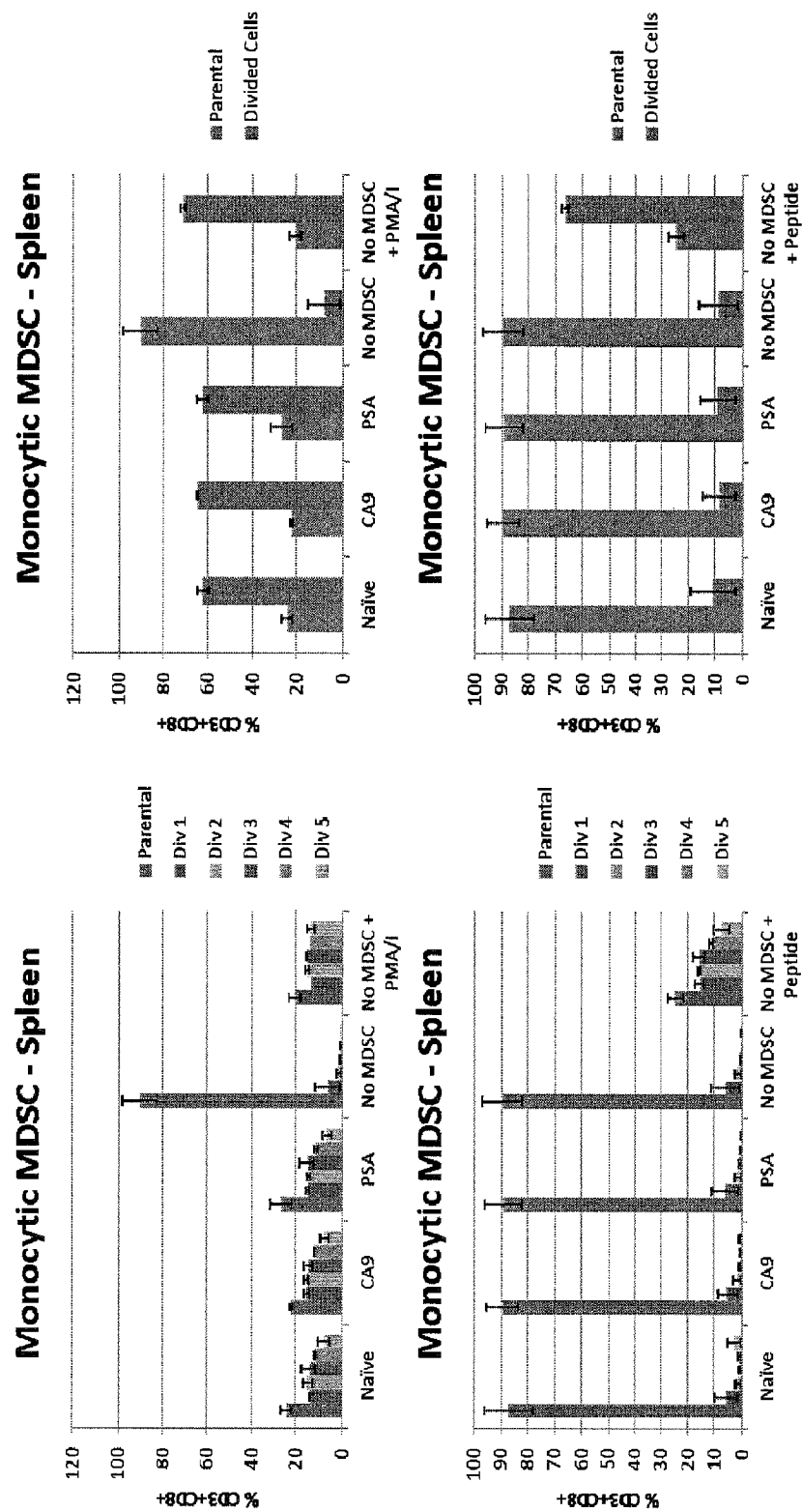


Figure 9

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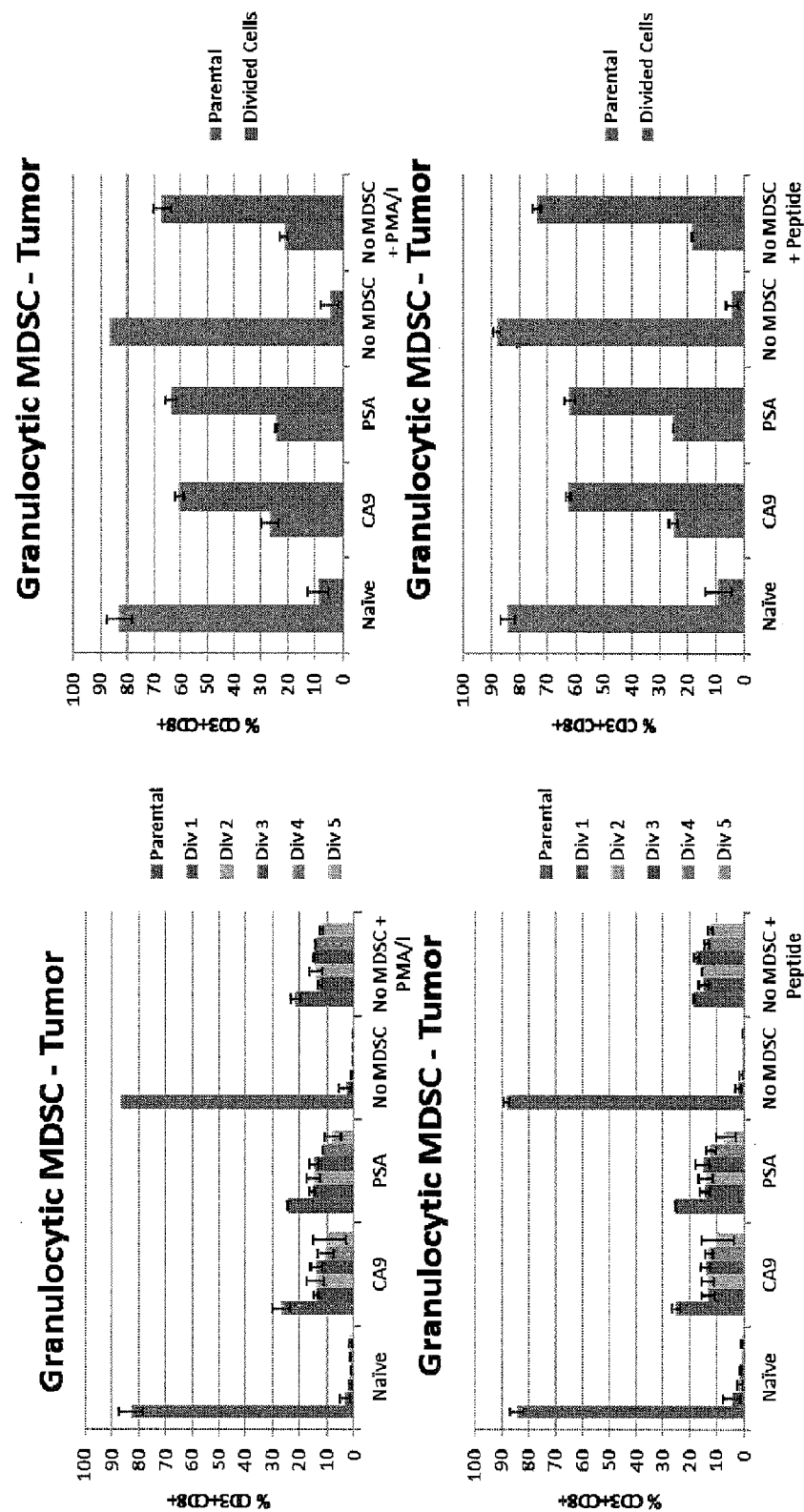


Figure 10

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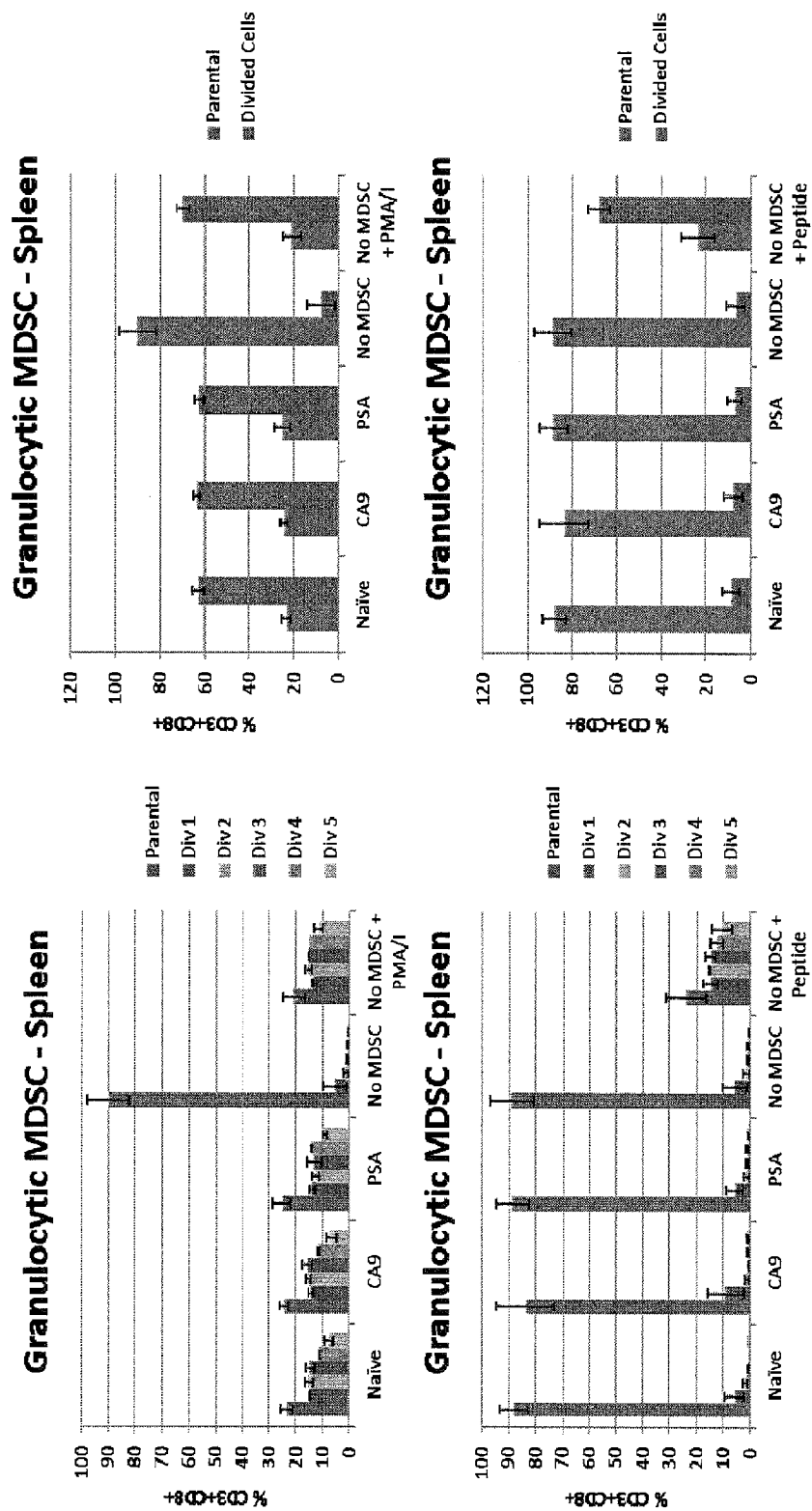


Figure 11

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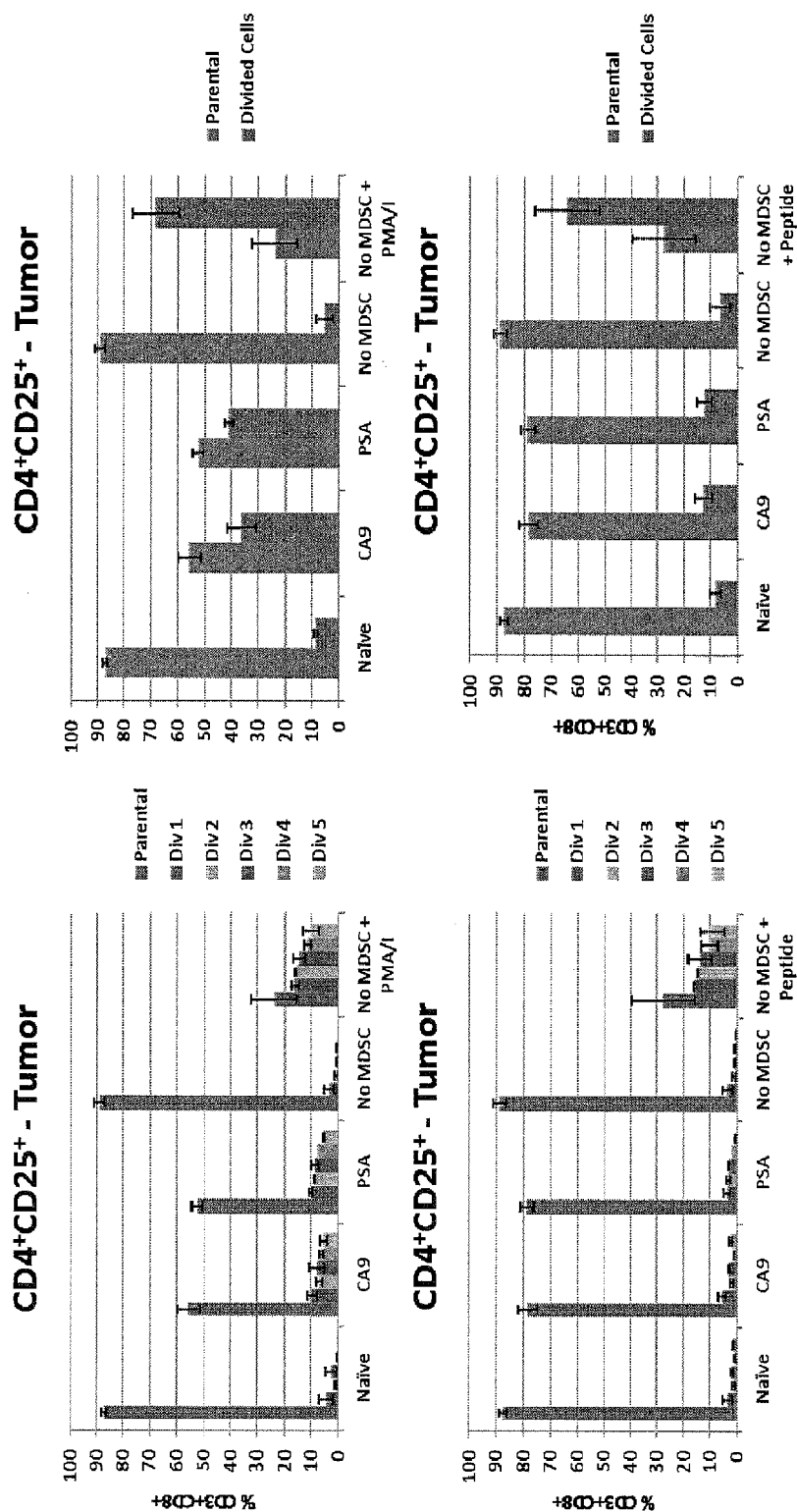


Figure 12

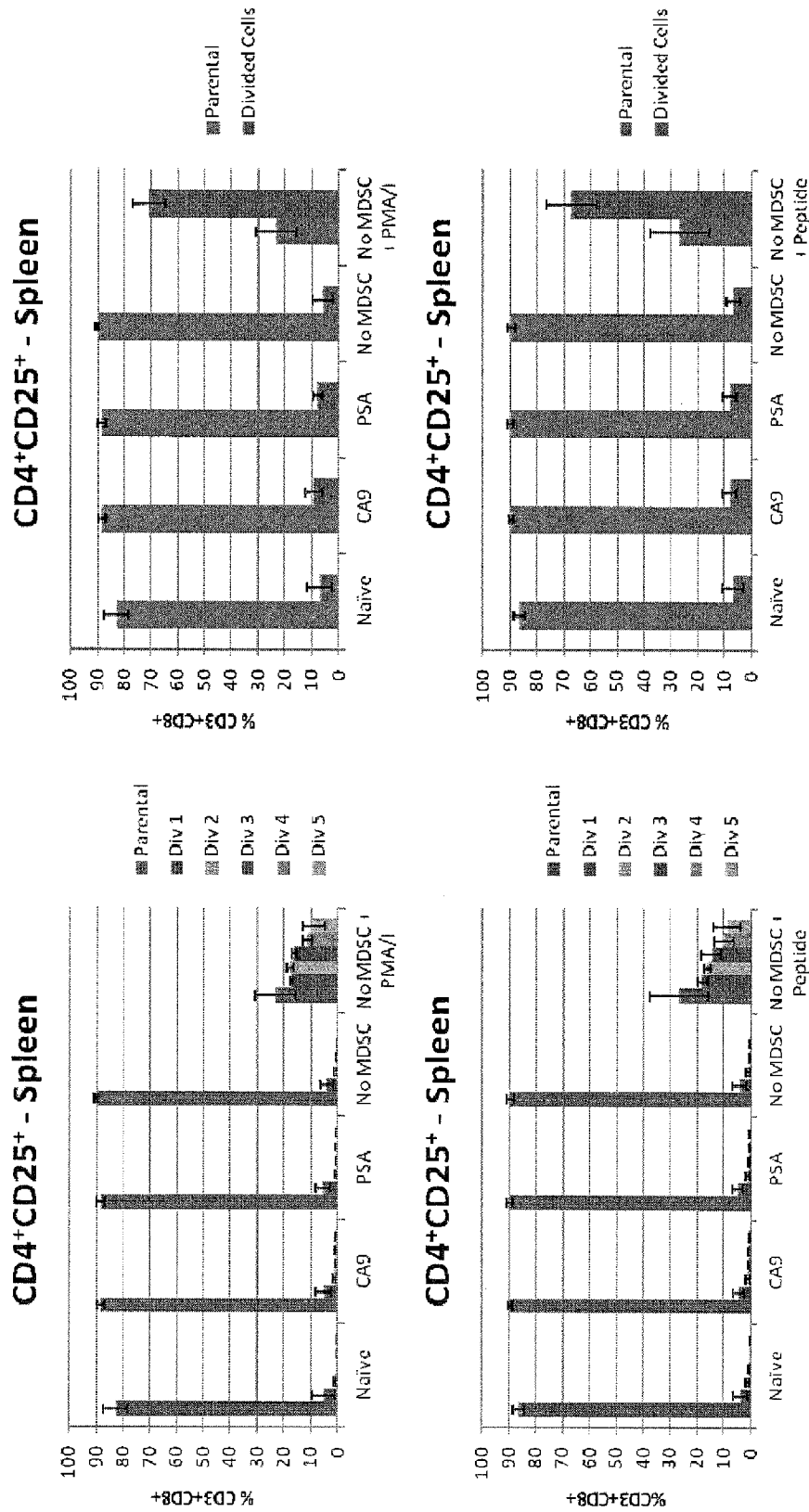


Figure 13

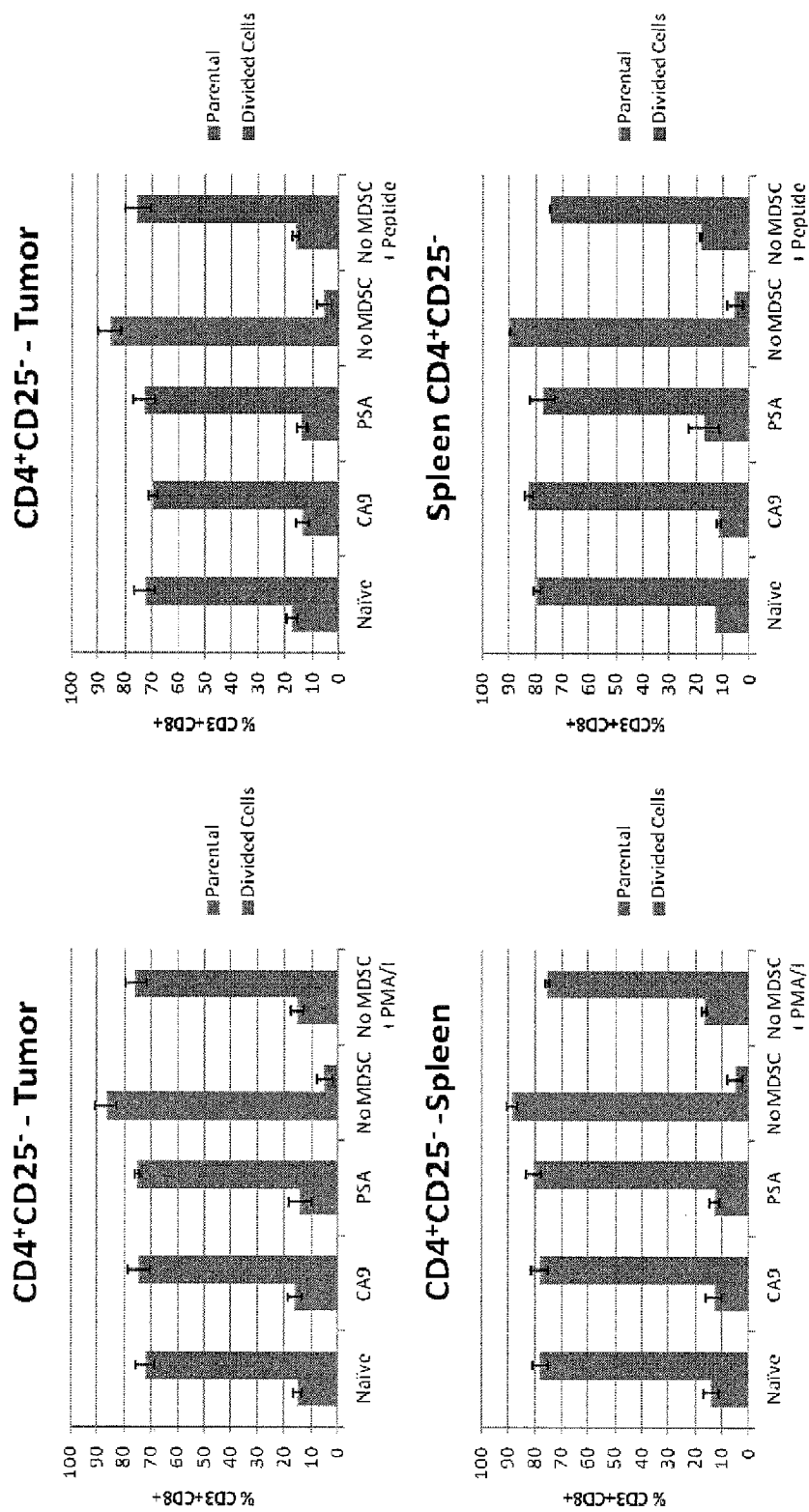


Figure 14

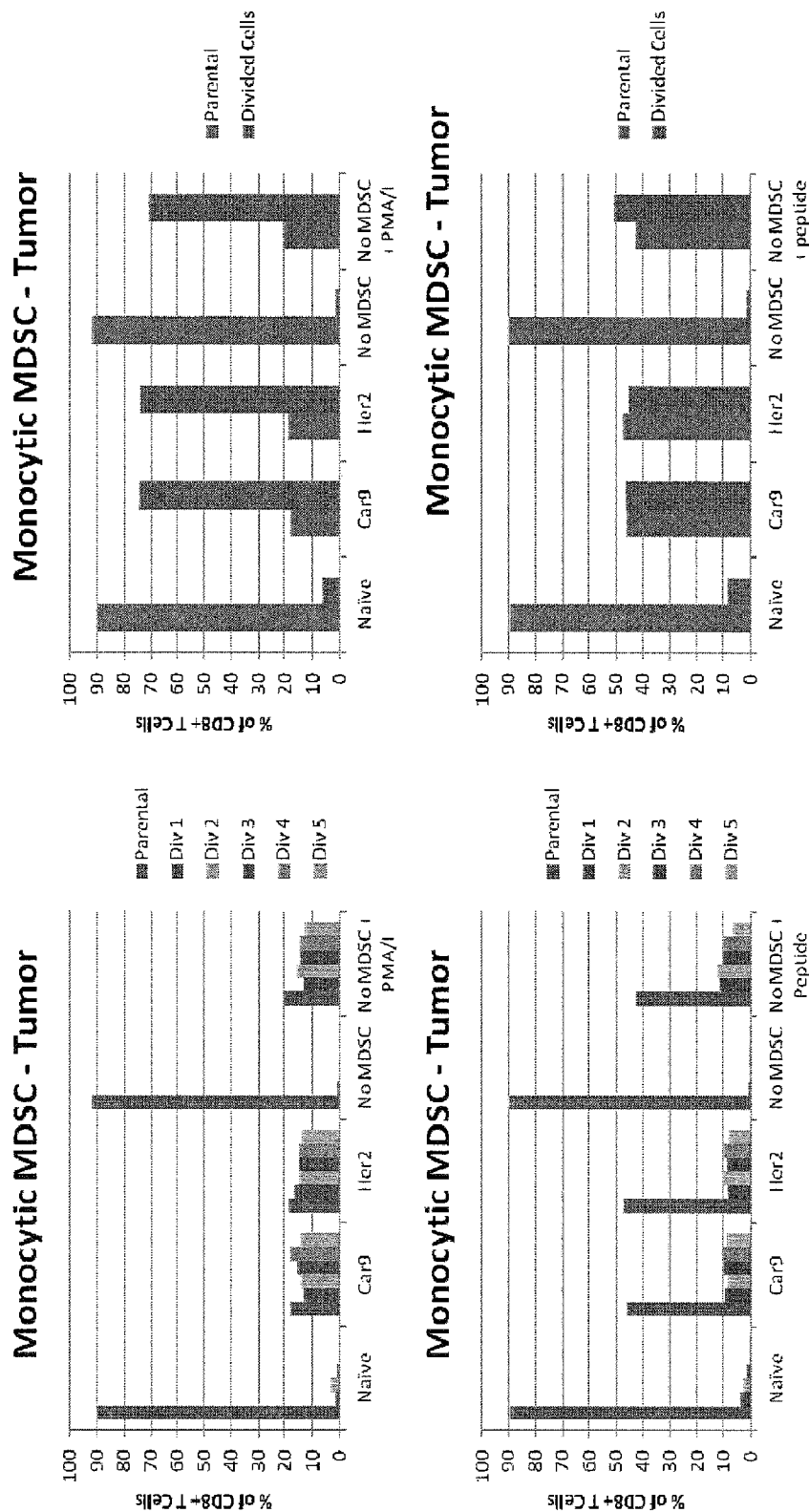


Figure 15

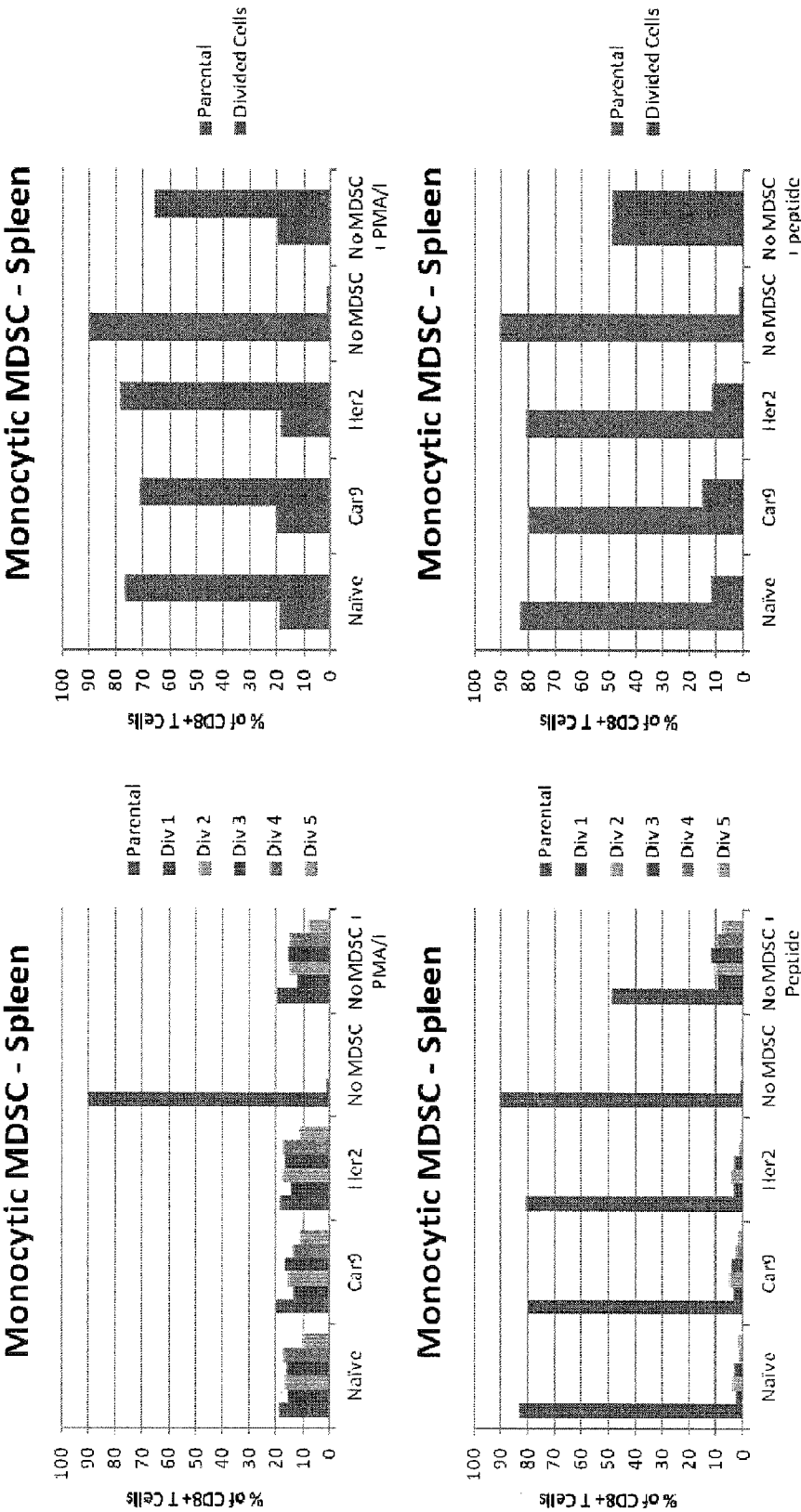


Figure 16

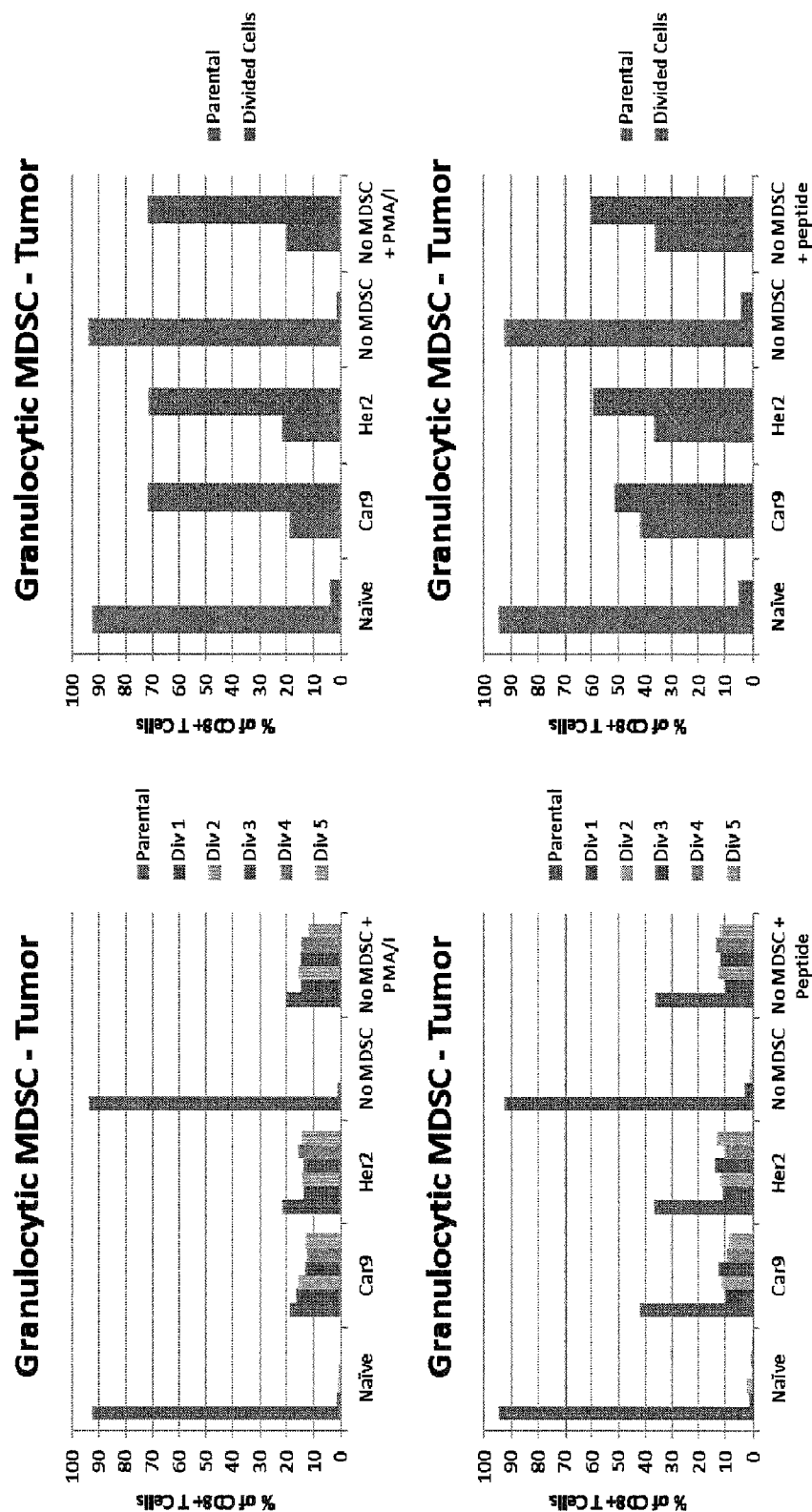


Figure 17

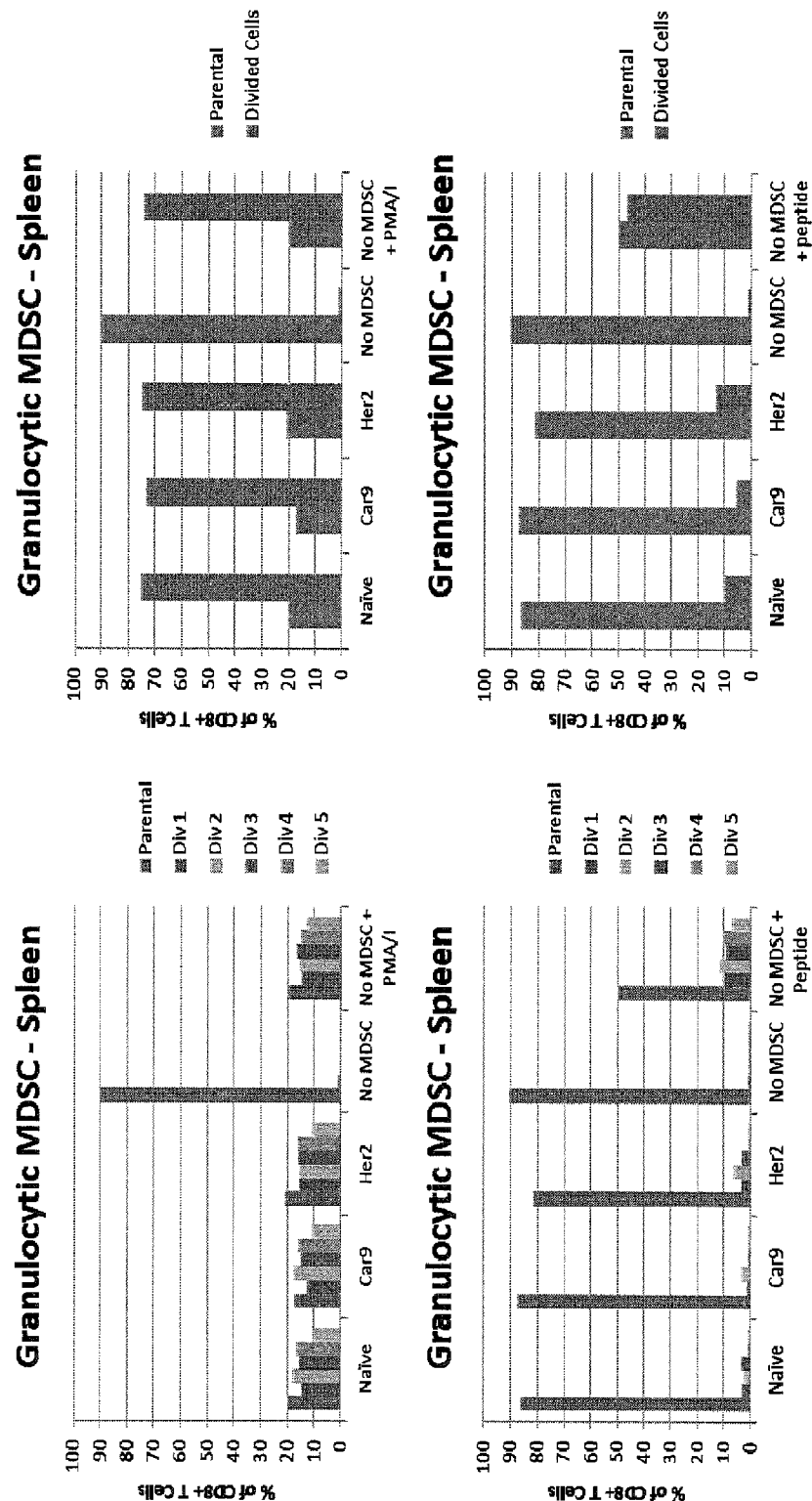


Figure 18

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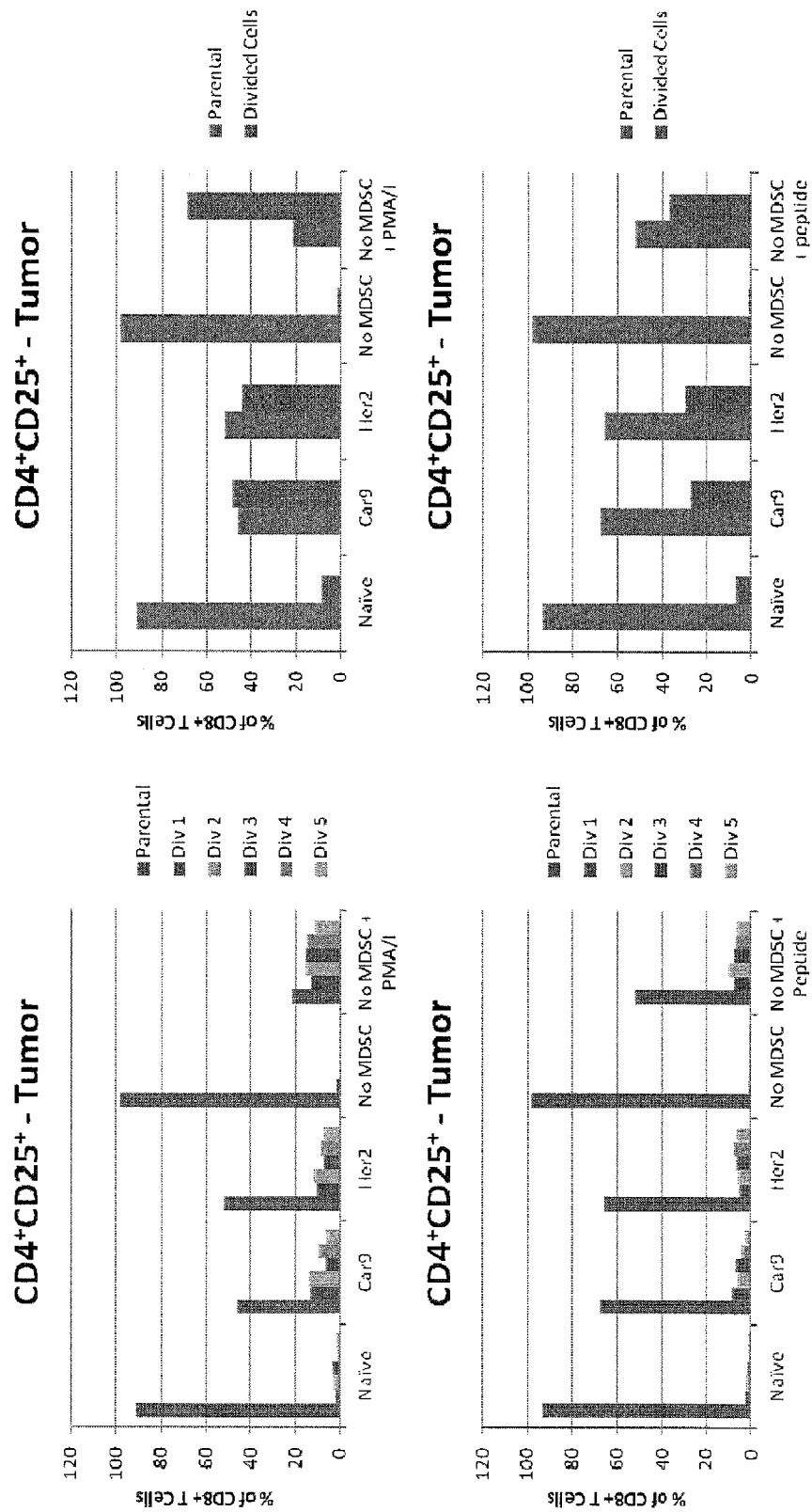


Figure 19

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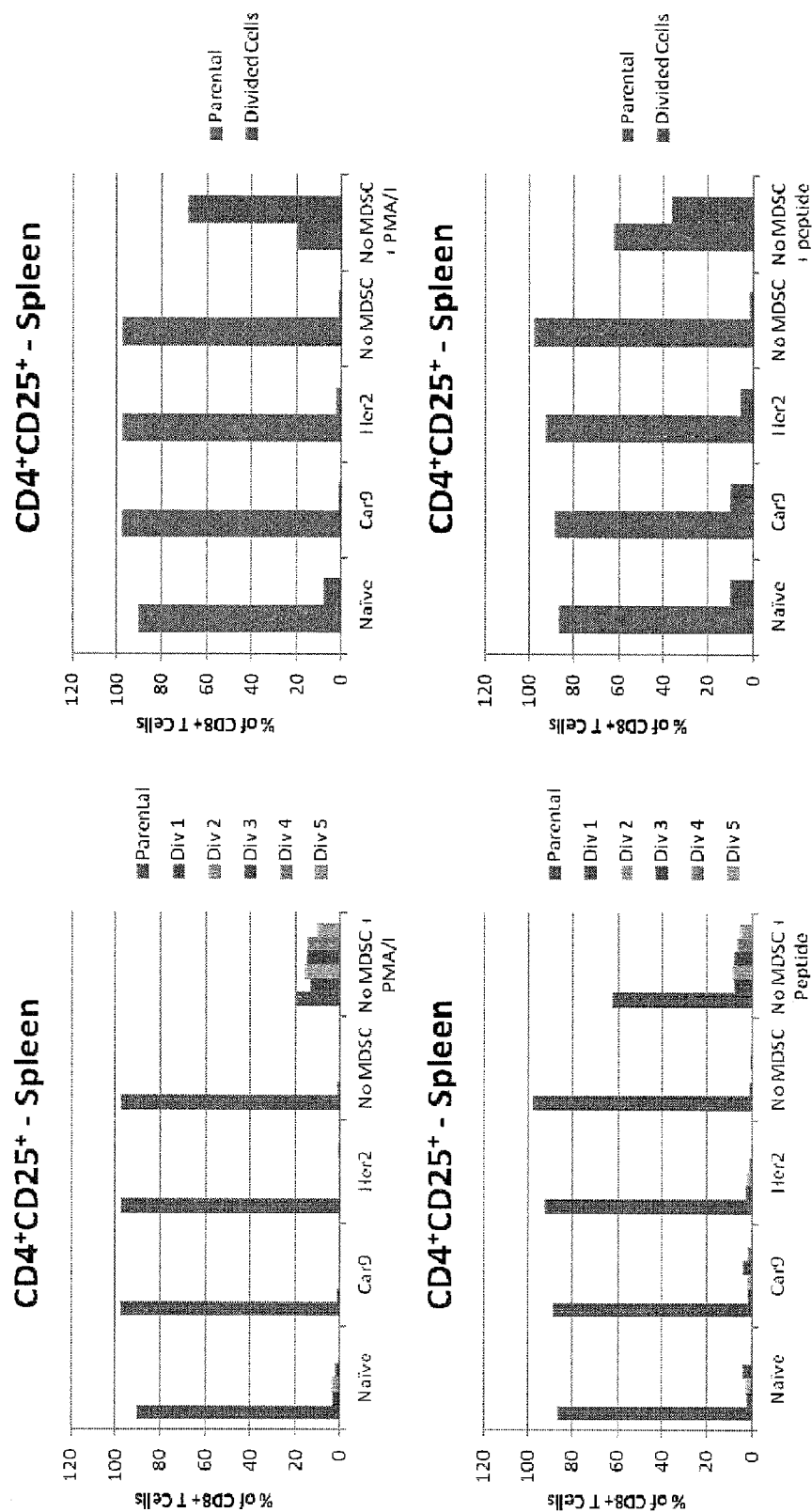


Figure 20

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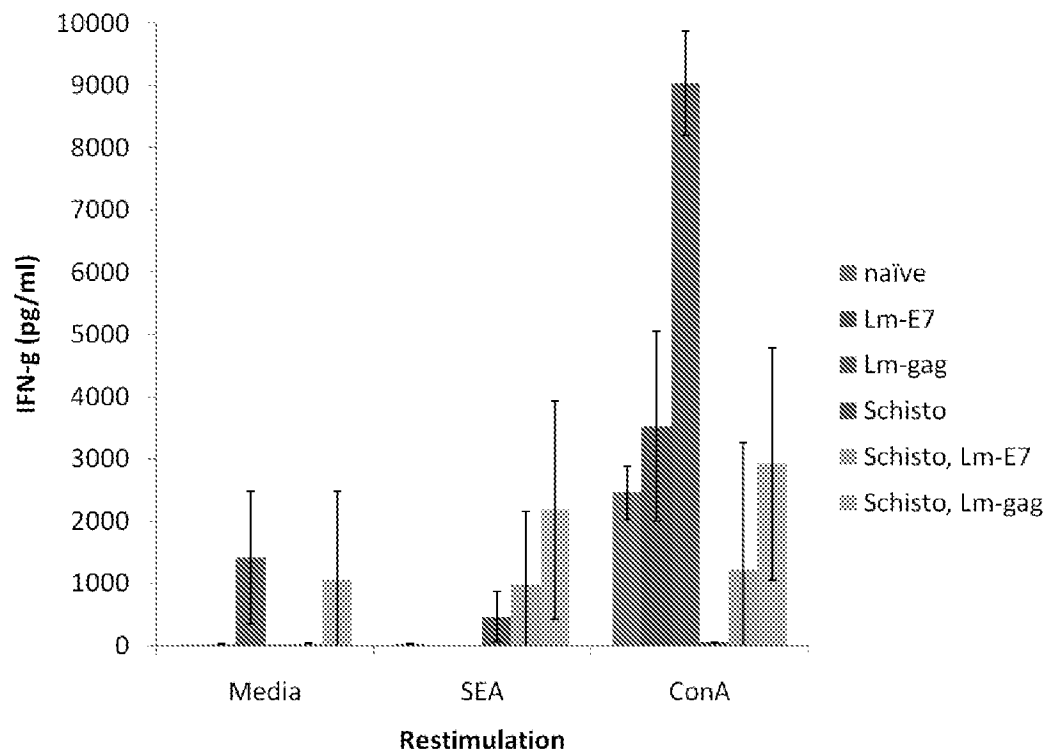


Figure 21

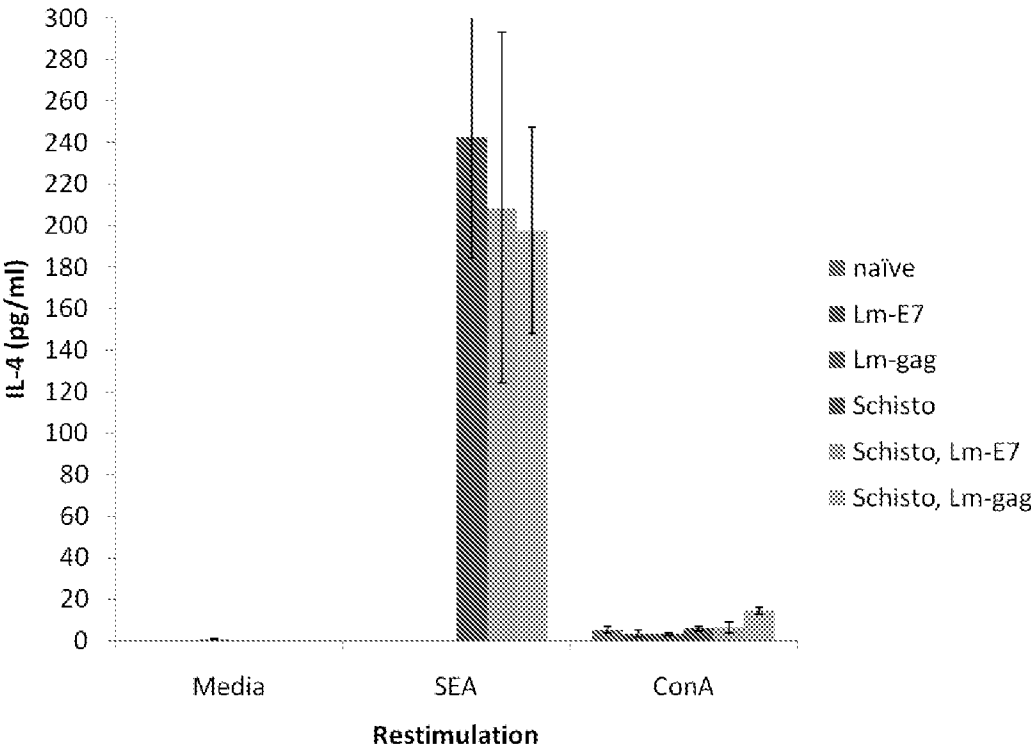


Figure 22

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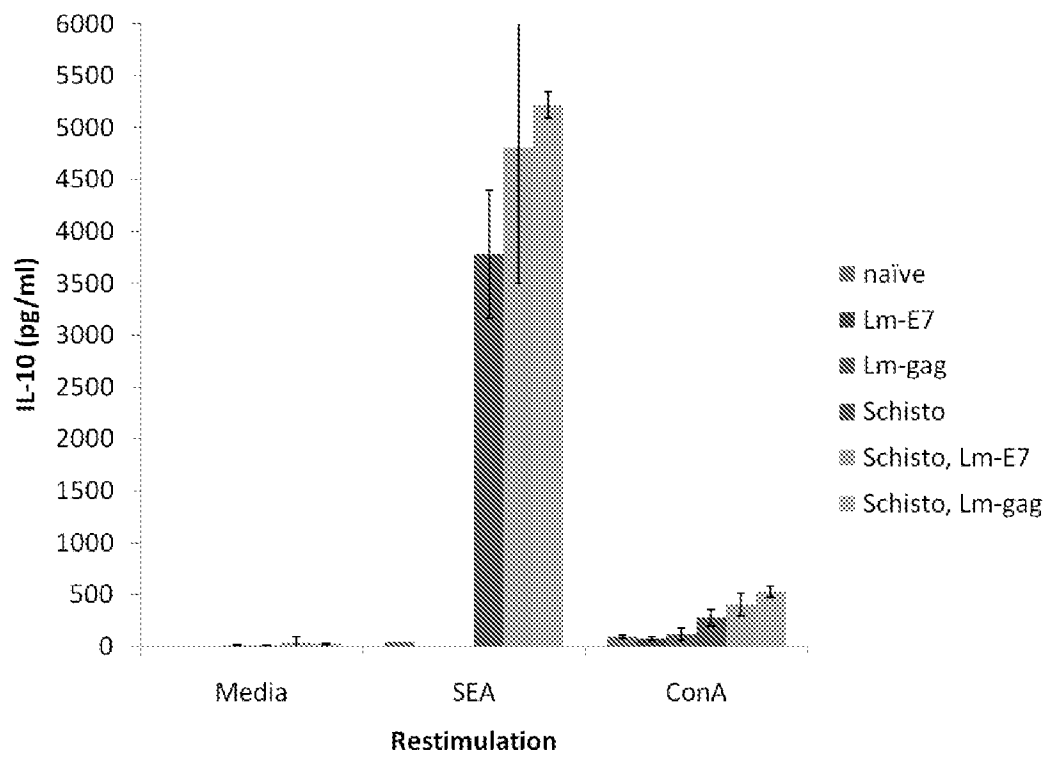


Figure 23

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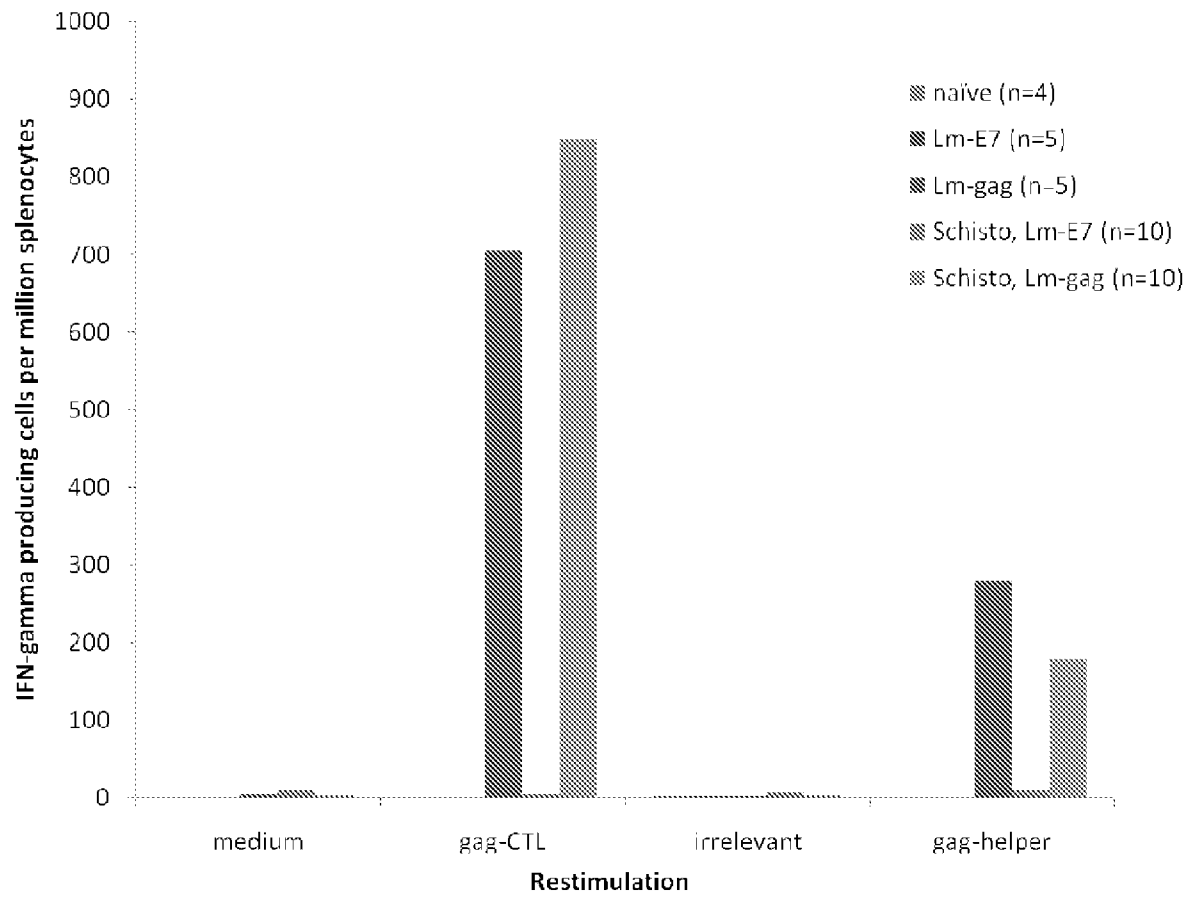


Figure 24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/28757

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61K 39/02 (2012.01) USPC - 424/234.1 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) USPC: 424/234.1				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC: 424/234.1 (keyword limited; terms below)				
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google; PubMed Search terms: listeria, based, vaccine, PEST, LLO, immunogenicity, cancer, disease, adjuvant, antigen-independent				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	WO 2009/143167 A2 (ADVAXIS) 26 November 2009 (26.11.2009) para [0006]-[0007], [0010], [0024]-[0025], [0028], [0031], [0041], [0157], [0161], [0189], [0193], [0197], [0199]-[0200], [0202], [0204], [0234], [0236]	1-4, 10-17, 23-30, 36-46, 52-58		
X	SHAHABI et al. "Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment." Bioeng. Bugs.; July-August 2010 (07-08.2010); Vol. 1, No. 4; pages 235-243; abstract; pg 237, col 1-2	1		
☐ Further documents are listed in the continuation of Box C.				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top;"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top;"> "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 </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search 5 August 2012 (05.08.2012)		Date of mailing of the international search report 27 AUG 2012		
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/28757

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

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

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

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

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

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: claims 1-58, drawn to a method of reconstituting an immune response in a subject by administering a live attenuated *Listeria* vaccine strain to the subject. The first invention (claims 1-4, 10-17, 23-30, 36-46, 52-58) is restricted to a nucleic acid encoding a PEST containing polypeptide. Should an additional fee(s) be paid, Applicant is invited to elect an additional gene(s) to be searched.

- Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 10-17, 23-30, 36-46, 52-58: limited to a PEST containing polypeptide

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of Box III: Lack of Unity of Invention

The inventions listed as Group I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Group I+ share the technical feature of a method of claim 1. However, this shared technical feature does not represent a contribution over prior art as being anticipated by an article titled "Live, attenuated strains of *Listeria* and *Salmonella* as vaccine vectors in cancer treatment" by Shahabi, et al. (Bioeng Bugs. 2010, 1(4):235-243) (hereinafter "Shahabi") that discloses that "a number of live, attenuated strains of *L. monocytogenes* have been generated that express and secrete a wide range of viral and tumor antigens including human papillomavirus (HPV)-16 E7 associated with cervix, head and neck cancer,... Her-2/neu that is overexpressed by about 30% of breast cancer,... high molecular weight-melanoma associated antigen involved with tumor angiogenesis,... and prostate specific antigen (PSA). ... Extensive preclinical testing of these strains using mouse models for cancer has shown that these vaccines are immunogenic and can cause regression of established tumors that express the target antigen. In addition, *L. monocytogenes* based vaccines can break the immunological tolerance toward self-antigens such as Her-2/ neu or HPV-16 E7 as demonstrated by the data obtained in Her-2/neu or HPV-16 transgenic animals, respectively" (pg 237, col 2). As said method was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Another special technical feature of the inventions listed as Group I+ is the specific nucleic acid/gene recited therein. The inventions do not share a special technical feature as 1) no significant structural similarities can readily be ascertained among the nucleic acid(s) /gene(s), and 2) Shahabi further discloses the claimed PEST-containing polypeptide (pg 237, col 1, "Several studies have demonstrated that T cell mediated immune responses against these antigens could be significantly enhanced when they were fused to a truncated non-hemolytic fragment of LLO... The immunogenic nature of LLO has been associated with the presence of PEST containing sequences at its aminoterminalus that may target the protein for rapid ubiquitin-mediated proteosome degradation.33-35"). Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.