SUPPRESSOR CELL FUNCTION INHIBITION FOLLOWING *LISTERIA* VACCINE TREATMENT

FIELD OF INVENTION

[0001] This invention provides methods and compositions for using a live attenuated *Listeria* for inhibiting cell-mediated suppression of anti-disease infiltrating T lymphocytes in a subject having the disease.

BACKGROUND OF THE INVENTION

[0002] *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.

[0003] Tregs play a critical role in the maintenance of peripheral self-tolerance. Naturally occurring $CD4^+CD25^{hi}$ Tregs are produced in the thymus and express FoxP3, a transcriptional factor required for establishment and maintenance of Treg lineage identity and suppressor function. Tregs can accumulate at a disease site, where they suppress the effector function of disease specific T cells. When this occurs it can result in an increase in disease despite the presence of appropriate antigens or T cells activated to attack those antigens. Increased densities of tumor-infiltrating $FoxP3^+$ Tregs have been associated with poor prognosis in various solid tumors, including pancreatic, ovarian, and hepatocellular carcinoma. Depletion of Tregs results in enhanced antitumor immunity and tumor rejection in murine models but may also result in the development of autoimmune diseases.

[0004] Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of early myeloid progenitors, immature granulocytes, macrophages, and dendritic cells at different stages of differentiation. These cells are of great interest because they have the capacity to suppress both the cytotoxic activities of natural killer (NK) and NKT cells, and the adaptive immune response mediated by $CD8^+$ T cells. While the mechanism of NK cell inhibition is currently not well-understood, multiple pathways are responsible for MDSC-mediated T cell suppression

including: 1) production of arginase 1/ARG1 and 2) upregulation of nitric oxide synthase 2 (NOS2). ARG1 and NOS2 metabolize L-arginine and either together, or separately, block translation of the T cell CD3 zeta chain, inhibit T cell proliferation, and promote T cell apoptosis. Additionally, MDSCs secrete immunosuppressive cytokines and induce regulatory T cell development. In mice, MDSCs are broadly defined as $CD11b^{+}Gr\text{-}1/Ly\text{-}6G^{+}$ cells, but the relative expression levels of Ly-6G and Ly-6C identify two specific subsets. Human MDSCs commonly express Siglec-3/CD33 and lack lineage markers and HLA-DR, but heterogeneous expression of CD14 and CD15 suggest that multiple subsets exist.

[0005] MDSCs are induced by pro-inflammatory cytokines and are found in increased numbers in infectious and inflammatory pathological conditions. They accumulate in the blood, bone marrow, and secondary lymphoid organs of tumor-bearing mice and their presence in the tumor microenvironment has been suggested to have a causative role in promoting tumor-associated immune suppression. Although it is now evident that MDSCs may serve as a target for preventing tumor progression, further characterization is necessary to determine effective mechanisms by which they can be inhibited.

[0006] The invention provides an effective mechanism of inhibiting suppressor cells such as Tregs and MDSCs by providing Listeria vaccines that once administered to tumor-bearing subject, proceed to suppress Tregs and MDSC function, thereby allowing anti-tumor T cells to replicate and inhibit tumor growth.

SUMMARY OF THE INVENTION

[0007] In one embodiment, the invention relates to a method of increasing a T infiltrating lymphocytes/suppressor cell ratio in a subject having a disease or in a disease site within the subject, the method comprising the step of administering to the subject a composition comprising a live attenuated *Listeria* vaccine strain.

[0008] In another embodiment, the invention relates to a method of reducing the percentage of suppressor cells in a disease in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to said subject.

[0009] 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

[00010] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, the inventions of which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein. The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[00011] **Figure 1** shows that Lm-E7 and Lm-LLO-E7 use different expression systems to express and secrete E7. Lm-E7 was generated by introducing a gene cassette into the orfZ domain of the L. monocytogenes genome (A). The hly promoter drives expression of the hly signal sequence and the first five amino acids (AA) of LLO followed by HPV-16 E7. B), Lm-LLO-E7 was generated by transforming the prfA- strain XFL-7 with the plasmid pGG-55. pGG-55 has the hly promoter driving expression of a nonhemolytic fusion of LLO-E7. pGG-55 also contains the prfA gene to select for retention of the plasmid by XFL-7 in vivo.

[00012] **Figure 2** shows that Lm-E7 and Lm-LLO-E7 secrete E7. Lm-Gag (lane 1), Lm-E7 (lane 2), Lm-LLO-NP (lane 3), Lm-LLO-E7 (lane 4), XFL-7 (lane 5), and 10403S (lane 6) were grown overnight at 37°C in Luria-Bertoni broth. Equivalent numbers of bacteria, as determined by OD at 600 nm absorbance, were pelleted and 18 ml of each supernatant was TCA precipitated. E7 expression was analyzed by Western blot. The blot was probed with an anti-E7 mAb, followed by HRP-conjugated anti-mouse (Amersham), and then developed using ECL detection reagents.

[00013] **Figure 3** shows that tumor immunotherapeutic efficacy of LLO-E7 fusions. Tumor size in millimeters in mice is shown at 7, 14, 21, 28 and 56 days post tumor-inoculation. Naive mice: open-circles; Lm-LLO-E7: filled circles; Lm-E7: squares; Lm-Gag: open diamonds; and Lm-

LLO-NP: filled triangles.

[00014] **Figure 4** shows that splenocytes from Lm-LLO-E7-immunized mice proliferate when exposed to TC-1 cells. C57BL/6 mice were immunized and boosted with Lm-LLO-E7, Lm-E7, or control rLm strains. Splenocytes were harvested 6 days after the boost and plated with irradiated TC-1 cells at the ratios shown. The cells were pulsed with ^{3}H thymidine and harvested. Cpm is defined as (experimental cpm) - (no-TC-1 control).

[00015] **Figure 5** shows (A) Western blot demonstrating that Lm-ActA-E7 secretes E7. Lane 1: Lm-LLO-E7; lane 2: Lm-ActA-E7.001; lane 3; Lm-ActA-E7-2.5.3; lane 4: Lm-ActA-E7-2.5.4. (B) Tumor size in mice administered Lm-ActA-E7 (rectangles), Lm-E7 (ovals), Lm-LLO-E7 (X), and naive mice (non-vaccinated; solid triangles).

[00016] **Figure 6** shows (A) schematic representation of the plasmid inserts used to create 4 LM vaccines. Lm-LLO-E7 insert contains all of the Listeria genes used. It contains the hly promoter, the first 1.3 kb of the hly gene (which encodes the protein LLO), and the HPV-16 E7 gene. The first 1.3 kb of hly includes the signal sequence (ss) and the PEST region. Lm-PEST-E7 includes the hly promoter, the signal sequence, and PEST and E7 sequences but excludes the remainder of the truncated LLO gene. Lm-ΔPEST-E7 excludes the PEST region, but contains the hly promoter, the signal sequence, E7, and the remainder of the truncated LLO. Lm-E7epi has only the hly promoter, the signal sequence, and E7. (B) Top panel: Listeria constructs containing PEST regions induce tumor regression. Bottom panel: Average tumor sizes at day 28 post-tumor challenge in 2 separate experiments. (C) Listeria constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes in the spleen. Average and SE of data from 3 experiments are depicted.

[00017] **Figure 7** shows (A) Induction of E7-specific IFN-gamma-secreting $CD8^{+}$ T cells in the spleens and the numbers penetrating the tumors, in mice administered TC-1 tumor cells and subsequently administered Lm-E7, Lm-LLO-E7, Lm-ActA-E7, or no vaccine (naive). (B) Induction and penetration of E7 specific $CD8^{+}$ cells in the spleens and tumors of the mice described for (A).

[00018] **Figure 8** shows Listeria constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes within the tumor. (A) representative data from 1 experiment. (B) average and SE of data from all 3 experiments.

[00019] **Figure 9** shows 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.

[00020] **Figure 10** shows the DNA sequence (SEQ ID NO: 81) 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).

[00021] **Figure 11** shows the sequence of DNA (SEQ ID NO: 82) 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.

[00022] **Figure 12** 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.

[00023] **Figure 13** shows the DNA sequence (SEQ ID NO: 83) 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 LmddΔactA 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.

[00024] **Figure 14** 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.

[00025] **Figure 15** shows (A) Plasmid map of pAdv164, which harbors *bacillus subtilis dal* gene

under the control of constitutive *Listeria* p60 promoter for complementation of the chromosomal *dal-dat* deletion in *LmddA* strain. It also contains the fusion of truncated $LLO_{(1-441)}$ to the chimeric human Her2/neu gene, which was constructed by the direct fusion of 3 fragments the Her2/neu: EC1 (aa 40-170), EC2 (aa 359-518) and ICI (aa 679-808). (B) Expression and secretion of tLLO-ChHer2 was detected in *Lm*-LLO-ChHer2 (Lm-LLO-138) and *LmddA*-LLO-ChHer2 (ADXS31-164) by western blot analysis of the TCA precipitated cell culture supernatants blotted with anti-LLO antibody. A differential band of ~104 KD corresponds to tLLO-ChHer2. The endogenous LLO is detected as a 58 KD band. *Listeria* control lacked ChHer2 expression.

[00026] **Figure 16** (A) Cytotoxic T cell responses elicited by Her2/neu *Listeria*-based vaccines in splenocytes from immunized mice were tested using NT-2 cells as stimulators and 3T3/neu cells as targets. Lm-control was based on the *LmddA* background that was identical in all ways but expressed an irrelevant antigen (HPV16-E7). (B) IFN-γ secreted by the splenocytes from immunized FVB/N mice into the cell culture medium, measured by ELISA, after 24 hours of *in vitro* stimulation with mitomycin C treated NT-2 cells. (C) IFN-γ secretion by splenocytes from HLA-A2 transgenic mice immunized with the chimeric vaccine, in response to *in vitro* incubation with peptides from different regions of the protein. A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide groups constituted the negative controls as listed in the figure legend. IFN-γ secretion was detected by an ELISA assay using cell culture supernatants harvested after 72 hours of co-incubation. Each data point was an average of triplicate data +/- standard error. * P value < 0.001.

[00027] **Figure 17** represents results from Her2/neu transgenic mice that were injected six times with each recombinant *Listeria*-ChHer2 or a control *Listeria* vaccine. Immunizations started at 6 weeks of age and continued every three weeks until week 21. Appearance of tumors was monitored on a weekly basis and expressed as percentage of tumor free mice. *$p<0.05$, N = 9 per group.

[00028] **Figure 18** shows FVB/N mice were inoculated s.c. with 1 x 10^6 NT-2 cells and immunized three times with each vaccine at one week intervals. Spleens were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. dot-plots of the Tregs from a representative experiment showing the frequency of $CD25^+/FoxP3^+$ T cells, expressed as

percentages of the total $CD3^+$ or $CD3^+CD4^+$ T cells across the different treatment groups.

[00029] **Figure 19** shows FVB/N mice were inoculated s.c. with 1 x 10^6 NT-2 cells and immunized three times with each vaccine at one week intervals. Tumors were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. (A). dot-plots of the Tregs from a representative experiment. (B). Frequency of $CD25^+/FoxP3^+$ T cells, expressed as percentages of the total $CD3^+$ or $CD3^+CD4^+$ T cells (left panel) and intratumoral CD8/Tregs ratio (right panel) across the different treatment groups. Data is shown as mean±SEM obtained from 2 independent experiments.

[00030] **Figure 20** shows a schematic representation of pAdv134 plasmid and dual plasmid. The restriction sites that will be used for cloning of antigen 1 (Xho I and SpeI) and antigen 2 (XbaI and SacI or BglII) genes are indicated. The black arrow represents the direction of transcription. p15 ori and RepR refer to *Listeria* and *E. coli* origin of replication. tLLO is truncated Listeriolysin O protein (1-441 aa) and tActA is truncated ActA (1-233 aa) protein. *Bacillus-dal* gene codes for D-alanine racemase which complements for the synthesis of D-alanine in *LmΔdal dat* strain.

[00031] **Figure 21** 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).

[00032] **Figure 22** 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.

[00033] **Figure 23** 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.

[00034] **Figure 24** 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.

[00035] **Figure 25** 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.

[00036] **Figure 26** 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.

[00037] **Figure 27** 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.

[00038] **Figure 28** shows suppressor assay data demonstrating that conventional CD4+ T cells have no effect on cell division regardless of whether they are found in the tumors or spleens of mice. Left-hand and Right-hand panels show pooled division cycles.

[00039] **Figure 29** 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.

[00040] **Figure 30** 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.

[00041] **Figure 31** 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.

[00042] **Figure 32** 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.

[00043] **Figure 33** 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.

[00044] **Figure 34** 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.

[00045] **Figure 35** shows suppressor assay data demonstrating that suppressive ability of the granulocytic MDSC is due to the overexpression of tLLO and is independent of the partnering fusion antigen (A-D). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00046] **Figure 36** shows suppressor assay data also demonstrating that suppressive ability of the monocytic MDSC is due to the overexpression of tLLO and is independent of the partnering fusion antigen (A-D). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00047] **Figure 37** shows suppressor assay data demonstrating that granulocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination (A, B). However, after non-specific stimulation, activated T cells (with PMA/ionomycin) are still capable of dividing (C, D). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00048] **Figure 38** shows suppressor assay data demonstrating that monocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination (A, B). However, after non-specific activation (stimulated by PMA/ionomycin), T cells are still capable of dividing (C, D). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00049] **Figure 39** shows suppressor assay data demonstrating that Tregs purified from the tumors of any of the *Lm*-treated groups have a slightly diminished ability to suppress the division of the responder T cells, regardless of whether the responder cells are antigen specific (A, B) or non-specifically (C, D) activated. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[00050] **Figure 40** shows suppressor assay data demonstrating that Tregs purified from the spleen are still capable of suppressing the division of both antigen specific (A, B) and non-specifically (C, D) activated responder T cells.

[00051] **Figure 41** shows suppressor assay data demonstrating that tumor Tcon cells are not capable of suppressing the division of T cells regardless of whether the responder cells are antigens specific (A, B) or non-specifically activated (C, D).

[00052] **Figure 42** shows suppressor assay data demonstrating that spleen Tcon cells are not capable of suppressing the division of T cells regardless of whether the responder cells are antigens specific (A, B) or non-specifically activated (C, D).

DETAILED DESCRIPTION OF THE INVENTION

[00053] In one embodiment, provided herein is a method of increasing the infiltrating T lymphocytes/suppressor cells ratio in a subject having a disease or in a disease site within the subject. In another embodiment, provided herein is a method of increasing the ratio of CD8+ T cells/suppressor cells in a subject having a disease or in a disease site within the subject. In another embodiment, the method of increasing the infiltrating T lymphocyte /suppressor cells or CD8+ T cells/ suppressor cells ratio comprises the step of administering to the subject a composition comprising a live attenuated *Listeria*, or recombinant Listeria strain of the present invention.

[00054] In one embodiment, provided herein is a method of increasing the infiltrating T lymphocyte /T regulatory cell ratio in a subject having a disease or in a disease site within the subject. In another embodiment, provided herein is a method of increasing the ratio of CD8+ T cells/T regulatory cells in a subject having a disease or in a disease site within the subject. In another embodiment, the method of increasing the infiltrating T lymphocyte /T regulatory cells

or CD8+ T cell/ T regulatory cell ratio comprises the step of administering to the subject a composition comprising a live attenuated *Listeria*, or recombinant Listeria strain of the present invention.

[00055] In one embodiment, provided herein is a method of increasing the infiltrating T lymphocyte/Myeloid-derived suppressor cell (MDSC) ratio in a subject having a disease or in a disease site within the subject. In another embodiment, provided herein is a method of increasing the ratio of CD8+ T cells/Myeloid-derived suppressor cells (MDSC) in a subject having a disease or in a disease site within the subject. In another embodiment, the method of increasing the infiltrating T lymphocyte/Myeloid-derived suppressor cells (MDSC) or CD8+ T cell/ Myeloid-derived suppressor cell (MDSC) ratio comprises the step of administering to the subject a composition comprising a live attenuated *Listeria*, or recombinant Listeria strain of the present invention.

[00056] In one embodiment, the infiltrating T lymphocyte is a Tumor infiltrating T lymphocyte (TIL).

[00057] In one embodiment, provided herein is a method of reducing the amount of cells that suppress an immune response against a disease. In another embodiment, the cells that suppress the immune response are suppressive cells. In another embodiment, the suppressive cells are myeloid-derived suppressor cells (MDSC). In another embodiment, the suppressive cells are T regulatory cells (Tregs).

[00058] 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 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 granulocytic as well.

[00059] In one embodiment, tumor MDSCs can unexpectedly inhibit both, the 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 17-20), the live attenuated *Listeria* provided herein reduces the percent of suppressor cells in a disease compared to the population of tumor infiltrating lymphocytes (TILs) at the disease site, for example, a tumor site.

[00060] *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* infection.

[00061] *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 induction of $CD4^+$ $T_{\gamma\delta}$ 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 myelopoesis and vascular endothelial cell function.

[00062] Lm infected macrophages produce TNF-α , IL-18 and IL-12, all of which are 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 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.

[00063] 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 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.

[00064] In one embodiment, LLO has an ability to induce epigenetic modifications affecting control of DNA expression. Extracellular LLO induces a dephosphorylation of the 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. In another embodiment, another genomic effect of LLO is its ability to increase NF-κβ translocation in association with the expression of ICAM and E-selectin, and the secretion of IL-8 and MCP-1. In another embodiment, another signaling cascade affected by LLO is the Mitogen Activated Protein Kinase (MAPK) pathway, resulting in increase of Ca^{2+} influx across the cell membrane, which facilitates the entry of *Listeria* into endothelial cells and their subsequent infection.

[00065] In one embodiment, LLO is a potent inducer of inflammatory cytokines such as IL-6, IL-8, IL-12, 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. In one embodiment, macrophages in the presence of LLO release IL-1α, TNF-α, IL-12 and IL-18, which in turn activate NK cells to release IFN-γ resulting in enhanced macrophage activation.

[00066] In one embodiment, LLO secreted by cytosolic *Lm* causes specific gene upregulation in macrophages resulting in significant IFN-γ transcription and secretion. In another embodiment, cytosolic LLO activates a potent type I interferon response to invasive *Lm* independent of Toll-like receptors (TLR) without detectable activation of NF-KB and MAPK.

[00067] In one embodiment, the *Listeria* (Lm) vaccine strains provided herein reduce the percentage of Tregs and MDSCs at sites of disease, with a corresponding shift in the ratio of effector to suppressor cells at sites of disease. In another embodiment, Lm vaccines provided herein are useful for improving immune responses by reducing the percentage of Tregs and MDSCs and the absolute number of MDSC at a specific site of disease in a subject. Such a site can be an inflammation site due to allergy, trauma, infection, disease or the site can be a tumor site.

[00068] 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 17-20 herein). In one embodiment, this effect is seen because splenic MDSCs are only suppressive in an antigen-specific manner. Hence, treatment with *Listeria* has the distinct advantage that it allows for tumor-specific inhibition of tumor suppressive cells such as Tregs and MDSCs. 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.

[00069] In another embodiment, both monocytic and granulocytic MDSCs purified from the tumors of truncated LLO-expressing *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 truncated LLO-expressing *Listeria* (See Example 21 herein). In one embodiment, this effect is seen because splenic MDSCs are only suppressive in an antigen-specific manner. Hence, treatment with truncated LLO-expressing *Listeria* has the distinct advantage that it allows for tumor-specific inhibition of tumor suppressive cells such as Tregs and MDSCs. Another unexpected advantage provided by the truncated LLO-expressing live attenuated Listeria of the methods and compositions provided herein is that there are lower amount of Tregs and MDSCs in the tumor, and the ones that persist lose the ability to suppress T cell replication, and this effect is observed even in the absence of an LLO fusion partner, such as a heterologous antigen.

[00070] In another embodiment, administering a truncated LLO-expressing live attenuated Listeria vaccine enhances an anti-tumor T cell response by suppressing Treg- and MDSC-mediated T cell suppression (see Example 21 herein).

[00071] 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.

[00072] 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 step of administering a live attenuated *Listeria* vaccine strain to said subject.

[00073] 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 immune response. In another embodiment, the immune response is a tumor infiltrating T-lymphocytes (TILs) immune response.

[00074] 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 *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.

[00075] 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 live attenuated *Listeria* vaccine strain to the subject.

[00076] In one embodiment, provided herein is a method of reducing the number of myeloid-derived suppressor cells in a disease in a subject, the method comprising the step of administering a live attenuated *Listeria* vaccine strain to said subject.

[00077] In one embodiment, the term "reducing the percentage of" is representative of the amount suppressor cells, either Tregs or MDSCs whose presence at a disease site is diminished or reduced in relation to the presence of T infiltrating cells as in an assay or in an immune response.

[00078] In another embodiment, the term "reducing the number of" refers to the absolute number of suppressor cells, either Tregs, or MDSCs who's absolute numbers have been diminished or reduced as a result of administration of the live attenuated Listeria provided herein or another reagents that achieve a similar effect, also described elsewhere herein.

[00079] In one embodiment, the suppressor cells provided herein are T regulatory cells (Tregs). In another embodiment, the suppressor cells are myeloid-derived suppressor cells (MDSCs).

[00080] In another embodiment, the live attenuated *Listeria* provided herein comprises a recombinant nucleic acid sequence 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 second polypeptide each comprise a heterologous antigen or a functional fragment thereof fused to an endogenous PEST-containing polypeptide.

[00081] In one embodiment, the heterologous antigen or functional fragments thereof and the endogenous PEST-containing polypeptide provided herein are translated in a single open reading frame. In another embodiment each heterologous antigenic polypeptides and the endogenous PEST-containing polypeptide provided herein are fused after being translated separately.

[00082] In another embodiment, the recombinant nucleic acid provided herein further comprises a third open reading frame encoding a third polypeptide, wherein the third polypeptide comprises a heterologous antigen or a functional fragment thereof fused to an endogenous PEST-containing polypeptide.

[00083] In another embodiment, the PEST-containing polypeptide is an N-terminal truncated LLO polypeptide, an N-terminal ActA polypeptide, or PEST-peptide, or a functional fragment thereof.

[00084] In another embodiment the first, the second or the third heterologous antigen or functional fragment thereof is expressed by or derived from an infectious pathogen, or a tumor cell.

[00085] In one embodiment, the first, the second or the third antigen is associated with the local tissue environment that is further associated with the development or metastasis of cancer, or is associated with tumor evasion or resistance to cancer, or is an angiogenic antigen.

[00086] In another embodiment, the heterologous antigen is an allergen that causes an allergic, inflammatory reaction in a host.

[00087] In another embodiment, the disease provided herein is a localized disease, i.e., to a specific disease site or is a systemic disease.

[00088] In another embodiment, the disease is an infectious disease, a respiratory or inflammatory disease, or a cancer or tumor.

[00089] In another 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 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, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (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 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, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human 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, 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.

[00090] 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 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.

[00091] In another embodiment, the respiratory or inflammatory disease is asthma.

[00092] The live attenuated *Listeria* strains are capable of alleviating asthma symptoms without co-administration of other therapeutic agents, such as anti-inflammatory agents or bronchodilators. In another embodiment, the methods provided herein further comprise the step of co-administering to a subject the live attenuated Listeria strain and one or more therapeutic agents. In another embodiment, the therapeutic agent is an anti-asthmatic agent. In another embodiment, the agent is an anti-inflammatory agent, a non-steroidal anti-inflammatory agent, an antibiotic, an antichlolinerginc agent, a bronchodilator, a corticosteroid, a short-acting beta-agonist, a long-acting beta-agonist, combination inhalers, an antihistamine, or combinations thereof.

[00093] In another embodiment, the present pharmaceutical composition may contain both the live attenuated *Listeria* strain and the co-administered therapeutic agents. The live attenuated *Listeria* strain and the co-administered therapeutic agents may also be in different pharmaceutical compositions.

[00094] In another embodiment, the agent includes inhaled corticosteroids, which include fluticasone (Flovent Diskus, Flovent HFA), budesonide (Pulmicort Flexhaler), mometasone (Asmanex), flunisolide (Aerobid), beclomethasone (Qvar) and others. They are the most commonly prescribed type of long-term asthma medication. Unlike oral corticosteroids, these corticosteroid medications have a relatively low risk of side effects and are generally safe for long-term use.

[00095] The agent can be a Leukotriene modifier. These oral medications include montelukast (Singulair), zafirlukast (Accolate) and zileuton (Zyflo, Zyflo CR). They help prevent asthma symptoms for up to 24 hours.

[00096] Moreover, the agent can be long-acting beta agonists (LABAs). These inhaled medications include salmeterol (Serevent Diskus) and formoterol (Foradil Aerolizer). LABAs open the airways and reduce inflammation. However, they've been linked to severe asthma attacks. LABAs should be taken only in combination with an inhaled corticosteroid.

[00097] 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.

[00098] In one embodiment, the heterologous antigen provided herein is HPV-E7. In another embodiment, the antigen is HPV-E6. In another embodiment, the antigen is Her-2/neu. In another embodiment, the antigen is NY-ESO-1. In another embodiment, the antigen is telomerase (TERT). In another embodiment, the antigen is SCCE. In another embodiment, the antigen is CEA. In another embodiment, the antigen is LMP-1. In another embodiment, the antigen is p53. In another embodiment, the antigen is carboxic anhydrase IX (CAIX). In another embodiment, the antigen is PSMA. In another embodiment, the antigen is prostate stem cell antigen (PSCA). In another embodiment, the antigen is HMW-MAA. In another embodiment, the antigen is WT-1. In another embodiment, the antigen is HIV-1 Gag. In another embodiment, the antigen is Proteinase 3. In another embodiment, the antigen is Tyrosinase related protein 2. In another embodiment, the antigen is PSA (prostate-specific antigen). In another embodiment, the antigen is selected from HPV-E7, HPV-E6, Her-2, NY-ESO-1, telomerase (TERT), SCCE, HMW-MAA, WT-1, HIV-1 Gag, CEA, LMP-1, p53, PSMA, PSCA, Proteinase 3, Tyrosinase related protein 2, Muc1, PSA (prostate-specific antigen), or a combination thereof.

[00099] In another embodiment, the heterologous antigen provided herein is a tumor-associated antigen, which in one embodiment, is one of the following tumor antigens: a MAGE (Melanoma-Associated Antigen E) protein, e.g. MAGE 1, MAGE 2, MAGE 3, MAGE 4, a tyrosinase; a mutant ras protein; a mutant p53 protein; p97 melanoma antigen, a ras peptide or p53 peptide associated with advanced cancers; the HPV 16/18 antigens associated with cervical cancers, KLH antigen associated with breast carcinoma, CEA (carcinoembryonic antigen) associated with colorectal cancer, gp100, a MART1 antigen associated with melanoma, or the PSA antigen associated with prostate cancer. In another embodiment, the antigen for the compositions and methods provided herein are melanoma-associated antigens, which in one embodiment are TRP-2, MAGE-1, MAGE-3, gp-100, tyrosinase, HSP-70, beta-HCG, or a combination thereof. It is to be understood that a skilled artisan would be able to use any heterologous antigen not mentioned herein but known in the art for use in the methods and compositions provided herein.

[000100] In one embodiment, the nucleic acid molecule provided herein further comprises a second open reading frame encoding a metabolic enzyme. In another embodiment, the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain. In another embodiment, the metabolic enzyme encoded by the second open reading frame is an alanine racemase enzyme. In one embodiment, the *Listeria* further comprises a third open reading frame encoding an additional metabolic enzyme. In another embodiment, the metabolic enzyme encoded by the third open reading frame is a D-amino acid transferase enzyme. In another embodiment, the nucleic acid molecule comprises a fourth reading frame encoding a heterologous antigen or fragment thereof.

[000101] In one embodiment, the nucleic acid molecule is integrated into the *Listeria* genome. In another embodiment, the nucleic acid molecule is in a plasmid in the recombinant *Listeria* vaccine strain. In another embodiment, the plasmid 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 the recombinant *Listeria*.

[000102] In one embodiment, provided herein is a nucleic acid molecule that 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 embodiment, the nucleic acid molecule is integrated into the *Listeria* genome and 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 inlA gene, and inlB gene, an inlC gene, inlJ gene, a PlbC gene or a PrfA gene. It is to be understood by a skilled artisan, the virulence gene can be any gene known in the art to be associated with virulence in the recombinant *Listeria*.

[000103] 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 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.

[000104] In one embodiment, the term "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.

[000105] In another 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.

[000106] 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.

[000107] 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. In another embodiment, the present invention further comprises a phage based chromosomal integration system for clinical applications, where a host strain that is auxotrophic for essential enzymes, including, but not limited to, d-alanine racemase can be used, for example *Lm*dal(-)dat(-). In another embodiment, in order to avoid a "phage curing step," a phage integration system based on PSA is used. 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 can be complemented. Each possibility represents a separate embodiment of the present invention.

[000108] 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.

[000109] 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.

[000110] In one embodiment, the present invention provides a method of producing a recombinant *Listeria* strain comprising an episomal expression plasmid comprising a first and at least a second nucleic acid encoding a first and at least a second polypeptide, wherein the first and the second polypeptide each comprise a heterologous antigen fused to an endogenous PEST-containing polypeptide, the method comprising the steps of a) recombinantly fusing in the plasmid the first and the second nucleic acid encoding the first and the second polypeptide each comprising a first and a second heterologous antigen fused to an endogenous PEST-containing polypeptide; b) transforming the recombinant *Listeria* with the episomal expression plasmid; and, c) expressing the first, and the at least second antigens under conditions conducive to antigenic expression in the recombinant *Listeria* strain.

[000111] In one embodiment, provided herein is a method of producing a recombinant *Listeria* strain comprising an episomal expression plasmid comprising a first, a second and a third nucleic acid encoding a first a second and a third polypeptide, wherein the first, the second and the third polypeptide each comprise a heterologous antigen fused to an endogenous PEST-containing polypeptide, the method comprising the steps of: a) recombinantly fusing in the plasmid the first, the second and the third nucleic acid encoding the first, the second and the third polypeptide each comprising a first, a second and a third heterologous antigen fused to an endogenous PEST-containing polypeptide; b) transforming the recombinant *Listeria* with the episomal expression plasmid; and, c) expressing the first, the second and the third antigens under conditions conducive to antigenic expression in the recombinant *Listeria* strain.

[000112] In another embodiment, the present invention provides a recombinant *Listeria* strain comprising at least one episomal recombinant nucleic acid molecule, the nucleic acid molecules 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 acids further comprise an open reading frame encoding a plasmid replication control region. In another embodiment, the plasmid control region regulates replication of the episomal recombinant nucleic acid molecule.

[000113] In another embodiment, the plasmid control region comprises an open reading frame encoding a transcription repressor that represses heterologous antigen expression from the first or

at least second nucleic acid molecule. In another embodiment, the plasmid control region comprises an open reading frame encoding transcription inducer that induces heterologous antigen expression from the first or at least second nucleic acid molecule. In another embodiment, the plasmid control region comprises an open reading frame encoding a transcription repressor that represses heterologous antigen expression from the first, second or third nucleic acid molecule. In another embodiment, the plasmid control region comprises an open reading frame encoding a transcription inducer that induces heterologous antigen expression from the first, second or third nucleic acid molecule.

[000114] In one embodiment, there are different types of transcription regulation, these include "negative control" and "positive control". In negative control, a regulatory protein or repressor protein binds to the operator and prevents RNA polymerase from binding properly to the promoter sequence. Alternatively, the repressor protein can be synthesized in an inactive form in that it cannot block RNA polymerase binding to the promoter, the repressor is then activated to prevent RNA polymerase binding to the promoter by the binding of a corepressor. This type of control is seen most often in anabolic pathways (e.g., arginine biosynthesis), where the corepressor is often the end product of the anabolic pathway. Alternatively, the repressor protein is synthesized in an active form, binds to the operator and prevents RNA polymerase from binding to promoter. When an inducer binds to the repressor, the repressor becomes inactive, therefore RNA polymerase is now free to initiate transcription. This type of control is seen most often in catabolic pathways (e.g., lactose catabolism). The inducer is often a form of the substrate that will be degraded. In positive control, a regulatory protein, called an activator protein, binds to the operator and the activator molecular stabilizes RNA polymerase binding to the promoter region. An example of this includes the arabinose catabolism. Regulatory proteins (for both positive and negative regulation) are encoded by regulatory genes and can be synthesized continuously at low levels. They can be made to be self-regulated whereby high concentrations of the regulatory protein (associated with high plasmid production) binds to its own operator and represses RNA polymerase from binding to the promoter sequence. This stops transcription until its level drops. Several examples of these types of regulation include the lactose operon, the arginine operon, the diphtheria toxin gene regulation system, etc. Transcription repressors and methods of use thereof are readily known in the art and are contemplated for use in the present invention.

[000115] In another embodiment, the plasmid replication regulation region enables the regulation of expression of exogenous heterologous antigen from each of the first or the at least second nucleic acid molecule. In another embodiment, the plasmid replication regulation region enables the regulation of expression of exogenous heterologous antigen from each of the first, second or third nucleic acid molecules.

[000116] In one embodiment, measuring metabolic burden is accomplished by any means know in the art at the time of the invention which include but are not limited to, measuring growth rates of the vaccine strain, optical density readings, colony forming unit (CFU) plating, and the like. In another embodiment, the metabolic burden on the bacterial cell is determined by measuring the viability of the bacterial cell. Methods of measuring bacteria viability are readily known and available in the art, some of which include but are not limited to, bacteria plating for viability count, measuring ATP, and flow cytometry. In ATP staining, detection is based on using the luciferase reaction to measure the amount of ATP from viable cells, wherein the amount of ATP in cells correlates with cell viability. As to flow cytometry, this method can be used in various ways, also known in the art, for example after employing the use of viability dyes which are excluded by live bacterial cells and are absorbed or adsorbed by a dead bacterial cells. A skilled artisan would readily understand that these and any other methods known in the art for measuring bacterial viability can be used in the present invention. It is to be understood that a skilled artisan would be able to implement the knowledge available in the art at the time of the invention for measuring growth rates of the vaccine strain or expression of marker genes by the vaccine strain that enable determining the metabolic burden of the vaccine strain expressing multiple heterologous antigens or functional fragments thereof.

[000117] In another embodiment, the "functional fragment" is an immunogenic fragment and elicits an immune response when administered to a subject alone or in a vaccine composition provided herein. In another embodiment, a functional fragment has biological activity as will be understood by a skilled artisan and as further provided herein.

[000118] In one embodiment, the term "at least second nucleic acid molecule" refers to two or more nucleic acid molecules, alternatively it refers to three, four, five, and so on nucleic acid molecules.

[000119] In another embodiment, the recombinant nucleic acid molecule further comprises a third open reading frame encoding a third polypeptide, wherein said third polypeptide comprises a heterologous antigen or a functional fragment thereof fused to an endogenous PEST-containing polypeptide.

[000120] In one embodiment, provided herein is a multivalent plasmid that delivers at least two antigens. In another embodiment, the plasmid is a dual plasmid. In another embodiment, provided herein is an episomal recombinant nucleic acid encoding the multivalent plasmid. In another embodiment, the episomal recombinant nucleic acid backbone is encoded by the sequence comprising SEQ ID NO: 1. In another embodiment, the episomal recombinant nucleic acid provided herein is encoded by the sequence consisting of SEQ ID NO: 1. In another embodiment, the episomal recombinant nucleic acid provided herein is encoded by the sequence set forth in SEQ ID NO: 1.

ggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcaccggtgcgtca
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gactataccaactatttgttaat (SEQ ID NO: 1).

[000121] In one embodiment, the multivalent plasmid backbone comprises at least two nucleic acid sequences encoding at least two antigens. In another embodiment, the recombinant episomal nucleic acid encodes a plasmid backbone sequence and at least two antigens. In another embodiment, the antigens are heterologous antigens to the bacteria host carrying the plasmid. In another embodiment, the antigens are heterologous antigens to the Listeria host carrying the plasmid. In another embodiment, the recombinant episomal nucleic acid sequence encoding the plasmid backbone and at least two heterologous antigens comprises SEQ ID NO: 2. In another embodiment, the recombinant episomal nucleic acid sequence encoding the plasmid backbone and at least two heterologous antigens consists of SEQ ID NO: 2.

ggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcaccggtgcgtca
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agtttacccagttttcacctaaaaaacaaatgatgagataatagctccaaaggctaaagaggactataccaactatttgttaat (SEQ ID NO: 2).

[000122] In another embodiment, one of the antigens encoded by a sequence within SEQ ID NO: 2 is E7 (bolded in SEQ ID NO:2). In another embodiment, the E7 sequence is set forth in SEQ ID NO: 3

[000123] Ctcgagcatggagatacacctacattgcatgaatatatgttagatttgcaaccagagacaactgatctctactgttatgagcaatta
aatgacagctcagaggaggaggatgaaatagatggtccagctggacaagcagaaccggacagagcccattacaatattgtaacctttttgttg
caagtgtgactctacgcttcggttgtgcgtacaaagcacacacgtagacattcgtactttggaagacctgttaatgggcacactaggaattgtgt
gccccatctgttctcagaaaccataaactagt(SEQ ID NO: 3).

[000124] In one embodiment, one of the antigens encoded by a sequence within SEQ ID NO: 2 is a chimeric Her2-neu antigen (italicized in SEQ ID NO: 2). In another embodiment, the chimeric Her2-neu sequence is set forth in SEQ ID NO: 4.

[000125] ctagtggtgatggtgatgatggagctcagatctgtctaagaggcagccatagggcataagctgtgtcaccagctgcaccgtgga
tgtcaggcagatgcccagaaggcgggagacatatggggagcccacaccagccatcacgtatgcttcgtctaagatttctttgttggctttggg
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gacaggctggcattggtgggcaggtaggtgagttccaggtttccctgcaccacctggcagccctggtagaggtggcggagcatgtccaggt
gggttctagat(SEQ ID NO: 4).

[000126] "Metabolic enzyme" refers, in another embodiment, to an enzyme involved in 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 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.

[000127] "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 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 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 *in vitro* and *in vitro*. Each possibility represents a separate embodiment of the present invention.

[000128] In another embodiment, the metabolic enzyme of the methods and compositions provided herein is an amino acid metabolism enzyme, where, in another embodiment, the 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.

[000129] 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.

[000130] In one embodiment, the live attenuated *Listeria* is a recombinant *Listeria*. In another embodiment, the 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. In another embodiment, the

recombinant *Listeria* comprises a mutation or a deletion of a genomic *actA* gene and a genomic *internalin C* gene.

[000131] In one embodiment, the recombinant *Listeria* strain has been passaged through an animal host. In another embodiment, the animal host is a non-human animal host. In another embodiment, the passaging maximizes efficacy of the strain as a vaccine vector. In another embodiment, the passaging stabilizes the immunogenicity of the *Listeria* strain. In another embodiment, the passaging stabilizes the virulence of the *Listeria* strain. In another embodiment, the passaging increases the immunogenicity of the *Listeria* strain. In another embodiment, the passaging increases the virulence of the *Listeria* strain. In another embodiment, the passaging removes unstable sub-strains of the *Listeria* strain. In another embodiment, the passaging reduces the prevalence of unstable sub-strains of the *Listeria* strain. In another embodiment, the passaging attenuates the strain, or in another embodiment, makes the strain less virulent. Methods for passaging a recombinant *Listeria* strain through an animal host are well known in the art, and are described, for example, in United States Patent Application Serial No. 10/541,614. Each possibility represents a separate embodiment of the methods and composition provided herein.

[000132] In one embodiment, this invention provides methods and compositions for preventing disease, treating disease and vaccinating a human subject.

[000133] In another embodiment, the present invention is directed to enhancing an anti-tumor immune response of a human. In another embodiment, the methods of enhancing an anti-tumor response in a subject by administering the compositions provided herein can be combined with other known anti-tumor or anti-cancer therapies. In another embodiment, *Lm*-LLO 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.

[000134] In another embodiment, the *Listeria* strain provided herein further comprises a third open reading frame encoding a metabolic enzyme.

[000135] 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.

[000136] In another embodiment, the recombinant *Listeria* is an attenuated auxotrophic strain.

[000137] In one embodiment the attenuated strain is Lm dal(-)dat(-) (*Lmdd*). In another embodiment, the attenuated strains is Lm dal(-)dat(-)ΔactA (*LmddA*). *LmddA* is based on a *Listeria* vaccine vector which is attenuated due to the deletion of virulence gene *actA* and retains the plasmid for a desired heterologous antigen or trunctated LLO expression *in vivo* and *in vitro* by complementation of *dal* gene.

[000138] In another embodiment the attenuated strain is *Lmdda*. In another embodiment, the attenuated strain is LmΔactA. 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 strain is the double mutant or triple mutant of any of the above-mentioned strains. 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. 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.

[000139] In one embodiment, translocation of *Listeria* to adjacent cells is inhibited by the deletion of the *actA* gene and/or the *inlC* gene, which are involved in the process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a vaccine backbone.

[000140] 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.

[000141] 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. In another embodiment, the recombinant *Listeria* vaccine strain comprises an adjuvant, wherein the adjuvant is a PEST amino acid sequence.

[000142] 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.

[000143] 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 herein. In another embodiment, the immune response leads to clearance of a tumor provided herein that is afflicting the subject.

[000144] In one embodiment, recombinant attenuated, *Listeria* expressing truncated listeriolysin O in combination with other therapeutic modalities are useful for enhancing an immune response, and for preventing, and treating a disease including cancer or solid tumors. In one embodiment, recombinant attenuated, *Listeria* expressing truncated ActA in combination with other therapeutic modalities are useful for enhancing an immune response, and for preventing, and treating a disease including cancer or solid tumors. In one embodiment, recombinant attenuated, *Listeria* expressing PEST amino acid sequence in combination with other therapeutic modalities are useful for enhancing an immune response, and for preventing, and treating a disease including cancer or solid tumors.

[000145] In another embodiment, provided herein is a method of improving the immunogenicity of

a therapeutic vaccine, the method comprising co-administering the vaccine and a live attenuated *Listeria* to a subject, wherein the live attenuated *Listeria* enhances the immunogenicity of the vaccine, thereby improving the immunogenicity of the vaccine. In another embodiment, the live attenuated *Listeria* is a recombinant *Listeria*. In one embodiment, the method enables the treatment of a tumor for which the vaccine is specific against.

[000146] In one embodiment, provided herein is a method of enhancing an immune response against a disease in an antigen-independent manner, the method comprising administering a live attenuated *Listeria* or recombinant *Listeria* to a subject.

[000147] In another embodiment, the live attenuated or recombinant Listeria provided herein expresses an LLO protein or a non-hemolytic fragment thereof. In another embodiment, Listeria provided herein is used alone or is combined 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 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.

[000148] 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*.

[000149] In one embodiment, the LLO protein is encoded by the following nucleic acid sequence set forth in (SEQ ID NO: 5).

atgaaaaaaataatgctagtttttattacacttatattagttagtctaccaattgcgcaacaaactgaagcaaaggatgcatctgcattcaataaaga
aaattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcgataagtat
atacaaggattggattacaataaaaacaatgtattagtataccacggagatgcagtgacaaatgtgccgccaagaaaaggttacaaagatgga
aatgaatatattgttgtggagaaaaagaagaaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttcgagcctaacctatcca
ggtgctctcgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcattaacactcagcattgatttgccag
gtatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgttaacaacgcagtaaatacattagtggaaagatggaatgaa
aaatatgctcaagcttatccaaatgtaagtgcaaaaattgattatgatgacgaaatggcttacagtgaatcacaattaattgcgaaatttggtacag
catttaaagctgtaaataatagcttgaatgtaaacttcggcgcaatcagtgaagggaaaatgcaagaagaagtcattagttttaaacaaatttacta
taacgtgaatgttaatgaacctacaagaccttccagatttttcggcaaagctgttactaaagagcagttgcaagcgcttggagtgaatgcagaaa
atcctcctgcatatatctcaagtgtggcgtatggccgtcaagtttatttgaaattatcaactaattcccatagtactaaagtaaaagctgcttttgatg
ctgccgtaagcggaaaatctgtctcaggtgatgtagaactaacaaatatcatcaaaaattcttccttcaaagccgtaatttacggaggttccgcaa
aagatgaagttcaaatcatcgacggcaacctcggagacttacgcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcc
cattgcttatacaacaaacttcctaaaagacaatgaattagctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacagatg
gaaaaattaacatcgatcactctggaggatacgttgctcaattcaacatttcttgggatgaagtaaattatgatctcgag (SEQ ID NO: 5).

[000150] In another embodiment, the LLO protein has the sequence SEQ ID NO: 6

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 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 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: 6)

The first 25 amino acids of the proprotein corresponding to this sequence are the signal 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. Each possibility represents a separate embodiment of the present invention.

[000151] 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 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.

[000152] 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.

[000153] In another embodiment, homologues of LLO from other species, including known lysins, such as streptolysin O, perfringolysin O, pneumolysin, etc, or fragments thereof may be used in the invention.

[000154] 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:

[000155] MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSEVNTGPRY ETAREVSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKGPNINNNNSEQTENAAINEEAS GADRPAIQVERRHPGLPSDSAAEIKKRRKAIASSDSELESLTYPDKPTKVNKKKVAKESV ADASESDLDSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKIDENPEVKKAI VDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPP PPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTEDELEIIRETASSLDSSFTRGDLAS LRNAINRHSQNFSDFPPIPTEEELNGRGGRP (SEQ ID No: 7). In another embodiment, an ActA AA sequence of methods and compositions of the present invention comprises the sequence set forth in SEQ ID No: 7. In another embodiment, the ActA AA sequence is a homologue of SEQ ID No: 7. In another embodiment, the ActA AA sequence is a variant of SEQ ID No: 7. In another embodiment, the ActA AA sequence is a fragment of SEQ ID No: 7. In another embodiment, the ActA AA sequence is an isoform of SEQ ID No: 5. Each possibility represents a separate embodiment of the present invention.

[000156] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide comprising the sequence:

ATGCGTGCGATGATGGTGGTTTTCATTACTGCCAATTGCATTACGATTAACCCCGACA
TAATATTTGCAGCGACAGATAGCGAAGATTCTAGTCTAAACACAGATGAATGGGAA
GAAGAAAAAACAGAAGAGCAACCAAGCGAGGTAAATACGGGACCAAGATACGAAA
CTGCACGTGAAGTAAGTTCACGTGATATTAAAGAACTAGAAAAATCGAATAAAGTG
AGAAATACGAACAAAGCAGACCTAATAGCAATGTTGAAAGAAAAAGCAGAAAAAG
GTCCAAATATCAATAATAACAACAGTGAACAAACTGAGAATGCGGCTATAAATGAA
GAGGCTTCAGGAGCCGACCGACCAGCTATACAAGTGGAGCGTCGTCATCCAGGATT
GCCATCGGATAGCGCAGCGGAAATTAAAAAAAGAAGGAAAGCCATAGCATCATCGG
ATAGTGAGCTTGAAAGCCTTACTTATCCGGATAAACCAACAAAAGTAAATAAGAAA
AAAGTGGCGAAAGAGTCAGTTGCGGATGCTTCTGAAAGTGACTTAGATTCTAGCATG
CAGTCAGCAGATGAGTCTTCACCACAACCTTTAAAAGCAAACCAACAACCATTTTTC
CCTAAAGTATTTAAAAAAATAAAAGATGCGGGGAAATGGGTACGTGATAAAATCGA
CGAAAATCCTGAAGTAAAGAAAGCGATTGTTGATAAAAGTGCAGGGTTAATTGACC
AATTATTAACCAAAAAGAAAAGTGAAGAGGTAAATGCTTCGGACTTCCCGCCACCA
CCTACGGATGAAGAGTTAAGACTTGCTTTGCCAGAGACACCAATGCTTCTTGGTTTT
AATGCTCCTGCTACATCAGAACCGAGCTCATTCGAATTTCCACCACCACCTACGGAT
GAAGAGTTAAGACTTGCTTTGCCAGAGACGCCAATGCTTCTTGGTTTTAATGCTCCTG
CTACATCGGAACCGAGCTCGTTCGAATTTCCACCGCCTCCAACAGAAGATGAACTAG
AAATCATCCGGGAAACAGCATCCTCGCTAGATTCTAGTTTTACAAGAGGGGATTTAG
CTAGTTTGAGAAATGCTATTAATCGCCATAGTCAAAATTTCTCTGATTTCCCACCAAT
CCCAACAGAAGAAGAGTTGAA CGGGAGAGGCGGTAGACCA (SEQ ID NO: 8). In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 8. In another embodiment, an ActA-encoding nucleotide of methods and compositions of the present invention comprises the sequence set forth in SEQ ID No: 8. In another embodiment, the ActA-encoding nucleotide is a homologue of SEQ ID No: 8. In another embodiment, the ActA-encoding nucleotide is a variant of SEQ ID No: 8. In another embodiment, the ActA-encoding nucleotide is a fragment of SEQ ID No: 8. In another embodiment, the ActA-encoding nucleotide is an isoform of SEQ ID No: 8. Each possibility represents a separate embodiment of the present invention.

[000157] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide comprising the sequence:

[000158] Tttatcacgtacccatttccccgcatcttttatttttttaaatactttagggaaaaatggtttttgatttgcttttaaaggttgtggtgtaga
ctcgtctgctgactgcatgctagaatctaagtcactttcagaagcatccacaactgactctttcgccacttttctcttatttgcttttgttggtttatct
ggataagtaaggctttcaagctcactatccgacgacgctatggcttttcttcttttttttaatttccgctgcgctatccgatgacagacctggatgac
gacgctccacttgcagagttggtcggtcgactcctgaagcctcttcatttatagccacatttcctgtttgctcaccgttgttattattgttattcgga
cctttctctgcttttgctttcaacattgctattaggtctgctttgttcgtatttttcactttattcgatttttctagttcctcaatatcacgtgaacttacttca
cgtgcagtttcgtatcttggtcccgtatttacctcgcttggctgctcttctgttttttcttcttcccattcatctgtgtttagactggaatcttcgctatct
gtcgctgcaaatattatgtcggggttaatcgtaatgcagttggcagtaatgaaaactaccatcatcgcacgcat (SEQ ID NO: 9). In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 9. In another embodiment, an ActA-encoding nucleotide of methods and compositions of the present invention comprises the sequence set forth in SEQ ID No: 9. In another embodiment, the ActA-encoding nucleotide is a homologue of SEQ ID No: 9. In another embodiment, the ActA-encoding nucleotide is a variant of SEQ ID No: 9. In another embodiment, the ActA-encoding nucleotide is a fragment of SEQ ID No: 9. In another embodiment, the ActA-encoding nucleotide is an isoform of SEQ ID No: 9. In another embodiment SEQ ID NO: 9 is used to arrive at the construct of SEQ ID NO: 2, also provided herein. Each possibility represents a separate embodiment of the present invention.

[000159] 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 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 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.

[000160] 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: 10:

MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSEVNTGPRYETARE
VSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKGPNINNNNSEQTENAAINEEASGADR
PAIQVERRHPGLPSDSAAEIKKRRKAIASSDSELESLTYPDKPTKVNKKKVAKESVADAS
ESDLDSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKIDENPEVKKAIVDKS
AGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTD
EELRLALPETPMLLGFNAPATSEPSSFEFPPPPTEDELEIIRETASSLDSSFTRGDLASLRNA
INRHSQNFSDFPPIPTEEELNGRGGRP. In another embodiment, the ActA fragment comprises the sequence set forth in SEQ ID NO: 10. 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.

[000161] In another embodiment, the recombinant nucleotide encoding a fragment of an ActA protein comprises the sequence set forth in SEQ ID NO: 11:

Atgcgtgcgatgatggtggttttcattactgccaattgcattacgattaaccccgacataatatttgcagcgacagatagcgaagattctagtct
aaacacagatgaatgggaagaagaaaaaacagaagagcaaccaagcgaggtaaatacgggaccaagatacgaaactgcacgtgaagta
agttcacgtgatattaaagaactagaaaaatcgaataaagtgagaaatacgaacaaagcagacctaatagcaatgttgaaagaaaaagcaga
aaaaggtccaaatatcaataataacaacagtgaacaaactgagaatgcggctataaatgaagaggcttcaggagccgaccgaccagctata
caagtggagcgtcgtcatccaggattgccatcggatagcgcagcggaaattaaaaaaagaaggaaagccatagcatcatcggatagtgag
cttgaaagccttacttatccggataaaccaacaaaagtaaataagaaaaaagtggcgaaagagtcagttgcggatgcttctgaaagtgactta
gattctagcatgcagtcagcagatgagtcttcaccacaaccttaaaagcaaaccaacaaccatttttccctaaagtatttaaaaaaataaaaga
tgcggggaaatgggtacgtgataaaatcgacgaaaatcctgaagtaaagaaagcgattgttgataaaagtgcagggttaattgaccaattatt
aaccaaaaagaaaagtgaagaggtaaatgcttcggacttcccgccaccacctacggatgaagagttaagacttgctttgccagagacacca
atgcttcttggttttaatgctcctgctacatcagaaccgagctcattcgaatttccaccaccacctacggatgaagagttaagacttgctttgcca
gagacgccaatgcttcttggttttaatgctcctgctacatcggaaccgagctcgttcgaatttccaccgcctccaacagaagatgaactagaaa
tcatccgggaaacagcatcctcgctagattctagttttacaagaggggatttagctagtttgagaaatgctattaatcgccatagtcaaaatttctc
tgatttcccaccaatcccaacagaagaagagttgaacgggagaggcggtagacca. In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 11. In another embodiment, the recombinant nucleotide comprises any other sequence that encodes a fragment of an ActA protein. Each possibility represents a separate embodiment of the present invention.

[000162] 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.

[000163] 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.

[000164] 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: 12). In another embodiment, the PEST sequence is KENSISSMAPPASPPASPK (SEQ ID No: 13). In another embodiment, fusion of an antigen to any LLO sequence that includes one of the PEST AA sequences enumerated herein can enhance cell mediated immunity against HMW-MAA.

[000165] 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: 14), KASVTDTSEGDLDSSMQSADESTPQPLK (SEQ ID NO: 15), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 16), or RGGIPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 17). In another embodiment, the PEST-like sequence is a variant of the PEST sequence described hereinabove, which in one

embodiment, is KESVVDASESDLDSSMQSADESTPQPLK (SEQ ID NO: 18), KSEEVNASDFPPPPTDEELR (SEQ ID NO: 19), or RGGRPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 20), 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: 21). 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: 22) at AA 35-51. In another embodiment, the PEST-like sequence is from *Streptococcus equisimilis* Streptolysin O, e.g. KQNTANTETTTTNEQPK (SEQ ID NO: 23) at AA 38-54. In another embodiment, the PEST-like sequence has a sequence selected from SEQ ID NO: 14-20. In another embodiment, the PEST-like sequence has a sequence selected from SEQ ID NO: 14-23. In another embodiment, the PEST sequence is another PEST AA sequence derived from a prokaryotic organism.

[000166] 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.

[000167] 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.

[000168] 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 another embodiment, a PEST-like sequence must contain at least 1 P, 1 D or E, and at least 1 S or T.

[000169] 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 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 Dootlittle, RF. J. Mol. Biol. 157, 105 (1982).

[000170] 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 desired PEST score is obtained as combination of local enrichment term and hydrophobicity term as expressed by the following equation:

[000171] PEST score = 0.55 * DEPST - 0.5 * hydrophobicity index.

[000172] It will be appreciated that the terms "PEST sequence", "PEST-like sequence" or "PEST-like sequence peptide" can encompass peptides having a score of at least +5, using the above algorithm. 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 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 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 represents a separate embodiment of the present invention.

[000173] 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:

[000174] 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.

[000175] Each method for identifying a PEST-like sequence represents a separate embodiment of the present invention.

[000176] 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.

[000177] It will be appreciated that the term "Fusion to a PEST sequence" encompass fusion to a protein 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. It will also be appreciated that the term "fusion" encompasses fusion to two peptides or protein fragments either linked together at their respective ends or embedded one within the other.

[000178] In another embodiment, provided herein is a vaccine comprising a recombinant form of *Listeria* of the present invention.

[000179] In another embodiment, provided herein, is a culture of a recombinant form of *Listeria* of the present invention.

[000180] 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.

[000181] In one embodiment, attenuated *Listeria* strains, such as LM ΔactA mutant, *L. monocytogenes* ΔplcA, or ΔActA, ΔINL-b, ΔINL-c 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 and mutant for the formation of lipoteichoic acids and those attenuated by a lack of a virulence gene (see examples herein).

[000182] 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.

[000183] 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.

[000184] Another embodiment is a plasmid such as pCR2.1 (Invitrogen, La Jolla, CA), which 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.

[000185] Such methods are well known in the art, and are described in, for example, Sambrook 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).

[000186] Antibiotic resistance genes are used in the conventional selection and cloning 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, cloramphenicol (CAT), neomycin, hygromycin, gentamicin and others well known in the art. Each gene represents a separate embodiment of the present invention.

[000187] Methods for transforming bacteria are well known in the art, and include calcium-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., 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 method represents a separate embodiment of the present invention.

[000188] 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.

[000189] It will be appreciated that the term "transforming," can be used identically with the term "transfecting," and refers to engineering a bacterial cell to take up a plasmid or other heterologous DNA molecule. It is also to be understood that the term "transforming" can refer to engineering a bacterial cell to express a gene of a plasmid or other heterologous DNA molecule.

[000190] 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 5 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.

[000191] 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.

[000192] 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.

[000193] In another embodiment, chemical synthesis is used to produce a single stranded oligonucleotide. This single stranded oligonucleotide is converted, in various embodiments, 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 using appropriate restriction enzymes. The fragments are then be ligated to produce the desired DNA sequence.

[000194] 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 comprising a suitable restriction site and an antisense primer comprising another restriction site, e.g. a non-identical restriction site to facilitate cloning.

[000195] 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 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.

[000196] 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.

[000197] 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.

[000198] 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.

[000199] The skilled artisan will appreciate that, in another embodiment, other auxotroph strains and complementation systems are adopted for the use with this invention.

[000200] 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 attenuated recombinant form of *Listeria* of the present invention.

[000201] 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.

[000202] 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.

[000203] In another embodiment, the immune response elicited by methods and compositions of the present invention 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.

[000204] In another embodiment, the immune response elicited by methods and compositions of the present invention comprises an innate immune response. In another embodiment, the immune response consists primarily of an innate immune response. In another embodiment, the only detectable component of the immune response is a innate immune response. It is to be

understood that the activation of an innate immune response involves the activation of macrophages such as M1 macrophages and also of dendritic cells (DC).

[000205] In another embodiment, the present invention provides a method of reducing an incidence of cancer or infectious disease or allergy, comprising administering a composition of the present invention. In another embodiment, the present invention provides a method of ameliorating cancer or infectious disease or allergy, comprising administering a composition of the present invention. Each possibility represents a separate embodiment of the present invention.

[000206] 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.

[000207] 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. In another embodiment, the vaccines of the present invention are used to treat people having 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. In another embodiment, the vaccines of the present invention are used prior to an alternative treatment in people having 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. In another embodiment, such treatments include chemotherapy, surgery, radiation, and the like. Prior to such treatments, the vaccines of the present invention are administered so that the 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.

[000208] In another embodiment, the vaccines and immunogenic compositions utilized in any 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.

[000209] 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 LD_{50}/dose. In another embodiment, the dosage is from about 0.4-4.9 LD_{50}/dose. In another embodiment the dosage is from about 0.5-0.59 LD_{50}/dose. In another embodiment the dosage is from about 0.6-0.69 LD_{50}/dose. In another embodiment the dosage is from about 0.7-0.79 LD_{50}/dose. In another embodiment the dosage is about 0.8 LD_{50}/dose. In another embodiment, the dosage is 0.4 LD_{50}/dose to 0.8 of the LD_{50}/dose.

[000210] In another embodiment, the dosage is 10^7 bacteria/dose. In another embodiment, the dosage is 1.5 x 10^7 bacteria/dose. In another embodiment, the dosage is 2 x 10^7 bacteria/dose. In another embodiment, the dosage is 3 x 10^7 bacteria/dose. In another embodiment, the dosage is 4 x 10^7 bacteria/dose. In another embodiment, the dosage is 6 x 10^7 bacteria/dose. In another embodiment, the dosage is 8 x 10^7 bacteria/dose. In another embodiment, the dosage is 1 x 10^8 bacteria/dose. In another embodiment, the dosage is 1.5 x 10^8 bacteria/dose. In another embodiment, the dosage is 2 x 10^8 bacteria/dose. In another embodiment, the dosage is 3 x 10^8 bacteria/dose. In another embodiment, the dosage is 4 x 10^8 bacteria/dose. In another embodiment, the dosage is 6 x 10^8 bacteria/dose. In another embodiment, the dosage is 8 x 10^8 bacteria/dose. In another embodiment, the dosage is 1 x 10^9 bacteria/dose. In another embodiment, the dosage is 1.5 x 10^9 bacteria/dose. In another embodiment, the dosage is 2 x 10^9 bacteria/dose. In another embodiment, the dosage is 3 x 10^9 bacteria/dose. In another embodiment, the dosage is 5 x 10^9 bacteria/dose. In another embodiment, the dosage is 6 x 10^9 bacteria/dose. In another embodiment, the dosage is 8 x 10^9 bacteria/dose. In another embodiment, the dosage is 1 x 10^{10} bacteria/dose. In another embodiment, the dosage is 1.5 x 10^{10} bacteria/dose. In another embodiment, the dosage is 2 x 10^{10} bacteria/dose. In another embodiment, the dosage is 3 x 10^{10} bacteria/dose. In another embodiment, the dosage is 5 x 10^{10} bacteria/dose. In another embodiment, the dosage is 6 x 10^{10} bacteria/dose. In another embodiment, the dosage is 8 x 10^{10} bacteria/dose. In another embodiment, the dosage is 8 x 10^9 bacteria/dose. In another embodiment, the dosage is 1 x 10^{11} bacteria/dose. In another

embodiment, the dosage is 1.5 x 10^{11} bacteria/dose. In another embodiment, the dosage is 2 x 10^{11} bacteria/dose. In another embodiment, the dosage is 3 x 10^{11} bacteria/dose. In another embodiment, the dosage is 5 x 10^{11} bacteria/dose. In another embodiment, the dosage is 6 x 10^{11} bacteria/dose. In another embodiment, the dosage is 8 x 10^{11} bacteria/dose. Each possibility represents a separate embodiment of the present invention.

[000211] 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.

[000212] 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 therapy. The additional therapy can be antibiotic-mediated therapy for infectious diseases, or chemotherapy, immunotherapy, radiation, or surgery for cancer, or any other type of disease therapy available in the art as will be understood by a skilled artisan. Each possibility represents a separate embodiment of the present invention.

[000213] 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, phosphotidylinositol-specific phospholipase, phospholipase C, and metalloprotease, respectively. Each possibility represents a separate embodiment of the present invention.

[000214] 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.

[000215] 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.

[000216] 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.

[000217] In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-76 of greater than 60%. In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-76 of greater than 70%. In another embodiment, the identity is greater than 75%. In another embodiment, the identity is greater than 78%. In another embodiment, the identity is greater than 80%. In another embodiment, the identity is greater than 82%. In another embodiment, the identity is greater than 83%. In another embodiment, the identity is greater than 85%. In another embodiment, the identity is greater than 87%. In another embodiment, the identity is greater than 88%. In another embodiment, the identity is greater than 90%. In another embodiment, the identity is greater than 92%. In another embodiment, the identity is greater than 93%. In another embodiment, the identity is greater than 95%. In another embodiment, the identity is greater than 96%. In another embodiment, the identity is greater than 97%. In another embodiment, the identity is greater than 98%. In another embodiment, the identity is greater than 99%. In another embodiment, the identity is 100%. Each possibility represents a separate embodiment of the present invention.

[000218] 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.

[000219] 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.

[000220] 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.

[000221] It will be well appreciated that the terms "contacting" or "administering," can encompass directly contacting the cancer cell, tumor, or site of disease with a composition of the present invention. In another embodiment, the terms refer to indirectly contacting the cancer cell, tumor, or site of disease 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 invention after which the composition is brought in contact with the cancer cell, tumor, or site of disease 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.

[000222] In another embodiment, the terms "gene" and "recombinant gene" refer to nucleic 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 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.

[000223] The pharmaceutical compositions containing vaccines and compositions of the 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-peritonealy, intra-ventricularly, intra-cranially, intra-vaginally or intra-tumorally.

[000224] In another embodiment of the methods and compositions provided herein, the 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 addition to the active compound and the inert carrier or diluent, a gelatin capsule.

[000225] 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 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.

[000226] It will be appreciated that the term "treating" can encompass curing a disease, preventing a disease, reducing the incidence of a disease, ameliorating symptoms of a disease, inducing remission of a disease, slowing the progression of a disease. The terms "reducing", "suppressing" and "inhibiting" refer in another embodiment to lessening or decreasing.

[000227] It will be well appreciated that the term "therapeutically effective dose" or "therapeutic effective amount" can encompass a dose that produces the desired effect for which it is administered. The exact dose will be ascertainable by one skilled in the art using known techniques.

[000228] It will be well appreciated that the term "about" can encompass 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%.

[000229] It will be well appreciated that the term "subject" can encompass a mammal including a human in need of therapy for, or susceptible to, a condition or its sequelae, and also 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.

[000230] 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 broad scope of the invention.

EXAMPLES

EXAMPLE 1: LLO-ANTIGEN FUSIONS INDUCE ANTI-TUMOR IMMUNITY

MATERIALS AND EXPERIMENTAL METHODS (EXAMPLES 1-2)

Cell lines

[000231] The C57BL/6 syngeneic TC-1 tumor was immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. TC-1, provided by T. C. Wu (Johns Hopkins University School of Medicine, Baltimore, MD) is a highly tumorigenic lung epithelial cell expressing low levels of with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. TC-1 was grown in RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100

μM nonessential amino acids, 1 mM sodium pyruvate, 50 micromolar (mcM) 2-ME, 400 microgram (mcg)/ml G418, and 10% National Collection Type Culture-109 medium at 37° with 10% CO_2. C3 is a mouse embryo cell from C57BL/6 mice immortalized with the complete genome of HPV 16 and transformed with pEJ-ras. EL-4/E7 is the thymoma EL-4 retrovirally transduced with E7.

L. monocytogenes strains and propagation

[000232] Listeria strains used were Lm-LLO-E7 (hly-E7 fusion gene in an episomal expression system; Figure 1A), Lm-E7 (single-copy E7 gene cassette integrated into Listeria genome), Lm-LLO-NP ("DP-L2028"; hly-NP fusion gene in an episomal expression system), and Lm-Gag ("ZY-18"; single-copy HIV-1 Gag gene cassette integrated into the chromosome). E7 was amplified by PCR using the primers 5'-GGCTCGAGCATGGAGATACACC-3' (SEQ ID No: 24; XhoI site is underlined) and 5'-GGGGACTAGTTTATGGTTTCTGAGAACA-3' (SEQ ID No: 25; SpeI site is underlined) and ligated into pCR2.1 (Invitrogen, San Diego, CA). E7 was excised from pCR2.1 by XhoI/ SpeI digestion and ligated into pGG-55. The hly-E7 fusion gene and the pluripotential transcription factor prfA were cloned into pAM401, a multicopy shuttle plasmid (Wirth R et al, J Bacteriol, 165: 831, 1986), generating pGG-55. The hly promoter drives the expression of the first 441 AA of the hly gene product, (lacking the hemolytic C-terminus, referred to below as "ΔLLO"), which is joined by the XhoI site to the E7 gene, yielding a hly-E7 fusion gene that is transcribed and secreted as LLO-E7. Transformation of a prfA negative strain of Listeria, XFL-7 (provided by Dr. Hao Shen, University of Pennsylvania), with pGG-55 selected for the retention of the plasmid *in vivo* (Figures 1A-B). The hly promoter and gene fragment were generated using primers 5'-GGGGGCTAGCCCTCCTTTGATTAGTATATTC-3' (SEQ ID No: 26; NheI site is underlined) and 5'-CTCCCTCGAGATCATAATTTACTTCATC-3' (SEQ ID No: 27; XhoI site is underlined). The prfA gene was PCR amplified using primers 5'-GACTACAAGGACGATGACCGACAAGTGATAACCCGGGATCTAAATAAATCCGTTT-3' (SEQ ID No: 28; XbaI site is underlined) and 5'-CCCGTCGACCAGCTCTTCTTGGTGAAG-3' (SEQ ID No: 29; SalI site is underlined). Lm-E7 was generated by introducing an expression cassette containing the hly promoter and signal sequence driving the expression and secretion of E7 into the orfZ domain of the LM genome. E7 was amplified by PCR using the primers 5'-GCGGATCCCATGGAGATACACCTAC-3' (SEQ ID No: 30; BamHI site is underlined) and 5'-GCTCTAGATTATGGTTTCTGAG-3' (SEQ ID No: 31; XbaI site is underlined). E7 was then

ligated into the pZY-21 shuttle vector. LM strain 10403S was transformed with the resulting plasmid, pZY-21-E7, which includes an expression cassette inserted in the middle of a 1.6-kb sequence that corresponds to the orfX, Y, Z domain of the LM genome. The homology domain allows for insertion of the E7 gene cassette into the orfZ domain by homologous recombination. Clones were screened for integration of the E7 gene cassette into the orfZ domain. Bacteria were grown in brain heart infusion medium with (Lm-LLO-E7 and Lm-LLO-NP) or without (Lm-E7 and ZY-18) chloramphenicol (20 μg/ml). Bacteria were frozen in aliquots at -80°C. Expression was verified by Western blotting (Figure 2).

Western blotting

[000233] Listeria strains were grown in Luria-Bertoni medium at 37°C and were harvested at the same optical density measured at 600 nm. The supernatants were TCA precipitated and resuspended in 1x sample buffer supplemented with 0.1 N NaOH. Identical amounts of each cell pellet or each TCA-precipitated supernatant were loaded on 4–20% Tris-glycine SDS-PAGE gels (NOVEX, San Diego, CA). The gels were transferred to polyvinylidene difluoride and probed with an anti-E7 monoclonal antibody (mAb) (Zymed Laboratories, South San Francisco, CA), then incubated with HRP-conjugated anti-mouse secondary Ab (Amersham Pharmacia Biotech, Little Chalfont, U.K.), developed with Amersham ECL detection reagents, and exposed to Hyperfilm (Amersham Pharmacia Biotech).

Measurement of tumor growth

[000234] Tumors were measured every other day with calipers spanning the shortest and longest surface diameters. The mean of these two measurements was plotted as the mean tumor diameter in millimeters against various time points. Mice were sacrificed when the tumor diameter reached 20 mm. Tumor measurements for each time point are shown only for surviving mice.

Effects of Listeria recombinants on established tumor growth

[000235] Six- to 8-wk-old C57BL/6 mice (Charles River) received 2 x 10^5 TC-1 cells s.c. on the left flank. One week following tumor inoculation, the tumors had reached a palpable size of 4–5 mm in diameter. Groups of eight mice were then treated with 0.1 LD_{50} i.p. Lm-LLO-E7 (10^7 CFU), Lm- E7 (10^6 CFU), Lm-LLO-NP (10^7 CFU), or Lm-Gag (5 x 10^5 CFU) on days 7 and 14.

^{51}Cr release assay

[000236] C57BL/6 mice, 6–8 wk old, were immunized i.p. with $0.1LD_{50}$ Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag. Ten days post-immunization, spleens were harvested. Splenocytes were established in culture with irradiated TC-1 cells (100:1, splenocytes:TC-1) as feeder cells; stimulated *in vitro* for 5 days, then used in a standard ^{51}Cr release assay, using the following targets: EL-4, EL-4/E7, or EL-4 pulsed with E7 H-2b peptide (RAHYNIVTF) (SEQ ID NO:32). E:T cell ratios, performed in triplicate, were 80:1, 40:1, 20:1, 10:1, 5:1, and 2.5:1. Following a 4-h incubation at 37°C, cells were pelleted, and 50 µl supernatant was removed from each well. Samples were assayed with a Wallac 1450 scintillation counter (Gaithersburg, MD). The percent specific lysis was determined as [(experimental counts per minute (cpm)- spontaneous cpm)/(total cpm - spontaneous cpm)] x 100.

TC-1-specific proliferation

[000237] C57BL/6 mice were immunized with 0.1 LD_{50} and boosted by i.p. injection 20 days later with 1 LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag. Six days after boosting, spleens were harvested from immunized and naive mice. Splenocytes were established in culture at 5 x 10^5/well in flat-bottom 96-well plates with 2.5 x 10^4, 1.25 x 10^4, 6 x 10^3, or 3 x 10^3 irradiated TC-1 cells/well as a source of E7 Ag, or without TC-1 cells or with 10 µg/ml Con A. Cells were pulsed 45 h later with 0.5 µCi [^{3}H]thymidine/well. Plates were harvested 18 h later using a Tomtec harvester 96 (Orange, CT), and proliferation was assessed with a Wallac 1450 scintillation counter. The change in cpm was calculated as experimental cpm - no Ag cpm.

Flow cytometric analysis

[000238] C57BL/6 mice were immunized intravenously (i.v.) with 0.1 LD_{50} Lm-LLO-E7 or Lm-E7 and boosted 30 days later. Three-color flow cytometry for CD8 (53-6.7, PE conjugated), CD62 ligand (CD62L; MEL-14, APC conjugated), and E7 H-2Db tetramer was performed using a FACSCalibur® flow cytometer with CellQuest® software (Becton Dickinson, Mountain View, CA). Splenocytes harvested 5 days after the boost were stained at room temperature (rt) with H-2Db tetramers loaded with the E7 peptide (RAHYNIVTF) (SEQ ID NO:32) or a control (HIV-Gag) peptide. Tetramers were used at a 1/200 dilution and were provided by Dr. Larry R. Pease (Mayo Clinic, Rochester, MN) and by the NIAID Tetramer Core Facility and the NIH AIDS Research and Reference Reagent Program. Tetramer$^+$, CD8$^+$, CD62L^{low} cells were analyzed.

B16F0-Ova experiment

[000239] 24 C57BL/6 mice were inoculated with 5 x 10^5 B16F0-Ova cells. On days 3, 10 and 17, groups of 8 mice were immunized with 0.1 LD_{50} Lm-OVA (10^6 cfu), Lm-LLO-OVA (10^8 cfu) and eight animals were left untreated.

Statistics

[000240] For comparisons of tumor diameters, mean and SD of tumor size for each group were determined, and statistical significance was determined by Student's t test. $p \leq 0.05$ was considered significant.

RESULTS

[000241] Lm-E7 and Lm-LLO-E7 were compared for their abilities to impact on TC-1 growth. Subcutaneous tumors were established on the left flank of C57BL/6 mice. Seven days later tumors had reached a palpable size (4–5 mm). Mice were vaccinated on days 7 and 14 with 0.1 LD_{50} Lm-E7, Lm-LLO-E7, or, as controls, Lm-Gag and Lm-LLO-NP. Lm-LLO-E7 induced complete regression of 75% of established TC-1 tumors, while tumor growth was controlled in the other 2 mice in the group (Figure 3). By contrast, immunization with Lm-E7 and Lm-Gag did not induce tumor regression. This experiment was repeated multiple times, always with very similar results. In addition, similar results were achieved for Lm-LLO-E7 under different immunization protocols. In another experiment, a single immunization was able to cure mice of established 5 mm TC-1 tumors.

[000242] In other experiments, similar results were obtained with 2 other E7-expressing tumor cell lines: C3 and EL-4/E7. To confirm the efficacy of vaccination with Lm-LLO-E7, animals that had eliminated their tumors were re-challenged with TC-1 or EL-4/E7 tumor cells on day 60 or day 40, respectively. Animals immunized with Lm-LLO-E7 remained tumor free until termination of the experiment (day 124 in the case of TC-1 and day 54 for EL-4/E7).

[000243] Thus, expression of an antigen as a fusion protein with ΔLLO enhances the immunogenicity of the antigen.

EXAMPLE 2: LM-LLO-E7 TREATMENT ELICITS TC-1 SPECIFIC SPLENOCYTE PROLIFERATION

[000244] To measure induction of T cells by Lm-E7 with Lm-LLO-E7, E7-specific proliferative responses, a measure of antigen-specific immunocompetence, were measured in immunized mice. Splenocytes from Lm-LLO-E7-immunized mice proliferated when exposed to irradiated TC-1 cells as a source of E7, at splenocyte: TC-1 ratios of 20:1, 40:1, 80:1, and 160:1 (Figure 4). Conversely, splenocytes from Lm-E7 and rLm control-immunized mice exhibited only background levels of proliferation.

EXAMPLE 3: ActA-E7 and PEST-E7 FUSIONS CONFER ANTI-TUMOR IMMUNITY

MATERIALS AND EXPERIMENTAL METHODS

Construction of Lm-ActA-E7

[000245] Lm-ActA-E7 is a recombinant strain of LM, comprising a plasmid that expresses the E7 protein fused to a truncated version of the actA protein. Lm-actA-E7 was generated by introducing a plasmid vector pDD-1, constructed by modifying pDP-2028, into Listeria. pDD-1 comprises an expression cassette expressing a copy of the 310 bp hly promoter and the hly signal sequence (ss), which drives the expression and secretion of ActA-E7; 1170 bp of the actA gene that comprises four PEST sequences (SEQ ID NO: 11) (the truncated ActA polypeptide consists of the first 390 AA of the molecule, SEQ ID NO: 10); the 300 bp HPV E7 gene; the 1019 bp prfA gene (controls expression of the virulence genes); and the CAT gene (chloramphenicol resistance gene) for selection of transformed bacteria clones (Sewell et al. (2004), Arch. Otolaryngol. Head Neck Surg., 130: 92-97).

[000246] The hly promoter (pHly) and gene fragment were PCR amplified from pGG55 (Example 1) using primer 5'-GGGGTCTAGACCTCCTTTGATTAGTATATTC-3' (Xba I site is underlined; SEQ ID NO: 33) and primer 5'-ATCTTCGCTATCTGTCGCCGCGGCGCGTGCTTCAGTTTGTTGCGC-'3 (Not I site is underlined. The first 18 nucleotides are the ActA gene overlap; SEQ ID NO: 34). The actA gene was PCR amplified from the LM 10403s wildtype genome using primer 5'-GCGCAACAAACTGAAGCAGCGGCCGCGGCGACAGATAGCGAAGAT-3' (NotI site is

underlined; SEQ ID NO: 35) and primer 5'-TGTAGGTGTATCTCCATGCTCGAGAGCTAGGCGATCAATTTC-3' (XhoI site is underlined; SEQ ID NO: 36). The E7 gene was PCR amplified from pGG55 (pLLO-E7) using primer 5'-GGAATTGATCGCCTAGCTCTCGAGCATGGAGATACACCTACA-3' (XhoI site is underlined; SEQ ID NO: 37) and primer 5'-AAACGGATTTATTTAGATCCCGGGTTATGGTTTCTGAGAACA-3' (XmaI site is underlined; SEQ ID NO: 38). The prfA gene was PCR amplified from the LM 10403s wild-type genome using primer 5'-TGTTCTCAGAAACCATAACCCGGGATCTAAATAAATCCGTTT-3' (XmaI site is underlined; SEQ ID NO: 39) and primer 5'-GGGGGTCGACCAGCTCTTCTTGGTGAAG-3' (SalI site is underlined; SEQ ID NO: 40). The hly promoter- actA gene fusion (pHly-actA) was PCR generated and amplified from purified pHly DNA and purified actA DNA using the upstream pHly primer (SEQ ID NO: 33) and downstream actA primer (SEQ ID NO: 36).

[000247] The E7 gene fused to the prfA gene (E7-prfA) was PCR generated and amplified from purified E7 DNA and purified prfA DNA using the upstream E7 primer (SEQ ID NO: 37) and downstream prfA gene primer (SEQ ID NO: 40).

[000248] The pHly-actA fusion product fused to the E7-prfA fusion product was PCR generated and amplified from purified fused pHly-actA DNA product and purified fused E7-prfA DNA product using the upstream pHly primer (SEQ ID NO: 33) and downstream prfA gene primer (SEQ ID NO: 40) and ligated into pCRII (Invitrogen, La Jolla, Calif.). Competent *E. coli* (TOP10'F, Invitrogen, La Jolla, Calif.) were transformed with pCRII-ActAE7. After lysis and isolation, the plasmid was screened by restriction analysis using BamHI (expected fragment sizes 770 bp and 6400 bp (or when the insert was reversed into the vector: 2500 bp and 4100 bp)) and BstXI (expected fragment sizes 2800 bp and 3900 bp) and also screened with PCR analysis using the upstream pHly primer (SEQ ID NO: 33) and the downstream prfA gene primer (SEQ ID NO: 40).

[000249] The pHly-actA-E7-prfA DNA insert was excised from pCRII by double digestion with Xba I and Sal I and ligated into pDP-2028 also digested with Xba I and Sal I. After transforming TOP10'F competent *E. coli* (Invitrogen, La Jolla, Calif.) with expression system pActAE7, chloramphenicol resistant clones were screened by PCR analysis using the upstream pHly primer

(SEQ ID NO: 33) and the downstream PrfA gene primer (SEQ ID NO: 40). A clone comprising pActAE7 was grown in brain heart infusion medium (with chloramphenicol (20 mcg (microgram)/ml (milliliter), Difco, Detroit, Mich.) and pActAE7 was isolated from the bacteria cell using a midiprep DNA purification system kit (Promega, Madison, Wis.). A prfA-negative strain of penicillin-treated Listeria (strain XFL-7) was transformed with expression system pActAE7, as described in Ikonomidis et al. (1994, J. Exp. Med. 180: 2209-2218) and clones were selected for the retention of the plasmid *in vivo*. Clones were grown in brain heart infusion with chloramphenicol (20 mcg/ml) at 37 °C. Bacteria were frozen in aliquots at -80 °C.

Immunoblot Verification of Antigen Expression

[000250] To verify that Lm-ActA-E7 secretes ActA-E7, (about 64 kD), *Listeria* strains were grown in Luria-Bertoni (LB) medium at 37°C. Protein was precipitated from the culture supernatant with trichloroacetic acid (TCA) and resuspended in 1x sample buffer with 0.1N sodium hydroxide. Identical amounts of each TCA precipitated supernatant were loaded on 4% to 20% Tris-glycine sodium dodecyl sulfate–polyacrylamide gels (NOVEX, San Diego, Calif). Gels were transferred to polyvinylidene difluoride membranes and probed with 1:2500 anti-E7 monoclonal antibody (Zymed Laboratories, South San Francisco, Calif), then with 1:5000 horseradish peroxidase–conjugated anti-mouse IgG (Amersham Pharmacia Biotech, Little Chalfont, England). Blots were developed with Amersham enhanced chemiluminescence detection reagents and exposed to autoradiography film (Amersham) (Figure 5A).

Construction of Lm-PEST-E7, Lm-ΔPEST-E7, and Lm-E7epi (Figure 6A)

[000251] Lm-PEST-E7 is identical to Lm-LLO-E7, except that it contains only the promoter and PEST sequence of the hly gene, specifically the first 50 AA of LLO. To construct Lm-PEST-E7, the hly promoter and PEST regions were fused to the full-length E7 gene using the SOE (gene splicing by overlap extension) PCR technique. The E7 gene and the hly-PEST gene fragment were amplified from the plasmid pGG-55, which contains the first 441 AA of LLO, and spliced together by conventional PCR techniques. To create a final plasmid, pVS16.5, the hly-PEST-E7 fragment and the prfA gene were subcloned into the plasmid pAM401, which includes a chloramphenicol resistance gene for selection *in vitro*, and the resultant plasmid was used to transform XFL-7.

[000252] Lm-ΔPEST-E7 is a recombinant Listeria strain that is identical to Lm- LLO-E7 except that it lacks the PEST sequence. It was made essentially as described for Lm-PEST-E7, except that the episomal expression system was constructed using primers designed to remove the PEST-containing region (bp 333–387) from the hly-E7 fusion gene. Lm-E7epi is a recombinant strain that secretes E7 without the PEST region or LLO. The plasmid used to transform this strain contains a gene fragment of the hly promoter and signal sequence fused to the E7 gene. This construct differs from the original Lm-E7, which expressed a single copy of the E7 gene integrated into the chromosome. Lm-E7epi is completely isogenic to Lm- LLO-E7, Lm-PEST-E7, and Lm-ΔPEST-E7 except for the form of the E7 antigen expressed.

RESULTS

[000253] To compare the anti-tumor immunity induced by Lm-ActA-E7 versus Lm-LLO-E7, 2 x 10^5 TC-1 tumor cells were implanted subcutaneously in mice and allowed to grow to a palpable size (approximately 5 millimeters [mm]). Mice were immunized i.p. with one LD_{50} of either Lm-ActA-E7 (5 x10^8 CFU), (crosses) Lm-LLO-E7 (10^8 CFU) (squares) or Lm-E7 (10^6 CFU) (circles) on days 7 and 14. By day 26, all of the animals in the Lm-LLO-E7 and Lm-ActA-E7 were tumor free and remained so, whereas all of the naive animals (triangles) and the animals immunized with Lm-E7 grew large tumors (Figure 5B). Thus, vaccination with ActA-E7 fusions causes tumor regression.

[000254] In addition, Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, and Lm-E7epi were compared for their ability to cause regression of E7-expressing tumors. S.c. TC-1 tumors were established on the left flank of 40 C57BL/6 mice. After tumors had reached 4-5 mm, mice were divided into 5 groups of 8 mice. Each groups was treated with 1 of 4 recombinant LM vaccines, and 1 group was left untreated. Lm-LLO-E7 and Lm-PEST-E7 induced regression of established tumors in 5/8 and 3/8 cases, respectively. There was no statistical difference between the average tumor size of mice treated with Lm-PEST-E7 or Lm-LLO-E7 at any time point. However, the vaccines that expressed E7 without the PEST sequences, Lm-ΔPEST-E7 and Lm-E7epi, failed to cause tumor regression in all mice except one (Figure 6B, top panel). This was representative of 2 experiments, wherein a statistically significant difference in mean tumor sizes at day 28 was observed between tumors treated with Lm-LLO-E7 or Lm-PEST-E7 and those treated with Lm-E7epi or Lm-ΔPEST-E7; $P < 0.001$, Student's t test; Figure 6B, bottom panel). In addition,

increased percentages of tetramer-positive splenocytes were seen reproducibly over 3 experiments in the spleens of mice vaccinated with PEST-containing vaccines (Figure 6C). Thus, vaccination with PEST-E7 fusions causes tumor regression.

EXAMPLE 4: FUSION OF E7 TO LLO, ActA, OR A PEST-LIKE SEQUENCE ENHANCES E7-SPECIFIC IMMUNITY AND GENERATES TUMOR-INFILTRATING E7-SPECIFIC $CD8^+$ CELLS

MATERIALS AND EXPERIMENTAL METHODS

[000255] 500 mcl (microliter) of MATRIGEL®, comprising 100 mcl of 2 x 10^5 TC-1 tumor cells in phosphate buffered saline (PBS) plus 400 mcl of MATRIGEL® (BD Biosciences, Franklin Lakes, N.J.) were implanted subcutaneously on the left flank of 12 C57BL/6 mice (n=3). Mice were immunized intraperitoneally on day 7, 14 and 21, and spleens and tumors were harvested on day 28. Tumor MATRIGELs were removed from the mice and incubated at 4 °C overnight in tubes containing 2 milliliters (ml) of RP 10 medium on ice. Tumors were minced with forceps, cut into 2 mm blocks, and incubated at 37 °C for 1 hour with 3 ml of enzyme mixture (0.2 mg/ml collagenase-P, 1 mg/ml DNAse-1 in PBS). The tissue suspension was filtered through nylon mesh and washed with 5% fetal bovine serum + 0.05% of NaN_3 in PBS for tetramer and IFN-gamma staining.

[000256] Splenocytes and tumor cells were incubated with 1 micromole (mcm) E7 peptide for 5 hours in the presence of brefeldin A at 10^7 cells/ml. Cells were washed twice and incubated in 50 mcl of anti-mouse Fc receptor supernatant (2.4 G2) for 1 hour or overnight at 4 °C. Cells were stained for surface molecules CD8 and CD62L, permeabilized, fixed using the permeabilization kit Golgi-stop® or Golgi-Plug® (Pharmingen, San Diego, Calif.), and stained for IFN-gamma. 500,000 events were acquired using two-laser flow cytometer FACSCalibur and analyzed using Cellquest Software (Becton Dickinson, Franklin Lakes, NJ). Percentages of IFN-gamma secreting cells within the activated ($CD62L^{low}$) $CD8^+$ T cells were calculated.

[000257] For tetramer staining, H-$2D^b$ tetramer was loaded with phycoerythrin (PE)-conjugated E7 peptide (RAHYNIVTF, SEQ ID NO: 32), stained at rt for 1 hour, and stained with anti-allophycocyanin (APC) conjugated MEL-14 (CD62L) and FITC-conjugated CD8β at 4 °C for 30 min. Cells were analyzed comparing $tetramer^+CD8^+$ $CD62L^{low}$ cells in the spleen and in the

tumor.

RESULTS

[000258] To analyze the ability of Lm-ActA-E7 to enhance antigen specific immunity, mice were implanted with TC-1 tumor cells and immunized with either Lm-LLO-E7 (1×10^7 CFU), Lm-E7 (1×10^6 CFU), or Lm-ActA-E7 (2×10^8 CFU), or were untreated (naïve). Tumors of mice from the Lm-LLO-E7 and Lm-ActA-E7 groups contained a higher percentage of IFN-gamma-secreting $CD8^+$ T cells (Figure 7A) and tetramer-specific $CD8^+$ cells (Figure 7B) than in Lm-E7 or naive mice.

[000259] In another experiment, tumor-bearing mice were administered Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, or Lm-E7epi, and levels of E7-specific lymphocytes within the tumor were measured. Mice were treated on days 7 and 14 with 0.1 LD_{50} of the 4 vaccines. Tumors were harvested on day 21 and stained with antibodies to CD62L, CD8, and with the E7/Db tetramer. An increased percentage of tetramer-positive lymphocytes within the tumor were seen in mice vaccinated with Lm-LLO-E7 and Lm-PEST-E7 (Figure 8A). This result was reproducible over three experiments (Figure 8B).

[000260] Thus, Lm-LLO-E7, Lm-ActA-E7, and Lm-PEST-E7 are each efficacious at induction of tumor-infiltrating $CD8^+$ T cells and tumor regression.

MATERIALS AND EXPERIMENTAL METHODS (SEE EXAMPLES 5-10)

Bacterial strains, transformation and selection

[000261] *E. coli* strain MB2159 was used for transformations, using standard protocols. Bacterial cells were prepared for electroporation by washing with H_2O.

[000262] *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.

Plasmid Constructions

[000263] 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 SalI- and XbaI-generated DNA ends to generate pDP1462.

[000264] 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 XbaI and PstI, treatment of the DNA ends with T4 DNA polymerase to make them blunt, and intramolecular ligation.

[000265] Plasmid pDP1499, containing the plcA promoter and a portion of the 3' end of plcA, 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 PstI and NsiI and intramolecular ligation.

[000266] pDP1526 (pKSV7::ΔplcA) 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).

[000267] 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

[000268] *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 μg 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.

[000269] 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 μg chloramphenicol per ml of media. Each integration strain was subsequently colony purified on BHI agar containing 10 μg 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.

[000270] Construction of DP-L1552 is achieved by integration of the pKSV7 derivative, pDP1526, to generate a merodiploid intermediate 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 ΔplcA for the wild-type allele on the chromosome.

[000271] 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 ΔplcA 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. The correct plcA deletion on the *L. monocytogenes* chromosome was confirmed by amplifying the deleted allele using PCR and sequencing across the deletion.

[000272] 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.

Construction of Lmdd

[000273] 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 upstream and downstream of the gene) surrounding the desired deletion. PCR analysis was used to confirm the structure of this chromosomal deletion.

[000274] 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

[000275] *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

[000276] 1 x 10^8 CFU were injected intraperitoneally (i.p.) into C57BL/6 mice. On day three, spleens were isolated and homogenized in PBS. An aliquot of the spleen suspension was 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

[000277] **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: 41) (reverse primer: 5'-GCT AGC CTA ATG GAT GTA TTT TCT AGG-3'; SEQ ID NO: 42) and a minimal p60 promoter sequence (forward primer: 5'-TTA ATT AAC AAA TAG TTG GTA TAG TCC-3'; SEQ ID No: 43) (reverse primer: 5'-GAC GAT GCC AGC CTG TCA CCA TGG AAA ACT CCT CTC-3'; SEQ ID No: 44) were 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 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: CAAATAGTTGGTATAGTCCTCTTTAGCCTTTGGAGTATTATCTCATCATTTGTTTTTTA GGTGAAAACTGGGTAAACTTAGTATTATCAATATAAAATTAATTCTCAAATACTTAA TTACGTACTGGGATTTTCTGAAAAAAGAGAGGAGTTTTCC (SEQ ID NO: 45) (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) .

[000278] **Removal of antibiotic resistance genes from pGG55**. The subsequent cloning strategy for removing the Chloramphenicol acetyltransferase (CAT) genes from pGG55 and 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: 46) and a 3'-primer that added a NheI site downstream of the sequence (GCTAGCCAGCAAAGAAAAACAAACACG, SEQ ID NO: 47). The PCR product was cloned into cloning vector pCR2.1 and sequence verified.

[000279] 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 PCR (primer 1, 5'-GTC GAC GGT CAC CGG CGC CAC TAA CTC AAC GCT AGT AG-3'; SEQ ID No: 48); (primer 2, 5'-TTA ATT AAG CTA GCC AGC AAA GAA AAA CAA ACA CG-3'; SEQ ID No: 49) 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 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 9). 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.

Preparation of DNA for real-time PCR

[000280] 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.

[000281] In order to obtain standard target DNA for real-time PCR, the LLO-E7 gene was PCR amplified from pGG55 (5'-ATGAAAAAAATAATGCTAGTTTTTATTAC-3' (SEQ ID NO: 50); 5'-GCGGCCGCTTAATGATGATGATGATGATGTGGTTTCTG AGAACAGATG-3' (SEQ ID NO: 51)) 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.

Real-time PCR

[000282] 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: 52); 5'-TGCCCATTAACAGGTCTTCCA-3' (SEQ ID NO: 53); 5'-FAM-TGCGTA CAAAGCACACACGTAGACATTCGTAC-TAMRA-3' (SEQ ID NO: 54) and the one-copy gene plcA (TGACATCGTTTGTGTTTGAGCTAG -3' (SEQ ID NO: 55), 5'-GCAGCGCTCTCTATACCAGGTAC-3' (SEQ ID NO: 56); 5'-TET-TTAATGTCCATGTTA TGTCTCCGTTATAGCTCATCGTA-TAMRA-3'; SEQ ID NO: 57) as a *Listeria* genome target.

[000283] 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 $p < 0.05$.

Growth measurements

[000284] 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 OD_{600} nm measurements to be the same for all strains.

Hemolytic Lysis Assay

[000285] 4 x 10^9 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 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 dilution.

Therapeutic efficacy of Lmdd-Tv3

[000286] 10^5 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 activated ras, which forms tumors upon subcutaneous implantation. Mice were immunized with 0.1 LD_{50} 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.

Generation of an ActA deletion mutant

[000287] 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 Lm*daldat* (Lmdd) background was constructed to avoid any polar effects on the expression of downstream genes. The Lm *dal dat* Δ*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: 58) and 4 (Adv304-ctaccatgtcttccgttgcttg) (SEQ ID NO: 59) as shown in the Fig. 12. The PCR analysis was performed on the chromosomal DNA isolated from Lmdd and Lm-ddΔ*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-ddΔ*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. 12 confirms that 1.8 kb region of *actA* was deleted in the strain, Lm-ddΔ*actA*. DNA sequencing was also performed on PCR products to confirm the deletion of *actA* containing region in the strain, Lm-ddΔ*actA* (Figure 13).

[000288] gcgccaaatcattggttgattggtgaggatgtctgtgtgcgtgggtcgcgagatgggcgaataagaagcattaaagatcctgaca aatataatcaagcggctcatatgaaagattacgaatcgcttccactcacagaggaaggcgactggggcggagttcattataatagtggtatcc cgaataaagcagcctataatactatcactaaacttggaaaagaaaaaacagaacagctttattttcgcgccttaaagtactatttaacgaaaaaat cccagtttaccgatgcgaaaaaagcgcttcaacaagcagcgaaagatttatatggtgaagatgcttctaaaaaagttgctgaagcttgggaag cagttggggttaactgattaacaaatgttagagaaaaattaattctccaagtgatattcttaaaataattcatgaatatttttttcttatattagctaatta agaagataactaactgctaatccaatttttaacggaacaaattagtgaaaatgaaggccgaattttccttgttctaaaaaggttgtattagcgtatc acgaggagggagtataa*gtgggattaaacagatttatgcgtgcgatgatggtggttttcattactgccaattgcattacgattaaccccgac* ***gtcgac****ccatacgacgttaatcttgcaatgttagctattggcgtgttctctttaggggcgtttatcaaaattattcaattaagaaaaaataatt* *a*aaaacacagaacgaaagaaaaagtgaggtgaatgatatgaaattcaaaaaggtggttctaggtatgtgcttgatcgcaagtgttctagtcttt ccggtaacgataaaagcaaatgcctgttgtgatgaatacttacaaacacccgcagctccgcatgatattgacagcaaattaccacataaactta gttggtccgcggataacccgacaaatactgacgtaaatacgcactattggctttttaaacaagcggaaaaaatactagctaaagatgtaaatca tatgcgagctaatttaatgaatgaacttaaaaaattcgataaacaaatagctcaaggaatatatgatgcggatcataaaaatccatattatgatact agtacatttttatctcatttttataatcctgatagagataatacttatttgccgggttttgctaatgcgaaaataacaggagcaaagtatttcaatcaat cggtgactgattaccgagaagggaa (SEQ ID NO: 60).

Production of Inflammatory cytokines:

[000289] Macrophages such as RAW 264.7 are infected with different *Listeria* backbones such as *Lm* prfA- (pGG55), 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:

[000290] To measure the *in vivo* cytokine production and recruitment of neutrophils, C57BL/6 mice are injected intraperitoneally with different 10^8 CFU of *Lm* prfA- (pGG55), Lm *dal dat*, Lm *dal dat actA,* Lm *dal dat actA* Δ *inlC* and Lm *dal dat* Δ *inlC*, *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:

[000291] 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 standardized 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:

[000292] 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 LmddA*inlC*142 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 for the assessment of therapeutic effects and immunogenicity. -

Impact on tumor microenvironment:

[000293] The ability of LmddA, LmddAΔactA, LmddAΔPlcA, LmddAΔPlcB, LmddAΔprfA, LmddA*inlC*142, 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 x 10^6 TPSA23 tumor cells on day 0 and are vaccinated on day 7, 14 and 21 with 10^8 CFU of LmddA*inlC*142, 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 ($Gr\text{-}1^+$ $CD11b^+$), myeloid derived

suppressor cells (MDSCs) ($Gr\text{-}1^+CD11b^+$), 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.

RESULTS

EXAMPLE 5: 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

[000294] 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 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 9). DNA was purified from several colonies.

EXAMPLE 6: PLASMIDS CONTAINING A METABOLIC ENZYME DO NOT INCREASE THE VIRULENCE OF BACTERIA

[000295] 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.

[000296] *In vivo* virulence was also measured by determining LD_{50} values, a more direct, and therefore accurate, means of measuring virulence. The LD_{50} 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.

EXAMPLE 7: INDUCTION OF ANTI-TUMOR IMMUNITY BY PLASMIDS CONTAINING A METABOLIC ENZYME

[000297] 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 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 14) with no statistically significant difference ($p < 0.05$) between vaccinated groups. All immunized mice were still alive after 63 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.

[000298] 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.

EXAMPLE 8: *inlC*-DELETION MUTANT GENERATE SIGNIFICANTLY HIGH LEVELS OF THE CHEMOKINES AND CYTOKINES.

[000299] *inlC* deletion mutant generates significantly high levels of the chemokines such as MIP-1α, 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 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 9: *inlC*-DELETION MUTANTS INDUCE NEUTROPHIL MIGRATION

[000300] 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 10: *inlC*-DELETION MUTANTS EFFECT A THERAPEUTIC ANTI-TUMOR RESPONSE

[000301] 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.

MATERIALS AND METHODS (EXAMPLES 11-16)

[000302] Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and DNA sequencing was done by Genewiz Inc, South Plainfield, NJ. Flow cytometry reagents were purchased from Becton Dickinson Biosciences (BD, San Diego, CA). Cell culture media, supplements and all other reagents, unless indicated, were from Sigma (St. Louise, MO). Her2/neu HLA-A2 peptides were synthesized by EZbiolabs (Westfield, IN). Complete RPMI 1640 (C-RPMI) medium contained 2mM glutamine, 0.1 mM non-essential amino acids, and 1mM sodium pyruvate, 10% fetal bovine serum, penicillin/streptomycin, Hepes (25mM). The polyclonal anti-LLO antibody was described previously and anti-Her2/neu antibody was purchased from Sigma.

Mice and Cell Lines

[000303] All animal experiments were performed according to approved protocols by IACUC at the University of Pennsylvania or Rutgers University. FVB/N mice were purchased from Jackson laboratories (Bar Harbor, ME). The FVB/N Her2/neu transgenic mice, which overexpress the rat Her2/neu onco-protein were housed and bred at the animal core facility at the University of Pennsylvania. The NT-2 tumor cell line expresses high levels of rat Her2/neu protein, was derived from a spontaneous mammary tumor in these mice and grown as described previously. DHFR-G8 (3T3/neu) cells were obtained from ATCC and were grown according to the ATCC recommendations. The EMT6-Luc cell line was a generous gift from Dr. John Ohlfest (University of Minnesota, MN) and was grown in complete C-RPMI medium. Bioluminescent work was conducted under guidance by the Small Animal Imaging Facility (SAIF) at the University of Pennsylvania (Philadelphia, PA).

***Listeria* constructs and antigen expression**

[000304] Her2/neu-pGEM7Z was kindly provided by Dr. Mark Greene at the University of Pennsylvania and contained the full-length human Her2/neu (hHer2) gene cloned into the pGEM7Z plasmid (Promega, Madison WI). This plasmid was used as a template to amplify three segments of hHer-2/neu, namely, EC1, EC2, and IC1, by PCR using pfx DNA polymerase (Invitrogen) and the oligos indicated in Table 1.

[000305] Table 1: Primers for cloning of Human her-2-Chimera

	DNA sequence	Base pair region	Amino acid region or junctions
Her-2-Chimera (F)	TGATCTCGAGACCCACCTGGACATGCTC (SEQ ID NO: 61)	120-510	40-170
HerEC1-EC2F (Junction)	CTACCAGGACACGATTTTGTGGAAG-AATATCCA GGAGTTTGCTGGCTGC (SEQ ID NO: 62)	510/1077	170/359
HerEC1-EC2R (Junction)	GCAGCCAGCAAACTCCTGGATATT-CTTCCACAA AATCGTGTCCTGGTAG (SEQ ID NO: 63)		
HerEC2-IC1F (Junction)	CTGCCACCAGCTGTGCGCCCGAGGG-CAGCAGAAGATCCGGAAGTACACGA (SEQ ID NO: 64)	1554/2034	518/679
HerEC2-IC1R (Junction)	TCGTGTACTTCCGGATCTTCTGCTG CCCTCGGGC GCACAGCTGGTGGCAG (SEQ ID NO: 65)		
Her-2-Chimera (R)	GTGGCCCGGGTCTAGATTAGTCTAAGAGGCAGCCATAGG (SEQ ID NO: 66)	2034-2424	679-808

[000306] The Her-2/neu chimera construct was generated by direct fusion by the SOEing PCR method and each separate hHer-2/neu segment as templates. Primers are shown in Table 2.

[000307]Sequence of primers for amplification of different segments human Her2 regions.

	DNA sequence	Base pair region	Amino acid region
Her-2-EC1(F)	CCGCCTCGAGGCCGCGAGCACCCAAGTG (SEQ ID NO: 67)	58-979	20-326
Her-2-EC1(R)	CGCGACTAGTTTAATCCTCTGCTGTCACCTC (SEQ ID NO: 68)		
Her-2-EC2(F)	CCGCCTCGAGTACCTTTCTACGGACGTG (SEQ ID NO: 69)	907-1504	303-501
Her- 2- EC2(R)	CGCGACTAGTTTACTCTGGCCGGTTGGCAG (SEQ ID NO: 70)		
Her-2- IC1(F)	CCGCCTCGAGCAGCAGAAGATCCGGAAGTAC (SEQ ID NO: 71)	2034-3243	679-1081
Her-2-IC1(R)	CGCGACTAGTTTAAGCCCCTTCGGAGGGTG (SEQ ID NO: 72)		

[000308] Sequence of primers for amplification of different segments human Her2 regions.

[000309] ChHer2 gene was excised from pAdv138 using XhoI and SpeI restriction enzymes, and cloned in frame with a truncated, non-hemolytic fragment of LLO in the *Lmdd* shuttle vector, pAdv134. The sequences of the insert, LLO and *hly* promoter were confirmed by DNA sequencing analysis. This plasmid was electroporated into electro-competent *actA, dal, dat* mutant *Listeria monocytogenes* strain, *LmddA* and positive clones were selected on Brain Heart infusion (BHI) agar plates containing streptomycin (250μg/ml). In some experiments similar *Listeria* strains expressing hHer2/neu (*Lm*-hHer2) fragments were used for comparative purposes. These have been previously described. In all studies, an irrelevant *Listeria* construct (*Lm*-control) was included to account for the antigen independent effects of *Listeria* on the immune system. *Lm*-controls were based on the same *Listeria* platform as ADXS31-164, but expressed a different antigen such as HPV16-E7 or NY-ESO-1. Expression and secretion of fusion proteins from *Listeria* were tested. Each construct was passaged twice *in vivo*.

Cytotoxicity assay

[000310] Groups of 3-5 FVB/N mice were immunized three times with one week intervals with 1 x 10^8 colony forming units (CFU) of *Lm*-LLO-ChHer2, ADXS31-164, *Lm*-hHer2 ICI or *Lm*-control (expressing an irrelevant antigen) or were left naïve. NT-2 cells were grown *in vitro*, detached by trypsin and treated with mitomycin C (250 μg/ml in serum free C-RPMI medium) at 37°C for 45 minutes. After 5 washes, they were co-incubated with splenocytes harvested from immunized or naïve animals at a ratio of 1:5 (Stimulator: Responder) for 5 days at 37°C and 5% CO_2. A standard cytotoxicity assay was performed using europium labeled 3T3/neu (DHFR-G8) cells as targets according to the method previously described. Released europium from killed target cells was measured after 4 hour incubation using a spectrophotometer (Perkin Elmer, Victor^2) at 590 nm. Percent specific lysis was defined as (lysis in experimental group-spontaneous lysis)/(Maximum lysis-spontaneous lysis).

Interferon-γ secretion by splenocytes from immunized mice

[000311] Groups of 3-5 FVB/N or HLA-A2 transgenic mice were immunized three times with one week intervals with 1 x 10^8 CFU of ADXS31-164, a negative *Listeria* control (expressing an irrelevant antigen) or were left naïve. Splenocytes from FVB/N mice were isolated one week after the last immunization and co-cultured in 24 well plates at 5 x 10^6 cells/well in the presence of mitomycin C treated NT-2 cells in C-RPMI medium. Splenocytes from the HLA-A2 transgenic mice were incubated in the presence of 1μM of HLA-A2 specific peptides or 1μg/ml of a recombinant His-tagged ChHer2 protein, produced in *E. coli* and purified by a nickel based affinity chromatography system. Samples from supernatants were obtained 24 or 72 hours later and tested for the presence of interferon-γ (IFN-γ) using mouse IFN-γ Enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's recommendations.

Tumor studies in Her2 transgenic animals

[000312] Six weeks old FVB/N rat Her2/neu transgenic mice (9-14/group) were immunized 6 times with 5 x 10^8 CFU of *Lm*-LLO-ChHer2, ADXS31-164 or *Lm*-control. They were observed twice a week for the emergence of spontaneous mammary tumors, which were measured using an electronic caliper, for up to 52 weeks. Escaped tumors were excised when they reached a size

$1cm^2$ in average diameter and preserved in RNAlater at -20°C. In order to determine the effect of mutations in the Her2/neu protein on the escape of these tumors, genomic DNA was extracted using a genomic DNA isolation kit, and sequenced.

Effect of ADXS31-164 on regulatory T cells in spleens and tumors

[000313] Mice were implanted subcutaneously (s.c.) with 1 x 10^6 NT-2 cells. On days 7, 14 and 21, they were immunized with 1 x 10^8 CFUs of ADXS31-164, *LmddA*-control or left naïve. Tumors and spleens were extracted on day 28 and tested for the presence of $CD3^+/CD4^+/FoxP3^+$ Tregs by FACS analysis. Briefly, splenocytes were isolated by homogenizing the spleens between two glass slides in C-RPMI medium. Tumors were minced using a sterile razor blade and digested with a buffer containing DNase (12U/ml), and collagenase (2mg/ml) in PBS. After 60 min incubation at RT with agitation, cells were separated by vigorous pipetting. Red blood cells were lysed by RBC lysis buffer followed by several washes with complete RPMI-1640 medium containing 10% FBS. After filtration through a nylon mesh, tumor cells and splenocytes were resuspended in FACS buffer (2% FBS/PBS) and stained with anti-CD3-PerCP-Cy5.5, CD4-FITC, CD25-APC antibodies followed by permeabilization and staining with anti-Foxp3-PE. Flow cytometry analysis was performed using 4-color FACS calibur (BD) and data were analyzed using cell quest software (BD).

Statistical analysis

[000314] The log-rank Chi-Squared test was used for survival data and student's *t*-test for the CTL and ELISA assays, which were done in triplicates. A p-value of less than 0.05 (marked as *) was considered statistically significant in these analyzes. All statistical analysis was done with either Prism software, V.4.0a (2006) or SPSS software, V.15.0 (2006). For all FVB/N rat Her2/neu transgenic studies we used 8-14 mice per group, for all wild-type FVB/N studies we used at least 8 mice per group unless otherwise stated. All studies were repeated at least once except for the long term tumor study in Her2/neu transgenic mouse model.

RESULTS

EXAMPLE 11: GENERATION OF *L. MONOCYTOGENES* STRAINS THAT SECRETE LLO FRAGMENTS FUSED TO Her-2 FRAGMENTS: CONSTRUCTION OF ADXS31-164.

[000315] Construction of the chimeric *Her2/neu* gene (ChHer2) was described previously. Briefly, *ChHer2* gene was generated by direct fusion of two extracellular (aa 40-170 and aa 359-433) and one intracellular fragment (aa 678-808) of the Her2/neu protein by SOEing PCR method. The chimeric protein harbors most of the known human MHC class I epitopes of the protein. *ChHer2* gene was excised from the plasmid, pAdv138 (which was used to construct *Lm*-LLO-ChHer2) and cloned into *LmddA* shuttle plasmid, resulting in the plasmid pAdv164 (Figure 15A). There are two major differences between these two plasmid backbones. 1) Whereas pAdv138 uses the chloramphenicol resistance marker (*cat*) for *in vitro* selection of recombinant bacteria, pAdv164 harbors the D-alanine racemase gene (*dal*) from *bacillus subtilis*, which uses a metabolic complementation pathway for *in vitro* selection and *in vivo* plasmid retention in *LmddA* strain which lacks the *dal-dat* genes. This vaccine platform was designed and developed to address FDA concerns about the antibiotic resistance of the engineered *Listeria* vaccine strains. 2) Unlike pAdv138, pAdv164 does not harbor a copy of the *prfA* gene in the plasmid (see sequence below and Fig. 15A), as this is not necessary for *in vivo* complementation of the *Lmdd* strain. The *LmddA* vaccine strain also lacks the *actA* gene (responsible for the intracellular movement and cell-to-cell spread of *Listeria*) so the recombinant vaccine strains derived from this backbone are 100 times less virulent than those derived from the *Lmdd*, its parent strain. *LmddA*-based vaccines are also cleared much faster (in less than 48 hours) than the *Lmdd*-based vaccines from the spleens of the immunized mice. The expression and secretion of the fusion protein tLLO-ChHer2 from this strain was comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (Figure 15B) as a band of ~104 KD was detected by an anti-LLO antibody using Western Blot analysis. The *Listeria* backbone strain expressing only tLLO was used as negative control.

[000316] pAdv164 sequence (7075 base pairs) (see Figure 15):

cggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcaccggtgcgtca

gcagaatatgtgatacaggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaatggcttacga
acggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgtttttccataggctccgc
ccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgacaggactataaagataccaggcgtttcccccctggcg
gctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcattccacgcctgacactcagttc
cgggtaggcagttcgctccaagctggactgtatgcacgaacccccccgttcagtccgaccgctgcgccttatccggtaactatcgtcttgagtc
caacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagttagtcttgaagtcatgcgccggttaaggc
taaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctcggttcaaagagttggtagctcagagaaccttcgaaaaaccg
ccctgcaaggcggtttttcgttttcagagcaagagattacgcgcagaccaaaacgatctcaagaagatcatcttattaatcagataaaatatttc
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aagctataaagcaagcatataatattgcgtttcatctttagaagcgaatttcgccaatattataattatcaaaagagaggggtggcaaacggtatt
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gcaacaaactgaagcaaaggatgcatctgcattcaataaagaaaattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaa
gacgccaatcgaaaagaaacacgcggatgaaatcgataagtatatacaaggattggattacaataaaaacaatgtattagtataccacggag
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gcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcccattgcttatacaacaaacttcctaaaagacaatgaattagct
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tgggcagtggactggccctcatccaccataacacccacctctgcttcgtgcacacggtgccctgggaccagctctttcggaacccgcaccaa
gctctgctccacactgccaaccggccagaggacgagtgtgtgggcgagggcctggcctgccaccagctgtgcgcccgagggcagcaga
agatccggaagtacacgatgcggagactgctgcaggaaacggagctggtggagccgctgacacctagcggagcgatgcccaaccaggc
gcagatgcggatcctgaaagagacggagctgaggaaggtgaaggtgcttggatctggcgcttttggcacagtctacaagggcatctggatc
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catacgtgatggctggtgtgggctccccatatgtctcccgccttctgggcatctgcctgacatccacggtgcagctggtgacacagcttatgcc
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gaaacagaacaagtaacattggagttagaaatggaagaagaaaaaagcaatgatttcgtgtgaataatgcacgaaatcattgcttatttttttaa
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caccataaattggtccatgcaaattcggccggcaattttcaggcgttttcccttcacaaggatgtcggtccctttcaattttcggagccagccgtc
cgcatagcctacaggcaccgtcccgatccatgtgtcttttccgctgtgtactcggctccgtagctgacgctctcgccttttctgatcagtttgaca

tgtgacagtgtcgaatgcagggtaaatgccggacgcagctgaaacggtatctcgtccgacatgtcagcagacgggcgaaggccatacatg
ccgatgccgaatctgactgcattaaaaaagccttttttcagccggagtccagcggcgctgttcgcgcagtggaccattagattctttaacggca
gcggagcaatcagctctttaaagcgctcaaactgcattaagaaatagcctctttcttttttcatccgctgtcgcaaaatgggtaaataccccttttgc
actttaaacgagggttgcggtcaagaattgccatcacgttctgaacttcttcctctgtttttacaccaagtctgttcatccccgtatcgaccttcaga
tgaaaatgaagagaaccttttttcgtgtggcgggctgcctcctgaagccattcaacagaataacctgttaaggtcacgtcatactcagcagcga
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cggccaagcatgaagcacctgcgtcaagagcagcctttgctgtttctgcatcaccatgcccgtaggcgtttgctttcacaactgccatcaagtg
gacatgttcaccgatatgttttttcatattgctgacattttcctttatcgcggacaagtcaatttccgcccacgtatctctgtaaaaaggttttgtgctc
atggaaaactcctctctttttttcagaaaatcccagtacgtaattaagtatttgagaattaattttatattgattaatactaagtttacccagttttcaccta
aaaaacaaatgatgagataatagctccaaaggctaaagaggactataccaactatttgttaattaa (SEQ ID NO: 73)

EXAMPLE 12: ADXS31-164 IS AS IMMUNOGENIC AS LM-LLO-ChHER2.

[000317] Immunogenic properties of ADXS31-164 in generating anti-Her2/neu specific cytotoxic T cells were compared to those of the *Lm*-LLO-ChHer2 vaccine in a standard CTL assay. Both vaccines elicited strong but comparable cytotoxic T cell responses toward Her2/neu antigen expressed by 3T3/neu target cells. Accordingly, mice immunized with a *Listeria* expressing only an intracellular fragment of Her2-fused to LLO showed lower lytic activity than the chimeras which contain more MHC class I epitopes. No CTL activity was detected in naïve animals or mice injected with the irrelevant *Listeria* vaccine (Figure 16A). ADXS31-164 was also able to stimulate the secretion of IFN-γ by the splenocytes from wild type FVB/N mice (Figure 16B). This was detected in the culture supernatants of these cells that were co-cultured with mitomycin C treated NT-2 cells, which express high levels of Her2/neu antigen (Figure 19C).

[000318] Proper processing and presentation of the human MHC class I epitopes after immunizations with ADXS31-164 was tested in HLA-A2 mice. Splenocytes from immunized HLA-A2 transgenics were co-incubated for 72 hours with peptides corresponding to mapped HLA-A2 restricted epitopes located at the extracellular (HLYQGCQVV SEQ ID NO: 74 or KIFGSLAFL SEQ ID NO: 75) or intracellular (RLLQETELV SEQ ID NO: 76) domains of the Her2/neu molecule (Figure 16C). A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide as negative controls. The data from this experiment show that ADXS31-164 is able to elicit anti-Her2/neu specific immune responses to human epitopes that are located at different domains of the targeted antigen.

EXAMPLE 13: ADXS31-164 WAS MORE EFFICACIOUS THAN LM-LLO-ChHER2 IN PREVENTING THE ONSET OF SPONTANEOUS MAMMARY TUMORS.

[000319] Anti-tumor effects of ADXS31-164 were compared to those of *Lm*-LLO-ChHer2 in Her2/neu transgenic animals which develop slow growing, spontaneous mammary tumors at 20-25 weeks of age. All animals immunized with the irrelevant *Listeria*-control vaccine developed breast tumors within weeks 21-25 and were sacrificed before week 33. In contrast, *Liseria*-Her2/neu recombinant vaccines caused a significant delay in the formation of the mammary tumors. On week 45, more than 50% o ADXS31-164 vaccinated mice (5 out of 9) were still tumor free, as compared to 25% of mice immunized with *Lm*-LLO-ChHer2. At week 52, 2 out of 8 mice immunized with ADXS31-164 still remained tumor free, whereas all mice from other experimental groups had already succumbed to their disease (Figure 17). These results indicate that despite being more attenuated, ADXS31-164 is more efficacious than *Lm*-LLO-ChHer2 in preventing the onset of spontaneous mammary tumors in Her2/neu transgenic animals.

EXAMPLE 14: MUTATIONS IN HER2/NEU GENE UPON IMMUNIZATION WITH ADXS31-164.

[000320] Mutations in the MHC class I epitopes of Her2/neu have been considered responsible for tumor escape upon immunization with small fragment vaccines or trastuzumab (Herceptin), a monoclonal antibody that targets an epitope in the extracellular domain of Her2/neu. To assess this, genomic material was extracted from the escaped tumors in the transgenic animals and sequenced the corresponding fragments of the *neu* gene in tumors immunized with the chimeric or control vaccines. Mutations were not observed within the Her-2/neu gene of any vaccinated tumor samples suggesting alternative escape mechanisms (data not shown).

EXAMPLE 15: ADXS31-164 CAUSES A SIGNIFICANT DECREASE IN INTRA-TUMORAL T REGULATORY CELLS.

[000321] To elucidate the effect of ADXS31-164 on the frequency of regulatory T cells in spleens and tumors, mice were implanted with NT-2 tumor cells. Splenocytes and intra-tumoral lymphocytes were isolated after three immunizations and stained for Tregs, which were defined as $CD3^+/CD4^+/CD25^+/FoxP3^+$ cells, although comparable results were obtained with either

FoxP3 or CD25 markers when analyzed separately. The results indicated that immunization with ADXS31-164 had no effect on the frequency of Tregs in the spleens, as compared to an irrelevant *Listeria* vaccine or the naïve animals (See Figure 18). In contrast, immunization with the *Listeria* vaccines caused a considerable impact on the presence of Tregs in the tumors (Figure 19A). Whereas in average 19.0% of all $CD3^+$ T cells in untreated tumors were Tregs, this frequency was reduced to 4.2% for the irrelevant vaccine and 3.4% for ADXS31-164, a 5-fold reduction in the frequency of intra-tumoral Tregs (Figure 19B). The decrease in the frequency of intra-tumoral Tregs in mice treated with either of the *LmddA* vaccines could not be attributed to differences in the sizes of the tumors. In a representative experiment, the tumors from mice immunized with ADXS31-164 were significantly smaller [mean diameter (mm) ±SD, 6.71±0.43, n=5] than the tumors from untreated mice (8.69±0.98, n=5, $p<0.01$) or treated with the irrelevant vaccine (8.41±1.47, n=5, p=0.04), whereas comparison of these last two groups showed no statistically significant difference in tumor size (p=0.73). The lower frequency of Tregs in tumors treated with *LmddA* vaccines resulted in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* vaccines. However, only the vaccine expressing the target antigen HER2/neu (ADXS31-164) was able to reduce tumor growth, indicating that the decrease in Tregs has an effect only in the presence on antigen-specific responses in the tumor.

EXAMPLE 16: CONSTRUCTION OF DUAL PLASMID THAT CONCOMITANTLY DELIVERS TWO HETEROLOGOUS ANTIGENS

[000322] DNA corresponding to the *actA* promoter region and 1-233 amino acids of N-terminus of ActA is amplified from *Listeria* genomic DNA by Polymerase Chain Reaction (PCR) using the following primers ActA-F- 5'-atcccgggtgaagcttgggaagcagttggg-3' (XmaI) (SEQ ID NO: 77) and ActA-R- attctagatttatcacgtacccatttccccgc(XbaI)(SEQ ID NO:78). The restriction sites used for cloning are underlined. XmaI/XbaI segment is cloned in plasmid pNEB193 to create pNEB193-ActA. Further antigen 2, which is Chimera Her2 is PCR amplified using the primers Ch-Her2-F –5'-attctagaacccacctggacatgctccgccac-3'(XbaI)(SEQ ID NO: 79) and Ch-Her2-R-5'-gtcgacactagtctagtggtgatggtgatgatggagctcagatctgtctaagaggcagccatagggc-3'(RE sites- SalI-SpeI-SacI-BglII)(SEQ ID NO: 80). The XbaI and SalI fragment of Ch-Her2 is cloned in the plasmid pNEB193-ActA to create pNEB193-ActA-Ch-Her2 plasmid. His tag DNA sequence is included in the Ch-Her2 reverse primer sequence between SacI and SpeI restriction site. The XmaI/SpeI

fragment corresponding to tActA-Ch-Her2-His from the plasmid pNEB193-ActA-Ch-Her2 is excised for cloning in XmaI/SpeI restricted pAdv134 to create dual plasmid.

[000323] A *Listeria*-based plasmid that delivers two recombinant antigens concomitantly as fusion proteins is then generated. The two fusion proteins that are expressed by this plasmid include tLLO-antigen 1 and tActA-antigen 2. The expression and secretion of the antigen 1 is under the control of *hly* promoter and LLO signal sequence and it is expressed as a fusion to non-hemolytic fragment of Listeriolysin O (truncated LLO or tLLO). The expression and secretion of antigen 2 is under the control of *actA* promoter and ActA signal sequence and it is expressed as fusion to 1-233 amino acids of ActA (truncated ActA or tActA).The construction of antibiotic –marker free plasmid pAdv134 has been described previously and it contains the gene cassette for the expression of tLLO-antigen 1 fusion protein. The SpeI and Xma I restriction sites present downstream of the tLLO-antigen 1 in pAdv134 are used for the cloning of *actA* promoter-tActA-antigen 2 DNA segment Fig. 20. The restriction sites XbaI, SacI and BglII are added in the cassette to facilitate cloning of the antigen 2 insert at XbaI/SacI or XbaI/BglII. A DNA sequence coding for His tag is added after SacI site to facilitate the detection of tActA-antigen 2-his fusion protein. The dual plasmid is able to concomitantly express and secrete two different antigens as fusion proteins.

MATERIALS AND METHODS (EXAMPLES 17-21)

MDSC and Treg Function

[000324] 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.

[000325] 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.

[000326] Briefly, single cell suspensions of both the tumors and the spleens were prepared. Spleens were crushed manually and red blood cells were lysed. Tumors were minced and incubated with collagenase/DNase. Alternatively, the GENTLEMACS^{TM} dissociator was used with the tumor dissociation kit.

[000327] MDSCs or Tregs were purified from tumors and spleens using a Miltenyi kit and columns or the autoMACs separator. Cells were then counted.

[000328] Single cell suspension was prepared and the red blood cells were lysed. Responder T cells were then labeled with CFSE.

[000329] Cells were plated together at a 2:1 ratio of responder T cells (from all division cycle stages) to MDSCs or Tregs at a density of $1x10^5$ 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% CO_2. Two days later, the cells were stained for FACS and analyzed on a FACS machine.

Analysis of T-cell responses

[000330] 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). 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 Immunospot analyzer (C.T.L.). Number of spots per million splenocytes were graphed.

[000331] 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.

[000332] 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 read at 405 nm. The lower limit of detection for the assays was 30 pg/ml.

RESULTS

EXAMPLE 17: SUPPRESSOR CELL FUNCTION AFTER *LISTERIA* VACCINE TREATMENT

[000333] 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 the number and percentages of infiltrating MDSCs and Treg 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, and the absolute number of MDSC, whereas the same effect is not observed in the spleens or the draining lymph nodes (TLDN) (Fig. 21).

[000334] 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. 22-34) 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 22-34).

[000335] 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 18: MDSCs FROM TPSA23 TUMORS BUT NOT SPLEEN ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[000336] 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/ionomycyn). Results 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 Lm-LLO-PSA and Lm-LLO-treated Groups in Figs. 22 & 24, 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 unstimulated/activated, remained in their parental (resting) state (Fig. 22 & 24), whereas T cells stimulated with PMA or ionomycin were observed to replicate (Fig. 22 & 24). 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.

[000337] 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. 22 & 24).

[000338] In addition, the observations discussed immediately above relating to Figures 22 and 18 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. 23 & 25). 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 19: TUMOR T REGULATORY CELLS' REDUCED SUPPRESSION

[000339] 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. 26), however, it was found that splenic Tregs are still suppressive (Fig. 27).

[000340] 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. 28).

EXAMPLE 20: MDSCs AND TREGS FROM 4T1 TUMORS BUT NOT SPLEEN ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[000341] 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. 29 & 31), that *Listeria* has no specific effect on splenic monocytic MDSCs (Figs. 30 & 32), that there is a decrease in the suppressive ability of Tregs from 4T1 tumors after *Listeria* vaccination (Fig. 33), and that *Listeria* has no effect on the suppressive ability of splenic Tregs (Fig. 34).

[000342] Finally, it was observed that *Listeria* has no effect on the suppressive ability of splenic Tregs.

EXAMPLE 21: CHANGE IN THE SUPPRESSIVE ABILITY OF THE GRANULOCITY AND MONOCYTIC MDSC IS DUE TO THE OVEREXPRESSION OF tLLO.

[000343] The LLO plasmid shows similar results as the *Listeria* vaccines with either the TAA or an irrelvant antigen (Figure 35). This means that the change in the suppressive ability of the granulocytic MDSC is due to the overexpression of tLLO and is independent of the partnering fusion antigen. The empty plasmid construct alone also led to a change in the suppressive ability of the MDSC, although not to exactly the same level as any of the vaccines that contain the truncated LLO on the plasmid. The average of the 3 independent experiments show that the difference in suppression between the empty plasmid and the other plasmids with tLLO (with and without a tumor antigen) are significant. Reduction in MDSC suppressive ability was identical regardless of the fact if antigen specific or non-specific stimulated responder T cells were used.

[000344] Similar to the granulocytic MDSC, the average of the 3 independent experiments shows that the differences observed in the suppressive ability of the monocytic MDSCs purified from the tumors after vaccination with the *Lm*-empty plasmid vaccine are significant when compared to the other vaccine constructs (Figure 36).

[000345] Similar to the above observations, granulocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination (Figure 37). However, after non-specific stimulation, activated T cells (with PMA/ionomycin) are still capable of dividing. None of these results are altered with the use of the LLO only or the empty plasmid vaccines showing that the *Lm*-based vaccines are not affecting the splenic granulocytic MDSC (Figure 37).

[000346] Similarly, monocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination. However, after non-specific activation (stimulated by PMA/ionomycin), T cells are still capable of dividing. None of these results are altered with the use of the LLO only or the empty plasmid vaccines showing that the Lm vaccines are not affecting the splenic monocytic MDSC (Figure 38).

[000347] Tregs purified from the tumors of any of the *Lm*-treated groups have a slightly diminished ability to suppress the division of the responder T cells, regardless of whether the responder cells are antigen specific or non-specifically activated. Especially for the non-specifically activated responder T cells, it looks as though the vaccine with the empty plasmid shows the same results as all the vaccines that contain LLO on the plasmid. Averaging this experiment with the others shows that the differences are not significant (Figure 39).

[000348] Tregs purified from the spleen are still capable of suppressing the division of both antigen specific and non-specifically activated responder T cells. There is no effect of *Lm* treatment on the suppressive ability of splenic Tregs (Figure 40).

[000349] Tcon cells are not capable of suppressing the division of T cells regardless of whether the responder cells are antigens specific or non-specifically activated, which is consistent with the fact that these cells are non-suppressive. Lm has no effect on these cells and there was no difference if the cells were purified from the tumors or the spleen of mice (Figures 41-42).

[000350] 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 broad scope of the invention.

CLAIMS:

1. Use of a recombinant *Listeria* strain comprising a recombinant nucleic acid, said recombinant nucleic acid comprising a first open reading frame encoding a truncated LLO polypeptide, wherein the truncated LLO is not fused to a heterologous antigen, for the preparation of a medicament for reducing suppression of activated T cells by T regulatory (Treg) suppressor cells in an antigen non-specific manner in a subject having a disease or a disease site.

2. The use according to claim 1, wherein said suppression of activated T cells comprises suppression of T cell replication of the activated T cells.

3. Use of a recombinant *Listeria* strain comprising a recombinant nucleic acid, said recombinant nucleic acid comprising a first open reading frame encoding a truncated LLO polypeptide, wherein the truncated LLO is not fused to a heterologous antigen, and for the preparation of a medicament for increasing a T-infiltrating lymphocyte/ MDSC ratio in an antigen independent manner in a subject having a disease or a disease site.

4. The use according to claim 3, wherein said MDSC cells at said disease site are less suppressive following administration of said *Listeria* strain.

5. The use according to claim 4, wherein said suppression by said MDSCs comprises suppression of activated T cell replication.

6. The use according to claim 4 or 5, wherein said increasing said ratio comprises reducing the percentage of myeloid-derived suppressor cells (MDSC) cells, or increasing the percentage of said T-infiltrating lymphocytes, or any combination thereof.

7. The use according to any one of claims 1 to 6, wherein said suppressor cells suppress an anti-disease T cell response.

8. The use according to any one of claims 1 to 7, wherein said nucleic acid further comprises an additional 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.

9. The use according to claim 8, wherein said metabolic enzyme encoded by said additional open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

10. The use according to any one of claims 1 to 9, wherein said recombinant *Listeria* comprises a mutation or a deletion of a genomic dal gene, dat gene, ActA gene, a PlcA gene, a PrfA gene or a PlcB gene.

11. The use according to any one of claims 1 to 10, wherein said nucleic acid is integrated into the *Listeria* genome, or is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.

12. The use according to any one of claims 1 to 11, wherein said disease is an infectious disease, a respiratory disease, an inflammatory disease, or a cancer or tumor.

13. The use according claim 12, wherein said tumor or cancer is a prostate tumor or cancer, a cervical tumor or cancer, a breast tumor or cancer, a Her2 containing tumor or cancer, a gastric tumor or cancer, a tumor or cancer of the pancreas, or a head and neck tumor or cancer.

14. The use according to claim 12, wherein said infectious disease is malaria, dengue fever, or human papillomavirus (HPV).