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(54) Title: LISTERIA-BASED IMMUNOMODULATION

(57) Abstract: This disclosure provides methods and compositions for using *Listeria monocytogenes* expressing truncated listeriolysin O (LLO) as an adjuvant for conditioning the tumor microenvironment prior to additional administration of an immunotherapy to a subject. In some instances, the subject is receiving cell based therapy. In other instances the subject is receiving a T cell engraftment.



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LISTERIA-BASED IMMUNOMODULATION

FIELD OF INTEREST

[001] This disclosure provides methods and compositions for using *Listeria monocytogenes* expressing truncated listeriolysin O (LLO) as an adjuvant for conditioning the tumor microenvironment prior to additional administration of an immunotherapy to a subject. In some instances, the subject is receiving cell based therapy. In other instances the subject is receiving a T cell engraftment.

BACKGROUND

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

[003] *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 before providing an antigen specific treatment.

[004] Because of its adjuvant properties, fusing a non-hemolytic form of LLO to a tumor antigen enhances immunogenicity whether delivered by *Listeria*, vaccinia, DNA, or as a protein.

[005] There is currently a need to improve T-cell therapies which, despite being successful with liquid tumors (e.g., leukemia), have failed for solid tumors, possibly due to the presence of an immunosuppressive environment at the tumor site.

[006] The present disclosure addresses this need by providing a method of pre-conditioning the tumor microenvironment prior to engraftment of T cells in order to improve the engraftment and the efficacy of the same as a part of T cell-based anti-tumor therapies.

SUMMARY

[007] In one aspect, the present disclosure relates to a method of pre conditioning a tumor microenvironment prior to administration of a cell based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), wherein said administration enhances an engraftment of cells from said administration of said cell based

therapy, thereby pre-conditioning said tumor microenvironment.

[008] In one embodiment, pre-conditioning the tumor environment with a *Listeria* or tLLO protein disclosed herein leads to tumor shrinkage. In another embodiment, pre-conditioning the tumor environment with a *Listeria* or tLLO disclosed herein makes a tumor more receptive to a cell-based therapy disclosed herein, including and in one embodiment, CAR T-cell engraftment.

[009] In one aspect, the present disclosure relates to a method of enhancing an engraftment of cells for providing a cell-based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), thereby enhancing an engraftment of said cells. In another embodiment, the method of enhancing an engraftment of cells decreases the time to full immunogenic engraftment.

[0010] In one aspect the present disclosure relates to a method of improving maturation of immunity in a subject, the method comprising administering a live attenuated *Listeria* vaccine strain to the subject. In another aspect, the subject is receiving a cell engraftment. In another embodiment, the method of improving maturation of immunity, decreases the time to full immunogenic engraftment.

[0011] In one aspect, the present disclosure relates to a method of decreasing the time to immune-competence in a subject receiving a cell engraftment, said method comprising administering a live attenuated *Listeria* vaccine strain to the subject.

[0012] In one aspect, a subject is receiving an engraftment of cells as a treatment for a tumor, cancer or a hematopoietic disease. In another aspect, a hematopoietic disease is a hematopoietic malignancy. In another aspect, an engraftment comprises a bone marrow transplant. In another aspect the tumor is a solid tumor or the cancer is a solid cancer. In another aspect, a bone marrow transplant is a hematopoietic stem cell transplantation (HSCT).

[0013] In one aspect, a subject is a human. In another aspect, a subject is a non-human mammal. In another aspect a subject is a human child.

[0014] Other features and advantages of the present disclosure 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 embodiments of the disclosure are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The subject matter regarded as the disclosure is particularly pointed out and distinctly claimed in the concluding portion of the specification. The disclosure, however, both as to organization and method of operation, together with objects, features, and advantages thereof, may best be understood by reference to the following detailed description when read with the accompanying drawings in which:

[0016] **Figures 1A and 1B** present schematic maps of *E. coli*-*Listeria* shuttle plasmids pGG55 (**Figure 1A**) and pTV3 (**Figure 1B**). 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.

[0017] **Figures 2A-2D**. **Figures 2A and 2B** show schematic representation of the Lm-dd (**Figure 2A**) and Lm-dd $\Delta actA$ (**Figure 2B**) strains. **Figures 2C and 2D** present gels showing the size of PCR products using oligo's 1/2 (**Figure 2C**) and oligo's 3/4 (**Figure 2D**) obtained using chromosomal DNA of the strains Lm-dd and Lm-dd $\Delta actA$ as template.

[0018] **Figure 3** depicts tumor regression in response to administration of *Lm* vaccine strains. Circles represent naive mice, inverted triangles represent mice administered Lmdd-TV3, and crosses represent mice administered Lm-LLOE7.

[0019] **Figures 4A and 4B** show a decrease in MDSCs and Tregs in tumors. The number of MDSCs (**Figure 4A**) and Tregs (**Figure 4B**) following *Lm* vaccination (LmddAPSA and LmddAE7).

[0020] **Figures 5A-5D** show suppressor assay data demonstrating that monocytic MDSCs from TPSA23 tumors (PSA expressing tumor) 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. In **Figures 5A and 5B** Phorbol-Myristate-Acetate and Ionomycin (PMA/I) represents non-specific stimulation. In **Figures 5C and 5D** the term "peptide" represents specific antigen stimulation. Percent (%) CD3+CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 5A and 5C** show individual cell division cycles for each group. **Figures 5B and 5D** show pooled division cycles.

[0021] **Figures 6A-6D** show suppressor assay data demonstrating that *Listeria* has no effect on

splenic monocytic MDSCs and they are only suppressive in an antigen-specific manner. In **Figures 6A and 6B** PMA/I represents non-specific stimulation. In **Figures 6C and 6D** the term “peptide” represents specific antigen stimulation. Percent (%) CD3+CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 6A and 6C** show individual cell division cycles for each group. **Figures 6B and 6D** show pooled division cycles.

[0022] **Figures 7A-7D** show 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. In **Figures 7A and 7B** PMA/I represents non-specific stimulation. In **Figures 7C and 7D** the term “peptide” represents specific antigen stimulation. Percent (%) CD3+CD7+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 7A and 7C** show individual cell division cycles for each group. **Figures 7B and 7D** show pooled percentage division.

[0023] **Figures 8A -8D** show suppressor assay data demonstrating that *Listeria* has no effect on splenic granulocytic MDSCs and they are only suppressive in an antigen-specific manner. In **Figures 8A and 8B** PMA/I represents non-specific stimulation. In **Figures 8C and 8D** the term “peptide” represents specific antigen stimulation. Percent (%) CD3+CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 8A and 8C** show individual cell division cycles for each group. **Figures 8B and 8D** show pooled percentage division.

[0024] **Figures 9A-D** show 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. In **Figures 9A and 9B** PMA/I represents non-specific stimulation. In **Figures 9C and 9D** the term “peptide” represents specific antigen stimulation. Percent (%) CD3+CD8+ represents % effector (responder) T cells. The No Treg group shows the lack of division of the responder T cells when they are left unstimulated and the last group (PMA/I or peptide added) shows the division of stimulated cells in the absence of Tregs. **Figures 9A and 9C** show individual cell division cycles for each group. **Figures 9B and 9D** show pooled percentage division.

[0025] **Figures 10A-10D** shows suppressor assay data demonstrating that splenic Tregs are still suppressive. In **Figures 10A and 10B** PMA/I represents non-specific stimulation. In **Figures 10C and 10D** the term “peptide” represents specific antigen stimulation. Percent (%) CD3+CD8+ represents % effector (responder) T cells. The No Treg group shows the lack of division of the responder T cells when they are left unstimulated and the last group (PMA/I or peptide added) shows the division of stimulated cells in the absence of Tregs. **Figures 10A and 10C** show individual cell division cycles for each group. **Figures 10B and 10D** show pooled percentage division.

[0026] **Figures 11A-11D** show suppressor assay data demonstrating that conventional CD4+ T cells have no effect on cell division regardless whether they are found in the tumors or spleens of mice. In **Figures 11A and 11B** PMA/I represents non-specific stimulation. In **Figures 11C and 11D** the term “peptide” represents specific antigen stimulation. Percent (%) CD3+CD8+ represents % effector (responder) T cells. The no Tregs group shows the lack of division of the responder T cells when they are left unstimulated and the last group (PMA/I or peptide added) shows the division of stimulated cells in the absence of Tregs. **Figures 11C-11D** show data from pooled percentage division.

[0027] **Figures 12A-12D** show suppressor assay data demonstrating that monocytic MDSCs from 4T1 tumors (Her2 expressing 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. In **Figures 12A and 12B** PMA/I represents non-specific stimulation. In **Figures 12C and 12D** the term “peptide” represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 12A and 12C** show individual cell division cycles for each group. **Figures 12B and 12D** show pooled percentage division.

[0028] **Figures 13A-13D** show suppressor assay data demonstrating that there is no *Listeria*-specific effect on splenic monocytic MDSCs. In **Figures 13A and 13B** PMA/I represents non-specific stimulation. In **Figures 13C and 13D** the term “peptide” represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSC. **Figures 13A and 13C** show individual cell division cycles for each group. **Figures 13B and**

13D show pooled percentage division.

[0029] **Figures 14A-14D** show suppressor assay data demonstrating that granulocytic MDSCs from 4T1 tumors (Her2 expressing 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. In **Figures 14A and 14B** PMA/I represents non-specific stimulation. In **Figures 14C and 14D** the term “peptide” represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 14A and 14C** show individual cell division cycles for each group. **Figures 14B and 14D** shows pooled percentage division.

[0030] **Figures 15A-15D** showed suppressor assay data demonstrating that there is no *Listeria*-specific effect on splenic granulocytic MDSCs. In **Figures 15A and 15B** PMA/I represents non-specific stimulation. In **Figures 15C and 15D** the term “peptide” represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) 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 (PMA/I or peptide added) shows the division of stimulated cells in the absence of MDSCs. **Figures 15A and 15C** show individual cell division cycles for each group. **Figures 15B and 15D** show pooled percentage division.

[0031] **Figures 16A-16D** show suppressor assay data demonstrating that decrease in the suppressive ability of Tregs from 4T1 tumors (Her2 expressing tumors) after *Listeria* vaccination. In **Figures 16A and 16B** PMA/I represents non-specific stimulation. In **Figures 16C and 16D** the term “peptide” represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) T cells. 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. **Figures 16A and 16C** show individual cell division cycles for each group. **Figures 16B and 16D** show pooled percentage division.

[0032] **Figures 17A-17D** show 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. In **Figures 17A and 17B** PMA/I represents non-specific stimulation. In **Figures 17C and 17D** the term “peptide” represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) T cells. **Figures 17A and 17C** show individual cell division cycles for each group. **Figures 17B and 17D** show pooled

percentage division.

[0033] **Figures 18A-18D** show 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. Left-hand panels (**Figures 18A and 18C**) show individual cell
5 division cycles for each group. Right-hand panels (**Figures 18B and 18D**) show pooled percentage division.

[0034] **Figures 19A-19D** show 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. Left-hand panels (**Figures 19A and 19C**) show individual cell
10 division cycles for each group. Right-hand panels (**Figures 19B and 19D**) show pooled percentage division.

[0035] **Figures 20A-20D** show 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 (**Figure 20A and 20B**). However, after non-specific
15 stimulation, activated T cells (with PMA/ionomycin) are still capable of dividing (**Figures 20C and 20D**). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled percentage division.

[0036] **Figures 21A-21D** show suppressor assay data demonstrating that monocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific
20 responder T cells after Lm vaccination (**Figures 21A and 21B**). However, after non-specific activation (stimulated by PMA/ionomycin), T cells are still capable of dividing (**Figures 21C and 21D**). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled percentage division.

[0037] **Figures 22A-22D** show suppressor assay data demonstrating that Tregs purified from the
25 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 (**Figures 22A and 22B**) or non-specifically (**Figures 22C and 22D**) activated. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled division cycles.

[0038] **Figures 23A-23D** show suppressor assay data demonstrating that Tregs purified from
30 the spleen are still capable of suppressing the division of both antigen specific (**Figures 23A-23B**) and non-specifically (**Figures 23C and 23D**) activated responder T cells.

[0039] **Figures 24A-24D** show 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 (**Figures 24A and 24B**) or non-specifically activated (**Figures 24C and 24D**).

[0040] **Figures 25A-25D** show 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 (**Figures 25A and 25B**) or non-specifically activated (**Figures 25C and 25D**).

[0041] **Figures 26A-C.** Immunization with *LmddA*-142 induces regression of Tramp-C1-PSA (TPSA) tumors. Mice were left untreated (n=8) (**Figure 26A**) or immunized i.p. with *LmddA*-142 (1×10^8 CFU/mouse) (n=8) (**Figure 26B**) or *Lm*-LLO-PSA (n=8) (**Figure 26C**) on days 7, 14 and 21. Tumor sizes were measured for each individual tumor and the values expressed as the mean diameter in millimeters. Each line represents an individual mouse.

[0042] **Figures 27A-B.** (**Figure 27A**) Analysis of PSA-tetramer⁺CD8⁺ T cells in the spleens and infiltrating T-PSA-23 tumors of untreated mice and mice immunized with either an *Lm* control strain or *Lm-ddA*-LLO-PSA (*LmddA*-142). (**Figure 27B**) Analysis of CD4⁺ regulatory T cells, which were defined as CD25⁺FoxP3⁺, in the spleens and infiltrating T-PSA-23 tumors of untreated mice and mice immunized with either an *Lm* control strain or *Lm-ddA*-LLO-PSA.

[0043] **Figures 28A-28B.** Surface phenotype profile of CD4⁺CD25⁺ T cells. Cells isolated from the different groups of vaccinated and control mice were triple-labeled with anti-CD4, anti-CD25, and anti-CTLA-4. **Figure 28A:** Representative plots show the gating strategy for flow cytometry data to identify suppressor T cells. These gates were applied to all the cells isolated from the different groups of mice. CD4⁺ T cells (R1) were identified and gated, and this gate was used to identify the CD4⁺CD25⁺ population (R2). This CD4⁺CD25⁺ population was further analyzed to identify the expression pattern of CTLA-4, with R3 showing the CD4⁺CD25⁺CTLA-4 hi population. **Figure 28B:** The table shows the percentages of the different cell populations (as determined by the R1, R2, and R3 gates described above) isolated from all the vaccinated and control mice. Each percentage is an average of at least four individually processed spleens from each group.

[0044] **Figures 29A-29C.** *Lm*-E7-vaccinated tumorbearing mice had more CD4⁺CD25⁺ T cells in the spleen and tumor. The lymphocyte population, prepared by Ficoll separation from the individual spleens from four mice, was stained for CD4 and CD25. The percentages of gated lymphocytes that were CD4⁺CD25⁺ were analyzed. **Figure 29A:** Mice were injected with TC-1 tumors on day 0 and the spleens analyzed on day 5 and day 10. **Figure 29B:** Non-tumor-bearing mice were injected with 0.1 LD₅₀ of either *Lm*-E7 or *Lm*-LLO-E7 on day 0. A booster dose of each vaccine was injected on day 7 and spleens were analyzed on day 11. **Figure 29C:** Mice were injected with TC-1 tumors on day 0 followed by 0.1 LD₅₀ of either *Lm*-E7 or *Lm*-LLO-E7 on days 7 and 14. Each of the organs was individually processed and stained for CD4 and CD25. The percentages of gated lymphocytes that were CD4⁺ or CD4⁺CD25⁺ were calculated.

*Statistically significant at $P < 0.04$. Data are shown from one experiment and are representative of results from at least four separate experiments.

[0045] **Figures 30A-30C.** $CD4^+ CD25^+$ T cells isolated from the vaccinated mice are capable of cell-mediated suppression. Mice were injected with TC-1 tumors on day 0 followed by 0.1 LD_{50} of either Lm-E7 or Lm-LLO-E7 on days 7 and 14 or left unvaccinated. **Figure 30A:** Splenocytes individually isolated from each group were separated by magnetic bead separation into $CD4^+ CD25^+$ and $CD4^+ CD25^-$ cells with $>90\%$ purity. **Figure 30B:** $CD4^+ CD25^-$ cells from the naive group were labeled with CFSE and incubated in a 96-well plate with anti-CD3 Ab and mitomycin C-treated antigen-presenting cells. $CD4^+ CD25^+$ cells from each vaccinated and control group were titrated into each of these wells at the following ratios: 0, 0.03, 0.25, or 1 to one $CD4^+ CD25^-$ cell. Following 3 days of incubation, cells were labeled with anti-CD4 fluorochrome conjugated antibody and the $CD4^+ CD25^-$ cells were analyzed for proliferation. The table indicates the exact percentage of CFSE $^+$ $CD4^+ CD25^-$ T cells in each dividing group. **Figure 30C:** Percentage of $CD4^+ CD25^-$ cells undergoing cell division at different ratios of $CD4^+ CD25^+$ cells from naive, Lm-E7, and Lm-LLO-E7 immunized mice.

*Statistically significant at $P < 0.04$. Each assay was performed in triplicate. Data are shown from one experiment and are representative of results from two separate experiments.

[0046] **Figure 31A-31C.** $CD4^+$ tumor-infiltrating T cells produce TGF and IL-10. Mice were injected with TC-1 tumors on day 0 followed by 0.1 LD_{50} of either Lm-E7 or Lm-LLO-E7 on days 7 and 14 or left unvaccinated. Splenocytes and tumor cells were incubated with or without anti-CD3/antigen-presenting cell stimulation for 3 days and supernatants were collected and analyzed for **(Figure 31A)** TGF by the MLEC luciferase assay and **(Figure 31B)** IL-10 by ELISA. **Figure 31C:** Tumor cells from the different groups were incubated as a whole with or without stimulation or were separated into a $CD4^+$ population by depleting the $CD4^+$ T cells using magnetic bead separation. *Statistically significant at $P < 0.04$. Data are shown from one experiment and are representative of results from two separate experiments.

[0047] **Figures 32A-32B.** **Figure 32A** presents a schematic flow chart of the protocol for use to determine the synergy between LM-LLO therapy and CAR-T cells. **Figure 32B** presents the analysis of these tissue-infiltrating cells (TIC).

[0048] **Figures 33A-33B.** **Figure 33A** presents a schematic flow chart of the protocol for use to determine the synergy between LM-LLO therapy and CAR-T cells, wherein the protocol includes a post CART cell boost of Lm-LLO (See, day 10). **Figure 33B** presents the analysis of these tissue-infiltrating cells (TIC).

[0049] It will be appreciated that for simplicity and clarity of illustration, elements shown in the

figures have not necessarily been drawn to scale. For example, the dimensions of some of the elements may be exaggerated relative to other elements for clarity. Further, where considered appropriate, reference numerals may be repeated among the figures to indicate corresponding or analogous elements.

DETAILED DESCRIPTION

[0050] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the disclosure. However, it will be understood by those skilled in the art that the present disclosure may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present disclosure.

[0051] A novel and heretofore unexplored use is to use a live attenuated *Listeria* vaccine strain devoid of antigen that enables the *Listeria* to secrete only the non-hemolytic, truncated form of LLO (*Lm*-LLO), as an adjuvant to pre-condition a tumor microenvironment.

[0052] In one embodiment, disclosed herein, is a method of pre-conditioning a tumor microenvironment prior to administration of a cell based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), wherein said administration enhances an engraftment of cells from said administration of said cell based therapy, thereby pre-conditioning said tumor microenvironment.

[0053] In one embodiment, disclosed herein is a method of enhancing an engraftment of cells for providing a cell-based therapy in a subject, the method of enhancing an engraftment of cells for providing a cell-based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), thereby enhancing an engraftment of said cells. In another embodiment, the method of enhancing an engraftment of cells decreases the time to full immunogenic engraftment. The term “full immunogenic engraftment” refers to the ability of engrafted cells to provide an anti-tumor response at maximum efficiency. In another embodiment, the term “maximum efficiency” refers to a level which is normal or enhanced under non-suppressive conditions which may occur as a result of a suppressor cell-mediated or regulatory T-cell-mediated suppression of what would otherwise be a normal or enhanced anti-tumor immune response.

[0054] In one embodiment, “pre-conditioning a tumor microenvironment” is achieved by administering a *Listeria* strain expressing disclosed hereina truncated LLO (tLLO), truncated ActA or a PEST sequence peptide. In another embodiment, pre-conditioning can be achieved by administering the tLLO polypeptide, truncated ActA polypeptide or PEST peptide alone in a pharmaceutical composition. It will be appreciated by the skilled artisan that pre-conditioning of a tumor microenvironment can be achieved as a result of administering a *Listeria* strain that expresses tLLO (Lm-LLO), truncated ActA (Lm-ActA), or PEST sequence peptide (LM-PEST) either from the chromosome or a plasmid, and wherein doing so leads to the upregulation of inflammatory factors such as TNF- β , IL-18 and IL-12, all of which are important in inducing the production of IFN- γ .

[0055] In one embodiment, the methods disclosed herein further comprise the step of administering an effective amount of a regimen of antibiotics following administration of the recombinant *Listeria* strain in order to prevent unwanted persistence of said recombinant *Listeria* strain.

[0056] In one embodiment, administering an antibiotic regimen disclosed herein prevents persistence of a *Listeria* strain on a tissue within a subject. In one embodiment, administering an antibiotic regimen disclosed herein prevents seeding of a *Listeria* strain on a tissue within a subject. In another embodiment, administering an antibiotic regimen disclosed herein prevents biofilm formation of a *Listeria* strain on a tissue within a subject. In another embodiment, the *Listeria* strain is administered to the subject as part of a *Listeria*-based immunotherapy disclosed herein. In one embodiment, the subject has a disease. In another embodiment, the subject is a normal subject free from disease.

[0057] *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.

[0058] *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 that 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 myelopoiesis and vascular endothelial cell function.

[0059] *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.

[0060] 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.

[0061] A poorly described property of LLO, is its ability to induce epigenetic modifications affecting control of DNA expression. Extracellular LLO induces a dephosphorylation of the histone protein H3 and a similar deacetylation of the histone H4 in early phases of *Listeria* infection. This epigenetic effect results in reduced transcription of certain genes involved in immune function, thus providing a mechanism by which LLO may regulate the expression of gene products required for immune responses. Another genomic effect of LLO is its ability to increase NF- κ B translocation in association with the expression of ICAM and E-selectin, and the secretion of IL-8 and MCP-1. Another signaling cascade affected by LLO is the Mitogen Activated Protein Kinase (MAPK) pathway, resulting in increase of Ca²⁺ influx across the cell membrane, which facilitates the entry of *Listeria* into endothelial cells and their subsequent infection.

[0062] 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. The proinflammatory cytokine-inducing property of LLO is thought to be a consequence of the activation of the TLR4 signal pathway. One evidence of the high Th1 cytokine-inducing activity of LLO is in that protective immunity to *Lm* can be induced with killed or avirulent *Lm* when administered together with LLO, whereas the protection is not generated in the absence of LLO. Macrophages

in the presence of LLO release IL-1 α , TNF- α , IL-12 and IL-18, which in turn activate NK cells to release IFN- γ resulting in enhanced macrophage activation.

[0063] IL-18 is also critical to resistance to *Lm*, even in the absence of IFN- γ , and is required for TNF- α and NO production by infected macrophages. A deficiency of caspase-1 impairs the ability of macrophages to clear *Lm* and causes a significant reduction in IFN- γ production and listericidal activity that can be reversed by IL-18. Recombinant IFN- γ injection restores innate resistance to listeriosis in caspase-1^{-/-} mice. Caspase-1 activation precedes the cell death of macrophages infected with *Lm*, and LLO deficient mutants that cannot escape the phagolysosome have an impaired ability to activate caspase-1.

[0064] In one embodiment, LLO secreted by cytosolic *Lm* causes specific gene upregulation in macrophages resulting in significant IFN- γ transcription and secretion. 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. One of the IFN I-specific apoptotic genes, TNF- α related apoptosis-inducing ligand (TRAIL), is up-regulated during *Lm* infection in the spleen. Mice lacking TRAIL are also more resistant to primary listeriosis coincident with lymphoid and myeloid cell death in the spleen.

[0065] In one embodiment, *Lm* also secretes P60, which acts directly on naïve DCs to stimulate their maturation in a manner that permits activation of NK cells. Both activated DCs and IFN- γ that is produced by NK cells can boost cellular (Th1-type) immune responses. ActA stimulates toll receptors, for example TLR-5, which plays a fundamental role in pathogen recognition and activation of innate immune response.

[0066] In one embodiment, the *Lm* vaccines disclosed herein reduce the number of Tregs and MDSCs in a disease further disclosed herein. In another embodiment, *Lm* vaccines disclosed herein are useful to improve immune responses by reducing the number of Tregs and MDSCs at a specific site in a subject. Such a site can be an inflammation site due to allergies, trauma, infection, disease or the site can be a tumor site.

[0067] In another embodiment, pre-conditioning a tumor environment allows for a more efficient anti-tumor response which includes but is not limited to, generation of robust MHC Class 2 restricted CD4⁺ T cell responses, and shifting of the phenotype of CD4⁺ T cells to Th-1.

[0068] In one embodiment, pre-conditioning a tumor environment allows for the infiltration of anti-tumor cytotoxic T (CD8⁺) cells and suppression of myeloid derived suppressor cells and suppression of regulatory T cells (Tregs) at the tumor site. In one embodiment, CD8⁺ T cells co-localize with macrophages and *Lm* in the T cell area of the spleen where they produce IFN- γ independent of antigen and IFN- γ production by CD8⁺ T cells depends partially on the

expression of LLO. In another embodiment, pre-conditioning a tumor microenvironment improves the anti-tumor response by improving maturation of immunity in a subject. In another embodiment, pre-conditioning a tumor microenvironment, decreases the time to immune-competence in a subject, allowing for a more efficient anti-tumor response. In another embodiment, pre-conditioning a tumor microenvironment improves the anti-tumor response by improving maturation of immunity in a subject, allowing for a more efficient cell based anti-tumor therapy. In another embodiment, pre-conditioning a tumor microenvironment decreases the time to immune-competence in a subject, allowing for a more efficient anti-tumor response, thereby allowing for a more efficient cell-based anti-tumor therapy.

[0069] In one embodiment, pre-conditioning the tumor microenvironment improves the function of engrafted T cells by reducing levels of myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs).

[0070] In one embodiment, the cells used in the cell-based therapy disclosed herein are immune cells. In one embodiment, the cell-based therapy disclosed herein is a T-cell based therapy. In another embodiment, the T-cell based therapy comprises using autologous T-cells. In another embodiment, the T-cell based therapy comprises using allogeneic T-cells. In another embodiment, the T-cell based therapy comprises using non-autologous T-cells. In another embodiment, the T-cell based therapy comprises using manipulated or recombinant T-cells, which in one embodiment are chimeric antigen receptor engineered T cell (CAR T cells). In another embodiment, the T-cell based therapy comprises using CD4+ T cells. In another embodiment, the T-cell based therapy comprises using CD8+ T cells.

[0071] In one embodiment, chimeric antigen receptor engineered T cells (CAR T cells) are generated by transducing a normal T cell to express a chimeric antigen receptor (CAR). CARs are molecules that combine antibody-based specificity for a desired antigen (e.g., tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-tumor cellular immune activity. It will be understood by a skilled artisan that methods of generating CAR T cells are well known in the art.

[0072] The CAR T cells of the present disclosure are genetically modified to stably express a desired CAR. In one embodiment, the T cell is genetically modified to stably express an antibody binding domain on its surface, conferring novel antigen specificity that is MHC independent.

[0073] In one embodiment, the antigen recognized by CAR T cells is a tumor associated antigen or a fragment thereof.

[0074] In one embodiment the *Listeria* strain comprises a nucleic acid molecule, wherein the

nucleic acid molecule comprises a first open reading frame encoding a tLLO. In one embodiment, disclosed herein is an immunogenic composition comprising chimeric antigen receptor-engineered T cells (CAR T cells), and a recombinant *Listeria* vaccine strain for comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a polypeptide, wherein the polypeptide comprises a tLLO fused to a heterologous antigen or fragment thereof. In another embodiment, the nucleic acid molecule comprising a first open reading frame encodes a tLLO alone, independent of or not fused to an additional protein or antigen.

[0075] In one embodiment, disclosed herein is an immunogenic composition comprising chimeric antigen receptor-engineered T cells (CAR T cells). In another embodiment, disclosed herein is an immunogenic composition comprising a recombinant *Listeria* vaccine strain for comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a polypeptide, wherein the polypeptide comprises a tLLO fused to a heterologous antigen or fragment thereof. In another embodiment, the nucleic acid molecule comprising a first open reading frame encodes a tLLO alone, independent of or not fused to an additional protein or antigen.

[0076] In one embodiment, compositions of this disclosure comprise CAR T cells. In one embodiment, a composition of this disclosure comprises an *Lm* strain and CAR T cells. In another embodiment, a composition of this disclosure comprises CAR T cells, wherein the composition does not include a *Listeria* strain as described herein.

[0077] In one embodiment, disclosed herein is a method of pre-conditioning a tumor microenvironment prior to administration of a T-cell based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), wherein said administration enhances an engraftment of T-cells from said administration of said T-cell based therapy, thereby pre-conditioning said tumor microenvironment.

[0078] In one embodiment, disclosed herein is a method of enhancing an engraftment of T-cells for providing a T-cell-based therapy in a subject, the method of enhancing an engraftment of cells for providing a T-cell-based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), thereby enhancing an engraftment of said T-cells.

[0079] In some embodiments of methods of this disclosure, the *Listeria* expresses said PEST-

containing polypeptide. In other embodiment, the *Listeria* expresses and secretes said PEST-containing polypeptide. In another embodiment, the PEST-containing polypeptide is a non-hemolytic LLO protein or immunogenic fragment thereof, an ActA protein or truncated fragment thereof, or a PEST-containing amino acid sequence. In another embodiment, the non-hemolytic LLO is a tLLO.

[0080] In one embodiment, disclosed herein is a method of facilitating recovery of immune responses after a subject receives a cell-based therapy, the method comprising administering a live attenuated *Listeria* vaccine strain to the subject. In another embodiment, the *Listeria* strain comprises a nucleic acid molecule, wherein the nucleic acid molecule comprises a first open reading frame encoding a PEST-containing polypeptide.

[0081] In one embodiment, disclosed herein is a method of improving maturation of an immune response in an antigen-independent manner in a subject, the method comprising administering a *Lm*-LLO to the subject.

[0082] In one embodiment, disclosed herein is a composition and method for bioengineering a live *Lm* bacterium that infects specific cells, including; antigen processing cells (APC), Kupffer cells, vascular endothelium, bone marrow, and others; as well as structures such as solid tumors and spleen. In another embodiment, the live *Lm* adjuvant then synthesizes and secretes a modified LLO fragment *in situ* where the adjuvant is needed and used to stimulate immune responses. . In another embodiment the live *Lm* synthesizes ActA. In another embodiment, unlike previous adjuvants, the instant disclosure administers the ability to make an adjuvant *in situ* and does not involve the systemic administration of an immune adjuvant.

[0083] In one embodiment, a *Listeria*-based adjuvant refers to a live-attenuated *Listeria* vaccine strain. In another embodiment, the *Listeria*-based adjuvant is an *Lm*-LLO. In another embodiment, *Lm*-LLO expresses a non-hemolytic LLO. In another embodiment, *Lm*-ActA expresses a truncated ActA protein. In another embodiment, *Lm*-LLO or *Lm*-ActA can be used alone, or prior to any therapy, including a cell-based therapy, in order to pre-condition a tumor microenvironment to make it more amenable to achieving more efficient anti-tumor responses. In another embodiment, methods of reducing regulatory T cells and myeloid derived suppressor cells are also disclosed herein and are further discussed in US Patent Publication No. 2015/0098964 which is incorporated by reference herein in its entirety.

[0084] In another embodiment, the *Listeria* strain disclosed herein further comprises a second open reading frame encoding a metabolic enzyme.

[0085] 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 second 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.

[0086] In another embodiment, the recombinant *Listeria* is an attenuated auxotrophic strain.

[0087] The present disclosure provides a number of *Listeria* species and strains for making or engineering an attenuated *Listeria* of the present disclosure. In one embodiment, the *Listeria* strain is *L. monocytogenes* 10403S wild type (see Bishop and Hinrichs (1987) J. Immunol. 139: 2005–2009; Lauer, et al. (2002) J. Bact. 184: 4177–4186.) In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4056 (phage cured) (see Lauer, et al. (2002) J. Bact. 184: 4177–4186). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4027, which is phage cured and deleted in the *hly* gene (see Lauer, et al. (2002) J. Bact. 184: 4177–4186; Jones and Portnoy (1994) Infect. Immunity 65: 5608–5613.). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4029, which is phage cured, deleted in *ActA* (see Lauer, et al. (2002) J. Bact. 184: 4177–4186; Skoble, et al. (2000) J. Cell Biol. 150: 527–538). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4042 (delta PEST) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4097 (LLO-S44A) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4364 (delta *lplA*; lipote protein ligase) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4405 (delta *inlA*) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4406 (delta *inlB*) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* CS-L0001 (delta *ActA*-delta *inlB*) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* CS-L0002 (delta *ActA*-delta *lplA*) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* CS-L0003 (L461T-delta *lplA*) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4038 (delta *ActA*-LLO L461T) (see Brockstedt, et al. (2004)

Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4384 (S44A-LLO L461T) (see Brockstedt, et al. (2004) Proc. Natl. Acad. Sci. USA 101: 13832–13837; supporting information). In another embodiment, the *Listeria* strain is *L. monocytogenes*. Mutation in lipoate protein (see O’Riordan, et al. (2003) Science 302: 462–464). In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4017 (10403S hly (L461T), having a point mutation in hemolysin gene (see U.S. Provisional Pat. Appl. Ser. No. 60/490,089 filed Jul. 24, 2003). In another embodiment, the *Listeria* strain is *L. monocytogenes* EGD (see GenBank Acc. No. AL591824). In another embodiment, the *Listeria* strain is *L. monocytogenes* EGD-e (see GenBank Acc. No. NC_003210. ATCC Acc. No. BAA-679). In another embodiment, the *Listeria* strain is any *Listeria* strain known in the art. In another embodiment, the *Listeria* strain is *L. monocytogenes* DP-L4029 deleted in uvrAB (see U.S. Provisional Pat. Appl. Ser. No. 60/541,515 filed Feb. 2, 2004; US Provisional Pat. Appl. Ser. No. 60/490,080 filed Jul. 24, 2003). In another embodiment, the *Listeria* strain is *L. monocytogenes* ActA-/inlB - double mutant (see ATCC Acc. No. PTA-5562). In another embodiment, the *Listeria* strain is *L. monocytogenes* lplA mutant or hly mutant (see U.S. Pat. Appl. Publ. No. 20040013690 of Portnoy, et. al). In another embodiment, the *Listeria* strain is *L. monocytogenes* DAL/DAT double mutant. (see U.S. Pat. Appl. No. 20050048081 of Frankel and Portnoy. The present disclosure encompasses reagents and methods that comprise the above Listerial strains, as well as these strains that are modified, e.g., by a plasmid and/or by genomic integration, to contain a nucleic acid encoding one of, or any combination of, the following genes: hly (LLO; listeriolysin); iap (p60); inlA; inlB; inlC; dal (alanine racemase); dat (D-amino acid aminotransferase); plcA; plcB; actA; or any nucleic acid that mediates growth, spread, breakdown of a single walled vesicle, breakdown of a double walled vesicle, binding to a host cell, uptake by a host cell. The present disclosure is not to be limited by the particular strains disclosed above.

[0088] In one embodiment the attenuated strain is *Lmdd*. (**Figure 2A**). In another embodiment the attenuated strain is *LmddA*. (**Figure 2B**). In another embodiment, the attenuated strain is *LmΔprfA*. In another embodiment, the attenuated strain is *LmΔplcB*. In another embodiment, the attenuated strain is *LmΔplcA*. In another embodiment, the attenuated strain is *LmddAΔinlC*. In another embodiment, the *LmddAΔinlC* mutant strain is created using EGD strain of *Lm*, which is different from the background strain 10403S, the parent strain for *Lm dal dat actA* (*LmddA*). In another embodiment, this strain exerts a strong adjuvant effect which is an inherent property of *Listeria*-based vaccines. In another embodiment, this strain is constructed from the EGD *Listeria*

backbone.

[0089] In another embodiment, the strain used in the disclosure 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 disclosed herein.

[0090] In one embodiment, translocation of *Listeria* to adjacent cells is inhibited by two separate mechanisms, deletion of *actA* and *inlB* genes, both of which are involved in the process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a vaccine backbone. In another embodiment, translocation of *Listeria* to adjacent cells is inhibited by two separate mechanisms, deletion of *actA* or *inlB* genes, both of which are involved in the process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a vaccine backbone.

[0091] Internalins are associated with increased virulence and their presence is associated with increased immunogenicity of *Listeria*, however, in the present disclosure, excising the *inlB* gene increases immunogenicity of the *Listeria* vaccine vector disclosed herein. In another embodiment, the present disclosure provides the novelty that the *inlB* genes are excised from a vector in which *actA* is deleted, thereby removing both, the ability to form actin flagella necessary for movement through the host cell membrane and into the neighboring cell, and the ability for transmembrane passage. Therefore, the combination of these two deletions yields the surprising result of decreased virulence and increased immunogenicity of a *Listeria* vaccine vector over a wild-type *Listeria* strain or a single mutant strain.

[0092] In another embodiment, the nucleic acid molecule disclosed herein is integrated into the *Listeria* genome. In another embodiment, the nucleic acid molecule is in a plasmid in the recombinant *Listeria* vaccine strain also disclosed herein. In another embodiment, the plasmid disclosed herein is stably maintained in the recombinant *Listeria* vaccine strain in the absence of antibiotic selection. In another embodiment, the plasmid does not confer antibiotic resistance upon said recombinant *Listeria*.

[0093] In one embodiment, the recombinant *Listeria* strain disclosed 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.

[0094] 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.

[0095] In another embodiment, the *Listeria*-based adjuvant is an LLO-expressing *Listeria* strain

or an LLO protein or a non-hemolytic fragment thereof. In another embodiment, *Listeria*-based adjuvant is used alone or is combined with an additional adjuvant. In another embodiment, the additional adjuvant is a non-nucleic acid adjuvant including aluminum adjuvant, Freund's adjuvant, MPL, emulsion, GM-CSF, QS21, SBAS2, CpG-containing oligonucleotide, a nucleotide molecule encoding an immune-stimulating cytokine, the adjuvant is or comprises a bacterial mitogen, or a bacterial toxin.

[0096] In another embodiment, the *Lm*-LLO of the present disclosure is co-administered with an additional adjuvant. In another embodiment, the additional adjuvant utilized in methods and compositions of the present disclosure 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.

[0097] In one embodiment, disclosed herein is a nucleic acid molecule that encodes a polypeptide comprising a tLLO of the present disclosure. In one embodiment, the polypeptide comprises a tumor associated antigen.

[0098] In one embodiment, a tumor-associated antigen is a naturally occurring tumor-associated antigen. In another embodiment, the tumor-associated antigen is a synthetic tumor-associated antigen. In yet another embodiment, the tumor-associated antigen is a chimeric tumor-associated antigen. In another embodiment, a tumor-associated antigen is a heterologous antigen. In another embodiment, a tumor-associated antigen is a self-antigen. In another embodiment, a tumor

associated antigen is an angiogenic antigen.

[0099] In another embodiment, the tumor associated antigen targeted by the cell-based therapy disclosed herein is expressed on the surface of the tumor cell.

[00100] It will be appreciated by the skilled artisan that an “antigen” or “antigenic polypeptide” may encompass to a polypeptide, peptide or recombinant peptide as described herein that is processed and presented on MHC class I and/or class II molecules present in a subject’s cells leading to the mounting of an immune response when present in, or in another embodiment, detected by, the host. In one embodiment, the antigen may be foreign to the host. In another embodiment, the antigen might be present in the host but the host does not elicit an immune response against it because of immunologic tolerance.

[00101] In one embodiment, the methods disclosed herein overcome or break tolerance to self.

[00102] In one embodiment, a tumor-associated antigen is a prostate specific antigen (PSA). In another embodiment, a tumor-associated antigen is a human papilloma virus (HPV) antigen. In yet another embodiment, a tumor-associated antigen is a Her2/neu chimeric antigen as described in US Patent Pub. No. US2011/014279, which is incorporated by reference herein in its entirety.

[00103] In one embodiment the HPV antigen is an HPV 16. In another embodiment, the HPV is an HPV-18. In another embodiment, the HPV is selected from HPV-16 and HPV-18. In another embodiment, the HPV is an HPV-31. In another embodiment, the HPV is an HPV-35. In another embodiment, the HPV is an HPV-39. In another embodiment, the HPV is an HPV-45. In another embodiment, the HPV is an HPV-51. In another embodiment, the HPV is an HPV-52. In another embodiment, the HPV is an HPV-58. In another embodiment, the HPV is a high-risk HPV type. In another embodiment, the HPV is a mucosal HPV type.

[00104] In one embodiment, the HPV E6 is from HPV-16. In another embodiment, the HPV E7 is from HPV-16. In another embodiment, the HPV-E6 is from HPV-18. In another embodiment, the HPV-E7 is from HPV-18.

[00105] In another embodiment, the tumor-associated antigen is HPV-E7. In another embodiment, the antigen is HPV-E6. In another embodiment, the antigen is Her-2. In another embodiment, the antigen is NY-ESO-1. In another embodiment, the antigen is telomerase. In another embodiment, the antigen is SCCE. 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 (TRP-2). In another embodiment, the antigen is CD20. In another embodiment, the antigen is PSA (prostate-specific antigen). In another embodiment, the antigen is selected from E7, E6, Her-2, NY-ESO-1, telomerase, EGFR-VIII, mesothelin, SCCE, WT-1, HIV-1 Gag, Proteinase 3, PSA (prostate-

specific antigen). In another embodiment, the antigen is a tumor-associated antigen. In another embodiment, the antigen is an infectious disease antigen.

[00106] In another embodiment, the tumor-associated antigen is an angiogenic antigen. In another embodiment, the angiogenic antigen is expressed on both activated pericytes and pericytes in tumor angiogenic vasculature, which in another embodiment, is associated with neovascularization *in vivo*. In another embodiment, the angiogenic antigen is HMW-MAA or a fragment thereof. In another embodiment, HMW-MAA is also known as CSPG4. In another embodiment, the angiogenic antigen is one known in the art and are provided in WO2010/102140, which is incorporated by reference herein.

[00107] In other embodiments, the antigen is a heterologous antigen derived from a fungal pathogen, bacteria, parasite, helminth, or viruses. In other embodiments, the antigen is selected from tetanus toxoid, hemagglutinin molecules from influenza virus, diphtheria toxoid, HIV gp120, HIV gag protein, IgA protease, insulin peptide B, *Spongospora subterranea* antigen, vibriose antigens, *Salmonella* antigens, pneumococcus antigens, respiratory syncytial virus antigens, *Haemophilus influenza* outer membrane proteins, *Helicobacter pylori* urease, *Neisseria meningitidis* pilins, *N. gonorrhoeae* pilins, the melanoma-associated antigens (TRP-2, MAGE-1, MAGE-3, gp-100, tyrosinase, MART-1, HSP-70, beta-HCG), human papilloma virus antigens E1 and E2 from type HPV-16, -18, -31, -33, -35 or -45 human papilloma viruses, the tumor antigens CEA, the ras protein, mutated or otherwise, the p53 protein, mutated or otherwise, Muc1, mesothelin, EGFRVIII or pSA.

[00108] In other embodiments, the antigen is associated with one of the following diseases; cholera, diphtheria, *Haemophilus*, hepatitis A, hepatitis B, influenza, measles, meningitis, mumps, pertussis, small pox, pneumococcal pneumonia, polio, rabies, rubella, tetanus, tuberculosis, typhoid, Varicella-zoster, whooping cough, yellow fever, the immunogens and antigens from Addison's disease, allergies, anaphylaxis, Bruton's syndrome, cancer, including solid and blood borne tumors, eczema, Hashimoto's thyroiditis, polymyositis, dermatomyositis, type 1 diabetes mellitus, acquired immune deficiency syndrome, transplant rejection, such as kidney, heart, pancreas, lung, bone, and liver transplants, Graves' disease, polyendocrine autoimmune disease, hepatitis, microscopic polyarteritis, polyarteritis nodosa, pemphigus, primary biliary cirrhosis, pernicious anemia, coeliac disease, antibody-mediated nephritis, glomerulonephritis, rheumatic diseases, systemic lupus erythematosus, rheumatoid arthritis, seronegative spondylarthritides, rhinitis, sjogren's syndrome, systemic sclerosis, sclerosing cholangitis, Wegener's granulomatosis, dermatitis herpetiformis, psoriasis, vitiligo, multiple sclerosis, encephalomyelitis, Guillain-Barre syndrome, myasthenia gravis, Lambert-Eaton

syndrome, sclera, episclera, uveitis, chronic mucocutaneous candidiasis, urticaria, transient hypogammaglobulinemia of infancy, myeloma, X-linked hyper IgM syndrome, Wiskott-Aldrich syndrome, ataxia telangiectasia, autoimmune hemolytic anemia, autoimmune thrombocytopenia, autoimmune neutropenia, Waldenstrom's macroglobulinemia, amyloidosis, chronic lymphocytic leukemia, non-Hodgkin's lymphoma, malarial circumsporozoite protein, microbial antigens, viral antigens, autoantigens, and listeriosis.

[00109] In another embodiment, the tumor-associated antigen is one of the following: 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 as disclosed 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.

[00110] In one embodiment, neoepitopes are generated and obtained as disclosed in any one of the following US applications (USSN 62/166,591; USSN 62/174,692; USSN 62/218,936; USSN 62/184,125, which are all incorporated by reference herein in their entirety.

[00111] In another embodiment, the *Listeria* strain comprises a nucleic acid molecule comprising an open reading frame encoding one or more peptides encoding one or more neoepitopes, wherein said one or more peptides are fused to an immunogenic protein or peptide. In another embodiment an immunogenic protein or peptide comprises a truncated LLO (tLLO), truncated ActA (tActA), or PEST amino acid sequence peptide.

[00112] In one embodiment, disclosed herein is a recombinant attenuated *Listeria* strain, wherein the *Listeria* strain comprises a nucleic acid sequence comprising one or more open reading frames encoding one or more peptides comprising one or more personalized neoepitopes, wherein the neo-epitope(s) comprises immunogenic epitopes present in a disease or condition-bearing tissue or cell of a subject having the disease or condition. In another embodiment, one or more neoepitopes are present in a disease or condition-bearing tissue or cell of a subject having the disease or condition.

[00113] In another embodiment, administering the *Listeria* strain to a subject having said disease or condition generates an immune response targeted to the subject's disease or condition.

[00114] In another embodiment, the strain is a personalized immunotherapy vector for said

subject targeted to said subject's disease or condition.

[00115] In another embodiment, the peptides comprise at least two different neo-epitopes amino acid sequences.

[00116] In another embodiment, the peptides comprise one or more neo-epitopes repeats of the same amino acid sequence.

[00117] In another embodiment, the *Listeria* strain comprises one neo-epitope. In another embodiment, the *Listeria* strain comprises the neo-epitopes in the range of about 1-100. Alternatively, the *Listeria* strain comprises the neo-epitopes in the range of about 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 5-15, 5-20, 5-25, 15-20, 15-25, 15-30, 15-35, 20-25, 20-35, 20-45, 30-45, 30-55, 40-55, 40-65, 50-65, 50-75, 60-75, 60-85, 70-85, 70-95, 80-95, 80-105 or 95-105. Alternatively, the *Listeria* strain comprises the neo-epitopes in the range of about 50-100. Alternatively, the *Listeria* strain comprises up to about 100 the neo-epitopes.

[00118] In another embodiment, the *Listeria* strain comprises above about 100 the neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 10 the neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 20 the neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 50 the neo-epitopes. Alternatively, the *Listeria* strain comprises about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 the neo-epitopes.

[00119] In one embodiment described herein, incorporation of amino acids in the range of about 5-30 amino acids flanking on each side of the detected mutation are generated. Additionally or alternatively, varying sizes of neo-epitope inserts are inserted in the range of about 8-27 amino acid sequence long. Additionally or alternatively, varying sizes of neo-epitope inserts are inserted in the range of about 5-50 amino acid sequence long.

[00120] In another embodiment, the neo-epitope sequences are tumor specific, metastases specific, bacterial infection specific, viral infection specific, and any combination thereof.

Additionally or alternatively, the neo-epitope sequences are inflammation specific, immune regulation molecule epitope specific, T-cell specific, an autoimmune disease specific, Graft-versus-host disease (GvHD) specific, and any combination thereof.

[00121] In another embodiment, one or more neo-epitopes comprise linear neo-epitopes. Additionally or alternatively, one or more neo-epitopes comprise a solvent-exposed epitope.

[00122] In another embodiment, one or more neo-epitopes comprise a T-cell epitope.

[00123] In one embodiment, the nucleic acid molecule disclosed herein is used to transform the *Listeria* in order to arrive at a recombinant *Listeria*. In another embodiment, the nucleic acid disclosed herein used to transform a *Listeria* that lacks a virulence gene. In another embodiment, the nucleic acid molecule is integrated into the *Listeria* genome that carries a non-functional virulence gene. In another embodiment, the *Listeria* comprises a mutation in a virulence gene. In yet another embodiment, the nucleic acid molecule is used to inactivate a gene (e.g. metabolic, virulence gene, or any other gene) present in the *Listeria* genome. In another embodiment, the *Listeria* already comprises an inactivation of a virulence gene. In yet another embodiment, the virulence gene disclosed herein is an *actA* gene, an *inlA* gene, an *inlB* gene, an *inlC* gene or a *prfA* gene. As will be understood by a skilled artisan, the virulence gene can be any gene known in the art to be associated with virulence in the recombinant *Listeria*.

[00124] In one embodiment, the metabolic gene, the virulence gene, etc. is lacking in a chromosome of the *Listeria* strain. In another embodiment, virulence gene, etc., or a fragment thereof is lacking in the chromosome and in any episomal genetic element of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc., or a fragment thereof 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. In another embodiment, the virulence gene is inactivated in the chromosome. In another embodiment, the virulence gene is not expressed.

[00125] In another embodiment, the nucleic acids and plasmids disclosed herein do not confer antibiotic resistance upon the recombinant *Listeria*.

[00126] “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 disclosure are composed of any type of nucleotide known in the art.

[00127] “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.

[00128] “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 vivo*.

[00129] In another embodiment, the metabolic enzyme of the methods and compositions disclosed 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.

[00130] 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.

[00131] The LLO utilized in the methods and compositions disclosed 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*.

[00132] In one embodiment, the LLO protein is encoded by the following nucleic acid sequence set forth in (SEQ ID NO: 1).

[00133] atgaaaaaataatgctagttttattacacttatattagttagtctaccaattgcgcaaaaactgaagcaaaggatgcatctgc
attcaataaagaaaattcaatttcacatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaacacgcggatg
5 aaatcgataagtatatacaaggattggattacaataaaaacaatgtattagtataccacggagatgcagtgacaaatgtgccccaagaaaa
ggttacaaagatggaaatgaatatattgttgaggagaaaaagaagaatccatcaatcaaaaataatgcagacattcaagttgtgaatgcaattt
cgagcctaacctatccaggtgctctcgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctcctgtaaaacgtgattcattaac
actcagcattgatttgccaggtatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgtaacaacgcagtaaatata
ttagtggaaagatggaatgaaaaatagctcaagcttatccaaatgtaagtgcataaattgattatgatgacgaatggcttacagtgaatcac
10 aattaattgcgaaatttggtacagcatttaaagctgtaataatagcttgaatgtaacttcggcgcaatcagtgagggaagaatgcaagaaga
agtcattagttttaacaaatttactataacgtgaatgtaatgaacctacaagacctccagatttttcggcaaaagctgttactaaagagcagttg
caagcgcttgagtgatgcagaaaatcctcctgcatatatactcaagtggtggcgatggccgtaagtttattgaaattatcaactaattcccat
agtactaaagtaaaagctgctttgatgctgccgtaagcggaaaaatctgtctcaggtgatgtagaactaacaatatcatcaaaaattctccttc
aaagccgtaatttacggagggtccgcaaaagatgaagttcaatcatcgacggcaacctcggagacttacgcgatatttgaaaaagcgcg
15 tacttttaatcgagaaacaccaggaggtccattgcttatacaacaacttcctaaaagacaatgaattagctgttattaaaaacaactcagaata
tattgaaacaactcaaaagcttatacagatggaaaaattaacatcgatcactctggaggatagcttctcaattcaacatttcttgggatgaagt
aaattatgatctcgag (SEQ ID NO: 1).

[00134] In another embodiment, the LLO protein has the sequence SEQ ID NO: 2

[00135] 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
20 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
25 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
30 L K K G A T F N R E T P G V P I A Y T T N F L K D N E L A V I K N N
S E Y I E T T S K A Y T D G K I N I D H S G G Y V A Q F N I S W D E
V N Y D L (SEQ ID NO: 2)

[00136] In another embodiment, the LLO protein has the sequence SEQ ID NO: 3:

[00137] MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSSISMAPPASPPASPKTPIEK

KHADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPrKGYKDGNEYIVVEKKKKKSNQNN
 ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSDLPGMTNQDNKIVVK
 NATKSNVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKA
 VNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN
 5 AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAADFDAVSGKSVSGDVELTNIKNSSFK
 AVIYGGS AKDEVQIIDGNLGLDRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIKNN
 SEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYDPEGNEIVQHKNWSENKSKL
 AHFTSSIYLPGNARNINVYAKECTGLAWWWRTVIDDRNLPLVKNRNISIWGTTLYPK
 YSNKVDNPIE (GenBank Accession No. P13128; SEQ ID NO: 3)

[00138] The first 25 amino acids of the proprotein corresponding to the sequences of SEQ ID NO: 2 and SEQ ID NO: 3 are the signal sequence and are cleaved from LLO when it is secreted by the bacterium. In one embodiment, the full length active LLO protein is 504 residues long.

[00139] In another embodiment, the LLO protein has an amino acid sequence encoded by the sequences set forth in GenBank Accession No. DQ054588, DQ054589, AY878649, U25452n

15 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.

[00140] 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.

[00141] In another embodiment, "truncated LLO" or "tLLO" refers to a fragment of LLO that
 20 comprises the PEST amino acid sequence 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-
 25 length LLO protein. In another embodiment, the LLO fragment is a non-hemolytic form of the LLO protein.

[00142] In another embodiment, the N-terminal fragment of an LLO protein utilized in compositions and methods of the present disclosure has the sequence:

[00143] MKKIMLVFITLILVSLPIAQQTAKDASAFNKENSISVAPPASPPASPKTPIEK
 30 KHADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPrKGYKDGNEYIVVEKKKKKSNQNN
 ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSDLPGMTNQDNKIVVK
 NATKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIAKFGTAFKA
 VNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN
 AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAADFDAVSGKSVSGDVELTNIKNSSFK

AVIYGGS AKDEVQIIDGNLGLDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIKNN
SEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYD (SEQ ID NO: 4).

[00144] In another embodiment, the LLO fragment corresponds to about AA 20-442 of an LLO protein utilized herein.

5 [00145] In another embodiment, the LLO fragment has the sequence:

[00146] MKKIMLVFITLILVSLPIAQQTAKDASAFNKENSISVAPPASPPASPKTPIEK
KHADEIDKYIQGLDYNKNNVLVYHGDAVTNVPVRKGYKDGNEYIVVEKKKKKINQNN
ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSDLPGMTNQDNKIVVK
NATKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIKFGTAFKA
10 VNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN
AENPPAYISSVAYGRQVYLKLSTNSHSTKVKA AFDAAVSGKSVSGDVELTNIKNSSFK
AVIYGGS AKDEVQIIDGNLGLDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIKNN
SEYIETTSKAYTD (SEQ ID NO: 5).

[00147] In another embodiment, “truncated LLO” or “ΔLLO” refers to a fragment of LLO that
15 comprises the PEST sequence domain. In another embodiment, the terms refer to an LLO
fragment that comprises a PEST sequence.

[00148] 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 cysteine 484. In another
embodiment, the terms refer to an LLO fragment that is not hemolytic. In another embodiment,
20 the LLO fragment is rendered non-hemolytic by deletion or mutation of the activation domain. In
another embodiment, the LLO fragment is rendered non-hemolytic by deletion or mutation of
cysteine 484. In another embodiment, the LLO fragment is rendered non-hemolytic by deletion
or mutation at another location. In another embodiment, the LLO is rendered non-hemolytic by a
deletion or mutation of the cholesterol binding domain (CBD) as detailed in US Patent No.
25 8,771,702, which is incorporated by reference herein.

[00149] In another embodiment, the LLO fragment consists of about the first 441 AA of the
LLO protein. In another embodiment, the LLO fragment consists of about the first 420 AA of
LLO. In another embodiment, the LLO fragment is a non-hemolytic form of the LLO protein.

[00150] In another embodiment, the LLO fragment consists of about residues 1-25. In another
30 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 1-175. 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.

[00151] In another embodiment, the LLO fragment contains residues of a homologous LLO protein that correspond to one of the above AA ranges. The residue numbers need not, in another embodiment, correspond exactly with the residue numbers enumerated above; e.g. if the homologous LLO protein has an insertion or deletion, relative to an LLO protein utilized herein, then the residue numbers can be adjusted accordingly. In another embodiment, the LLO fragment is any other LLO fragment known in the art.

[00152] In another embodiment, a homologous LLO refers to identity to an LLO sequence (e.g. to one of SEQ ID No: 2-5) of greater than 65%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 72%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 75%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 78%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 80%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 82%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 83%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 85%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 87%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 88%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 90%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 92%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 93%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 95%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 96%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 97%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 98%. In another

embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of greater than 99%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-5 of 100%.

[00153] In one embodiment, the live attenuated *Listeria* or recombinant *Listeria* disclosed herein expresses an ActA protein or a fragment thereof. In another embodiment of the methods and compositions of the present disclosure, a fragment of an ActA protein is fused to the heterologous antigen or a fragment thereof also disclosed herein. In another embodiment, the fragment of an ActA protein has the sequence:

[00154] In one embodiment, an ActA protein comprises the sequence set forth in SEQ ID NO: 6:

[00155] MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKTEEQPS
EVNTGPRYETAREVSSRDIKELEKSNKVRNTNKADLIAMLEKEAEGPNINNNNSEQT
ENAAINEEASGADRP AIQVERRHPGLPSDSAAEIKKRRKAIASSDSELES LTYPDKPTKV
NKKKVAKESVADASESDLSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRD
KIDENPEVKKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPTDEELRLALPETPMLLGFN
APATSEPSSFEPPTDEELRLALPETPMLLGFNAPATSEPSSFEPPTDELEIIRETA
SSLDSSFTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGRPTSEEFSSLNSGDFTDD
ENSETTEEEIDRLADLRDRGTGKHSRNAGFLPLNPFASSPVPSLSPKVSKISDRALISDIT
KKTPFKNPSQPLNVFNKKT TTKTVTKKPTPVKTAPKLAELPATKPQETVLRENKTPFIE
KQAETNKQSINMPSLPVIQKEATESDKEEMKPQTEEKMVEESESANNANGKNRSAGIE
EGKLIAKSAEDEKAKEEPPGNHTTLILAMLAIGVFSLGAFIKIIQLRKNN. (SEQ ID No: 6).

The first 29 AA of the proprotein corresponding to this sequence are the signal sequence and are cleaved from ActA protein when it is secreted by the bacterium. In one embodiment, an ActA polypeptide or peptide comprises the signal sequence, AA 1-29 of SEQ ID NO: 6 above. In another embodiment, an ActA polypeptide or peptide does not include the signal sequence, AA 1-29 of SEQ ID NO: 6 above. In another embodiment, an ActA AA sequence of methods and compositions of the present disclosure comprises the sequence set forth in SEQ ID No: 6. In another embodiment, the ActA AA sequence is a homologue of SEQ ID No: 6. In another embodiment, the ActA AA sequence is a variant of SEQ ID No: 6. In another embodiment, the ActA AA sequence is a fragment of SEQ ID No: 6. In another embodiment, the ActA AA sequence is an isoform of SEQ ID No: 6.

[00156] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide comprising the sequence:

[00157] atgcgtgcgatgatggtggttttcattactgccaatgcattacgattaaccccgacataatatttgcagcgacagatagcgaa

gattctagtctaaacacagatgaatgggaagaagaaaaaacagaagagcaaccaagcgaggtaaatacgggaccaagatacgaaactg
cacgtgaagtaagttcacgtgatattaagaactagaaaaatcgaataaagtgagaaatacgaacaaagcagacctaatagcaatgttgaaa
gaaaaagcagaaaaaggtccaatatcaataataacaacagtgaacaaactgagaatcgggctataaatgaagaggcttcaggagccgac
cgaccagctatacaagtggagcgtcgtcatccaggattgccatcgatagcgcagcggaaattaaaaaagaaggaaagccatagcatca
5 tcggatagttagcttgaaagccttacttatccggataaaccaacaaaagtaataagaaaaaagtggcgaaagagtcagttgcggatgcttc
tgaaagtgacttagattctagcatgcagtcagcagatgagcttcaccacaacctttaaaagcaaaccaacaaccattttccctaaagtattta
aaaaataaaaagatcggggaaatgggtacgtgataaaatcgacgaaaatcctgaagtaagaaagcgattgttgataaaagtgcagggtt
aattgaccaattattaacaaaaaagaaaagtgaagaggtaaatgcttcggacttcccgccaccactacggatgaagagttaaactgtctt
gccagagacaccaatgcttcttggttttaagtctcctgctacatcagaaccgagctcattcgaatttccaccaccactacggatgaagagtta
10 agacttgcttggcagagacgccaatgcttcttggttttaagtctcctgctacatcggaaccgagctcgttcgaatttccaccgctccaacaga
agatgaactagaaatcatccgggaaacagcatcctcgttagattctagttttacaagaggggatttagctagtttgagaaatgctattaatcgc
catagtcaaaatttctctgatttcccaccaatcccaacagaagaagagttgaacgggagagggcggttagacca (SEQ ID NO: 7). In

another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 7. In
another embodiment, an ActA-encoding nucleotide of methods and compositions of the present
15 disclosure comprises the sequence set forth in SEQ ID No: 7. In another embodiment, the ActA-
encoding nucleotide is a homologue of SEQ ID No: 7. In another embodiment, the ActA-
encoding nucleotide is a variant of SEQ ID No: 7. In another embodiment, the ActA-encoding
nucleotide is a fragment of SEQ ID No: 7. In another embodiment, the ActA-encoding
nucleotide is an isoform of SEQ ID No: 7.

[00158] In another embodiment, the ActA fragment is encoded by a recombinant nucleotide
comprising the sequence:

[00159] ttatcacgtaccatttccccgcatctttattttttaatacttttagggaaaaatggttttgatttgcttttaagggtgtggtgtag
actcgtctgctgactgcatgctagaatctaagtcactttcagaagcatccacaactgacttttcgccacttttctcttatttgctttgttggttatct
ggataagtaaggctttcaagtcactatccgacgacgctatggcttttctcttttttaatttccgctgcgctatccgatgacagacctggatgac
25 gacgctccacttgagagttggtcggctgactcctgaagcctcttcatttatagccacatttctgttgctcaccgttggtattattgttattcgga
cctttctctgcttttgcttcaacattgctattaggtctgctttgttcgtatttttactttattcgaattttctagttcctcaatatcacgtgaacttactca
cgtgcagtttcgtatcttgggtccgtatttacctgcttggctgctcttctgttttttcttccattcatctgtgtttagactggaattctcgtatct
gtcgtgcaaatattatgtcggggtaatcgtaatgcagttggcagtaatgaaaactaccatcatcgacgcat (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
30 disclosure 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.

[00160] In another embodiment of methods and compositions of the present disclosure, 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 disclosure 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.

[00161] An N-terminal fragment of an ActA protein utilized in methods and compositions of the present disclosure has, in another embodiment, the sequence set forth in SEQ ID NO: 9:

[00162] MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKTEEQPSEVNTGPRYETAREVSSRDIKELEKSNKVRNTNKADLIAMLEKEAEKGPNINNNNSEQTENAAINEEASGADRPAIQVERRHPGLPSDSAAEIKRRKAIASSDSELESLTYPDKPTKVNKKKVAKESVADASESDLDSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKIDENPEVKKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSEPSSEFFPPPPTDEELRLALPETPMLLGFNAPATSEPSSSEFFPPPPTDELEIIRETASSLDSSFTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGGRP. In another embodiment, an ActA AA sequence of methods and compositions of the present disclosure comprises the sequence set forth in SEQ ID No: 9. In another embodiment, the ActA AA sequence is a homologue of SEQ ID No: 9. In another embodiment, the ActA AA sequence is a variant of SEQ ID No: 9. In another embodiment, the ActA AA sequence is a fragment of SEQ ID No: 9. In another embodiment, the ActA AA sequence is an isoform of SEQ ID No: 9.

[00163] In another embodiment, the recombinant nucleotide encoding a fragment of an ActA protein comprises the sequence set forth in SEQ ID NO: 10

[00164] Atgcgtgcgatgatggtggtttcattactgccaattgcattacgattaaccccgacataatattgcagcgacagatagcgaa gattctagtctaaacacagatgaatgggaagaagaaaaacagaagagcaaccaagcgaggtaaatacgggaccaagatacgaaactg cacgtgaagtaagttcacgtgatattaagaactagaaaaatcgataaagtgagaaatacgaacaagcagacctaatagcaatgttgaaa gaaaaagcagaaaaaggtccaaatatcaataataacaacagtgaacaaactgagaatgcggctataaatgaagaggcttcaggagccgac cgaccagctatacaagtggagcgtcgtcatccaggattgccatcgatagcgcagcggaaattaaaaaagaaggaaagccatagcatca tcggatagtgcgttgaaagccttacttatccggataaaccaacaaaagtaataagaaaaagtggcgaaagagtcagttgcggatgcttc tgaaagtgacttagattctagcatgcagtcagcagatgagtcctcaccacaacctttaaaagcaaaccaacaaccatttttcctaaagtattta

aaaaataaaagatgcggggaaatgggtacgtgataaaatcgacgaaaatcctgaagtaaagcgcgattgttgataaaagtgcagggtt
aattgaccaattattaacaaaaagaaaagtgaagaggtaaatgcttcggacttcccgccaccacctacggatgaagagttaagacttgcttt
gccagagacaccaatgcttcttggttttaatgctcctgctacatcagaaccgagctcattcgaatttcaccaccacctacggatgaagagtta
agacttgctttgccagagacgccaatgcttcttggttttaatgctcctgctacatcggaaccgagctcgttcgaatttcaccgcctccaacaga
5 agatgaactagaaatcatccgggaaacagcatcctcgctagattctagttttacaagaggggatttagctagtttgagaaatgctattaatcg
catagtcaaaatttctctgatttcccaccaatcccaacagaagaagagttgaacgggagaggcggttagacca (SEQ ID NO: 10).

In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 10. In another embodiment, the recombinant nucleotide comprises any other sequence that encodes a fragment of an ActA protein.

- 10 [00165] 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 disclosure comprises the sequence set forth in Genbank Accession No. AF103807.
- 15 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.

- 20 [00166] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 11:

[00167] MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKTEEQPS
EVNTGPRYETAREVSSRDIKELEKSNKVRNTNKADLIAM LKEKA EKG (SEQ ID NO: 11).

- In another embodiment, an ActA AA sequence of methods and compositions of the present disclosure comprises the sequence set forth in SEQ ID No: 11. In another embodiment, the ActA AA sequence is a homologue of SEQ ID No: 11. In another embodiment, the ActA AA sequence is a variant of SEQ ID No: 11. In another embodiment, the ActA AA sequence is a fragment of SEQ ID No: 11. In another embodiment, the ActA AA sequence is an isoform of SEQ ID No: 11.

- 30 [00168] In another embodiment, an ActA protein comprises SEQ ID NO: 12

MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDS
SSLNTDEWEEEEKTEEQPSEVNTGPRYETAREVSSR
DIEELEKSNKVKNTNKADLIAM LKAKAEKGPN
NNNNGEQTGNVAINEEASGVDRPTLQVERRHPG

L S S D S A A E I K K R R K A I A S S D S E L E S L T Y P D K P T K A
 N K R K V A K E S V V D A S E S D L D S S M Q S A D E S T P Q P L K
 A N Q K P F F P K V F K K I K D A G K W V R D K I D E N P E V K K
 A I V D K S A G L I D Q L L T K K K S E E V N A S D F P P P P T D E
 5 E L R L A L P E T P M L L G F N A P T P S E P S S F E F P P P P T D E
 E L R L A L P E T P M L L G F N A P A T S E P S S F E F P P P P T E D
 E L E I M R E T A P S L D S S F T S G D L A S L R S A I N R H S E N F
 S D F P P I P T E E E L N G R G G R P T S E E F S S L N S G D F T D D
 E N S E T T E E E I D R L A D L R D R G T G K H S R N A G F L P L N
 10 P F I S S P V P S L T P K V P K I S A P A L I S D I T K K A P F K N P S
 Q P L N V F N K K T T T K T V T K K P T P V K T A P K L A E L P A T
 K P Q E T V L R E N K T P F I E K Q A E T N K Q S I N M P S L P V I Q
 K E A T E S D K E E M K P Q T E E K M V E E S E S A N N A N G K N
 R S A G I E E G K L I A K S A E D E K A K E E P G N H T T L I L A M
 15 L A I G V F S L G A F I K I I Q L R K N N (SEQ ID NO: 12). The first 29 AA of
 the proprotein corresponding to this sequence are the signal sequence and are cleaved from ActA
 protein when it is secreted by the bacterium. In one embodiment, an ActA polypeptide or
 peptide comprises the signal sequence, AA 1-29 of SEQ ID NO: 12. In another embodiment, an
 ActA polypeptide or peptide does not include the signal sequence, AA 1-29 of SEQ ID NO: 12.
 20 [00169] In another embodiment, a truncated ActA protein comprises SEQ ID NO: 13.
 [00170] A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P
 R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L
 K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G (SEQ ID
 NO: 13). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 13 is referred to
 25 as ActA/PEST1. In another embodiment, a truncated ActA comprises from the first 30 to amino
 acid 122 of the full length ActA sequence. In another embodiment, SEQ ID NO: 13 comprises
 from the first 30 to amino acid 122 of the full length ActA sequence. In another embodiment, a
 truncated ActA comprises from the first 30 to amino acid 122 of SEQ ID NO: 13. In another
 embodiment, SEQ ID NO: 13 comprises from the first 30 to amino acid 122 of SEQ ID NO: 12.
 30 [00171] In another embodiment, a truncated ActA protein comprises SEQ ID NO: 14
 [00172] A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P
 R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L
 K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D R P
 T L Q V E R R H P G L S S D S A A E I K K R R K A I A S S D S E L E S

L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q
 S A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D
 K (SEQ ID NO: 14). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 14 is
 referred to as ActA/PEST2. In another embodiment, a truncated ActA comprises from amino
 acid 30 to amino acid 229 of the full length ActA sequence. In another embodiment, SEQ ID
 NO: 14 comprises from about amino acid 30 to about amino acid 229 of the full length ActA
 sequence. In another embodiment, a truncated ActA comprises from about amino acid 30 to
 amino acid 229 of SEQ ID NO: 12. In another embodiment, SEQ ID NO: 14 comprises from
 amino acid 30 to amino acid 229 of SEQ ID NO: 12.

[00173] In another embodiment, a truncated ActA protein comprises SEQ ID NO: 15

[00174] A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P
 R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L
 K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D R P
 T L Q V E R R H P G L S S D S A A E I K K R R K A I A S S D S E L E S
 L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q
 S A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D
 K I D E N P E V K K A I V D K S A G L I D Q L L T K K K S E E V N A
 S D F P P P P T D E E L R L A L P E T P M L L G F N A P T P S E P S S
 F E F P P P P T D E E L R L A L P E T P M L L G F N A P A T S E P S S

(SEQ ID NO: 15). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 15 is
 referred to as ActA/PEST3. In another embodiment, this truncated ActA comprises from the first
 30 to amino acid 332 of the full length ActA sequence. In another embodiment, SEQ ID NO: 15
 comprises from the first 30 to amino acid 332 of the full length ActA sequence. In another
 embodiment, a truncated ActA comprises from about the first 30 to amino acid 332 of SEQ ID
 NO: 12. In another embodiment, SEQ ID NO: 20 comprises from the first 30 to amino acid 332
 of SEQ ID NO: 12.

[00175] In another embodiment, a truncated ActA protein comprises SEQ ID NO: 16

[00176] A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P
 R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L
 K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D R P
 T L Q V E R R H P G L S S D S A A E I K K R R K A I A S S D S E L E S
 L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q
 S A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D
 K I D E N P E V K K A I V D K S A G L I D Q L L T K K K S E E V N A

S D F P P P P T D E E L R L A L P E T P M L L G F N A P T P S E P S S
 F E F P P P P T D E E L R L A L P E T P M L L G F N A P A T S E P S S
 F E F P P P P T E D E L E I M R E T A P S L D S S F T S G D L A S L R
 S A I N R H S E N F S D F P L I P T E E E L N G R G G R P T S E (SEQ ID

5 NO: 16). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 16 is referred to as ActA/PEST4. In another embodiment, this truncated ActA comprises from the first 30 to amino acid 399 of the full length ActA sequence. In another embodiment, SEQ ID NO: 16 comprises from the first 30 to amino acid 399 of the full length ActA sequence. In another embodiment, a truncated ActA comprises from the first 30 to amino acid 399 of SEQ ID NO: 12.
 10 In another embodiment, SEQ ID NO: 16 comprises from the first 30 to amino acid 399 of SEQ ID NO: 12.

[00177] In another embodiment, a truncated ActA sequence disclosed herein is further fused to an hly signal peptide at the N-terminus. In another embodiment, the truncated ActA fused to hly signal peptide comprises SEQ ID NO: 17

15 [00178] M K K I M L V F I T L I L V S L P I A Q Q T E A S R A T D S E
 D S S L N T D E W E E E K T E E Q P S E V N T G P R Y E T A R E V S
 S R D I E E L E K S N K V K N T N K A D L I A M L K A K A E K G P N
 N N N N N G E Q T G N V A I N E E A S G V D R P T L Q V E R R H P
 G L S S D S A A E I K K R R K A I A S S D S E L E S L T Y P D K P T K
 20 A N K R K V A K E S V V D A S E S D L D S S M Q S A D E S T P Q P L
 K A N Q K P F F P K V F K K I K D A G K W V R D K. In another embodiment, a truncated ActA as set forth in SEQ ID NO: 17 is referred to as LA229.

[00179] In another embodiment, a truncated ActA fused to hly signal peptide is encoded by a sequence comprising SEQ ID NO: 18

25 Atgaaaaaaataatgctagttttattacacttatattagttagttctaccaattgcgcaaaaactgaagcatctagagcgacagatagcgaag
 attccagtctaaacacagatgaatgggaagaagaaaaacagaagagcagccaagcgaggtaaatacgggaccaagatacgaactgc
 acgtgaagtaagttcacgtgatatagggaactagaaaaatcgaataaagtgaataaacgaacaaagcagacctaataagcaatgttgaaa
 gcaaaagcagagaaaaggtccgaataacaataaacaacgggtgagcaaacaggaaatgtggctataaatgaagaggcttcaggagtcga
 ccgaccaactctgcaagtggagcgtcgtcatccaggtctgtcatcgatagcgcagcggaattaaaaaagaagaaaagccatagcgtc
 30 gtcggatagtgagcttgaaagccttacttatccagataaaccaacaaaagcaataagagaaaagtgcgaaagagtcagttgtggatgctt
 ctgaaagtgacttagattctagcatgcagtcagcagacgagtcctacaccacaacctttaaagcaaatcaaaaaccattttccctaaagtattt
 aaaaaataaaagatgcggggaaatgggtacgtgataaa (SEQ ID NO: 18). In another embodiment, SEQ ID
 NO: 18 comprises a sequence encoding a linker region (see bold, italic text) that is used to create
 a unique restriction enzyme site for XbaI so that different polypeptides, heterologous antigens,

etc. can be cloned after the signal sequence. Hence, it will be appreciated by a skilled artisan that signal peptidases act on the sequences before the linker region to cleave signal peptide.

[00180] In another embodiment, the ActA fragment is any other ActA fragment known in the art. In another embodiment, a recombinant nucleotide of the present disclosure 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.

[00181] In another embodiment, “truncated ActA” or “ Δ ActA” refers to a fragment of ActA that comprises the PEST sequence domain. In another embodiment, the terms refer to an ActA fragment that comprises a PEST sequence.

[00182] In another embodiment, the PEST amino acid(AA) sequence is another PEST AA sequence derived from a prokaryotic organism. In another embodiment, the PEST AA sequence is any other PEST AA sequence known in the art.

[00183] In another embodiment, the ActA fragment consists of about the first 100 AA of the ActA protein.

[00184] In another embodiment, the ActA fragment consists of about residues 1-25. In another embodiment, the ActA fragment consists of about residues 1-50. In another embodiment, the ActA fragment consists of about residues 1-75. In another embodiment, the ActA fragment consists of about residues 1-100. In another embodiment, the ActA fragment consists of about residues 1-125. In another embodiment, the ActA fragment consists of about residues 1-150. In another embodiment, the ActA fragment consists of about residues 1-175. In another embodiment, the ActA fragment consists of about residues 1-200. In another embodiment, the ActA fragment consists of about residues 1-225. In another embodiment, the ActA fragment consists of about residues 1-250. In another embodiment, the ActA fragment consists of about residues 1-275. In another embodiment, the ActA fragment consists of about residues 1-300. In another embodiment, the ActA fragment consists of about residues 1-325. In another embodiment, the ActA fragment consists of about residues 1-338. In another embodiment, the ActA fragment consists of about residues 1-350. In another embodiment, the ActA fragment consists of about residues 1-375. In another embodiment, the ActA fragment consists of about residues 1-400. In another embodiment, the ActA fragment consists of about residues 1-450. In another embodiment, the ActA fragment consists of about residues 1-500. In another embodiment, the ActA fragment consists of about residues 1-550. In another embodiment, the ActA fragment consists of about residues 1-600. In another embodiment, the ActA fragment consists of about residues 1-639. In another embodiment, the ActA fragment consists of about residues 30-100. In another embodiment, the ActA fragment consists of about residues 30-125.

In another embodiment, the ActA fragment consists of about residues 30-150. In another embodiment, the ActA fragment consists of about residues 30-175. In another embodiment, the ActA fragment consists of about residues 30-200. In another embodiment, the ActA fragment consists of about residues 30-225. In another embodiment, the ActA fragment consists of about residues 30-250. In another embodiment, the ActA fragment consists of about residues 30-275. In another embodiment, the ActA fragment consists of about residues 30-300. In another embodiment, the ActA fragment consists of about residues 30-325. In another embodiment, the ActA fragment consists of about residues 30-338. In another embodiment, the ActA fragment consists of about residues 30-350. In another embodiment, the ActA fragment consists of about residues 30-375. In another embodiment, the ActA fragment consists of about residues 30-400. In another embodiment, the ActA fragment consists of about residues 30-450. In another embodiment, the ActA fragment consists of about residues 30-500. In another embodiment, the ActA fragment consists of about residues 30-550. In another embodiment, the ActA fragment consists of about residues 1-600. In another embodiment, the ActA fragment consists of about residues 30-604.

[00185] In another embodiment, the ActA fragment contains residues of a homologous ActA protein that correspond to one of the above AA ranges. The residue numbers need not, in another embodiment, correspond exactly with the residue numbers enumerated above; e.g. if the homologous ActA protein has an insertion or deletion, relative to an ActA protein utilized herein, then the residue numbers can be adjusted accordingly. In another embodiment, the ActA fragment is any other ActA fragment known in the art.

[00186] In another embodiment, a homologous ActA refers to identity to an ActA sequence (e.g. to one of SEQ ID No: 6, 9, or 11-17) of greater than 70%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 72%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 75%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 78%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 80%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 82%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 83%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 85%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 87%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater

than 88%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 greater than 90%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 92%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 93%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 95%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 96%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 97%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 98%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of greater than 99%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 6, 9, or 11-17 of 100%. Each possibility represents a separate embodiment of the present disclosure .

[00187] In one embodiment, the live attenuated *Listeria* or recombinant *Listeria* disclosed herein expresses a PEST sequence peptide. In another embodiment of methods and compositions of the present disclosure, a PEST AA sequence is fused to the heterologous antigen or fragment. In another embodiment, the PEST AA sequence is KENSISSMAPPASPPASPKTPIEKKHAEIDK (SEQ ID NO: 19). In another embodiment, the PEST sequence is KENSISSMAPPASPPASPK (SEQ ID NO: 20).

[00188] 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: 21), KASVTDTSEGLDSSMQSADESTPQPLK (SEQ ID NO: 22), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 23), or RGGIPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 24). In another embodiment, the PEST AA sequence is a variant of the PEST sequence described hereinabove, which in one embodiment, is KESVVDASEDLSSMQSADESTPQPLK (SEQ ID NO: 25, KSEEVNASDFPPPPTDEELR (SEQ ID NO: 26), or RGGRPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 27), as would be understood by a skilled artisan. In another embodiment, the PEST AA sequence is from *Listeria seeligeri* cytolysin, encoded by the lso gene. In another embodiment, the PEST sequence is RSEVTISPAETPESPPATP (SEQ ID NO: 28). 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: 29) at AA 35-51. In another embodiment, the PEST AA sequence is from *Streptococcus equisimilis*

Streptolysin O, e.g. KQNTANTETTTTNEQPK (SEQ ID NO: 30) at AA 38-54. In another embodiment, the PEST AA sequence has a sequence selected from SEQ ID NO: 19-30. In another embodiment, the PEST sequence is another PEST AA sequence derived from a prokaryotic organism.

[00189] 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.

[00190] 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.

[00191] 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 AA sequence must contain at least 1 P, 1 D or E, and at least 1 S or T.

[00192] 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 Doolittle, RF. J. Mol. Biol. 157, 105 (1982).

[00193] 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:

[00194] $\text{PEST score} = 0.55 * \text{DEPST} - 0.5 * \text{hydrophobicity index}.$

[00195] In another embodiment, "PEST sequence", "PEST AA sequence" or "PEST AA sequence peptide" refers to a peptide 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.

[00196] 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:

[00197] 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.

[00198]

[00199] In another embodiment, the PEST sequence is any other PEST sequence known in the

art.

[00200] “Fusion to a PEST sequence” refers, in another embodiment, to 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. Thus, in another embodiment, “fusion” refers to two peptides or protein fragments either linked together at their respective ends or embedded one within the other.

[00201] In another embodiment, disclosed herein, is a composition comprising a recombinant form of *Listeria* of the present disclosure .

[00202] In another embodiment, disclosed herein is a vaccine comprising a recombinant form of *Listeria* of the present disclosure .

[00203] In another embodiment, disclosed herein is a culture of a recombinant form of *Listeria* of the present disclosure .

[00204] In another embodiment, the *Listeria* of methods and compositions of the present disclosure 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*.

[00205] In one embodiment, attenuated *Listeria* strains, such as *Lm* delta-*actA* mutant (Brundage et al, 1993, Proc. Natl. Acad. Sci., USA, 90:11890-11894), *L. monocytogenes* delta-*plcA* (Camilli et al, 1991, J. Exp. Med., 173:751-754), or delta-ActA, delta INL-b (Brockstedt et al, 2004, PNAS, 101:13832–13837) are used in the present disclosure . In another embodiment, attenuated *Listeria* strains are constructed by introducing one or more attenuating mutations, as will be understood by one of average skill in the art when equipped with the disclosure herein. Examples of such strains include, but are not limited to *Listeria* strains auxotrophic for aromatic amino acids (Alexander et al, 1993, Infection and Immunity 10 61:2245-2248) and mutant for the formation of lipoteichoic acids (Abachin et al, 2002, Mol. Microbiol. 43:1-14) and those attenuated by a lack of a virulence gene (see examples herein).

[00206] In another embodiment, the nucleic acid molecule of methods and compositions of the present disclosure is operably linked to a promoter/regulatory sequence. In another embodiment, the first open reading frame of methods and compositions of the present disclosure is operably linked to a promoter/regulatory sequence. In another embodiment, the second open reading frame of methods and compositions of the present disclosure 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.

[00207] The skilled artisan, when equipped with the present disclosure and the methods disclosed 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 disclosure . As is contemplated in the present disclosure , 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 disclosure . In another embodiment, a commercially available plasmid is used in the present disclosure . 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.

[00208] 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.

[00209] 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).

[00210] 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 disclosure include, but are not limited to, gene products that confer resistance to ampicillin, penicillin, methicillin, streptomycin, erythromycin, kanamycin, tetracycline, chloramphenicol (CAT), neomycin, hygromycin, gentamicin and others well known in the art.

[00211] 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 disclosure is transformed by electroporation.

[00212] 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).

[00213] “Transforming,” in one embodiment, is used identically with the term “transfecting,” and refers to engineering a bacterial cell to take up a plasmid or other heterologous DNA molecule. In another embodiment, “transforming” refers to engineering a bacterial cell to express a gene of a plasmid or other heterologous DNA molecule.

[00214] Plasmids and other expression vectors useful in the present disclosure 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 disclosure 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 disclosure 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.

[00215] 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.

[00216] In another embodiment, the present disclosure further comprises a phage based chromosomal integration system for clinical applications. A host strain that is auxotrophic for essential enzymes, including, but not limited to, d-alanine racemase will be used, for example Lmdal(-)dat(-). In another embodiment, in order to avoid a "phage curing step," a phage integration system based on PSA is used (Lauer, et al., 2002 J Bacteriol, 184:4177-4186). This requires, in another embodiment, continuous selection by antibiotics to maintain the integrated gene. Thus, in another embodiment, the current disclosure enables the establishment of a phage based chromosomal integration system that does not require selection with antibiotics. Instead, an auxotrophic host strain will be complemented.

[00217] The recombinant proteins of the present disclosure 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 disclosure, 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 disclosure 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.

[00218] 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.

5 [00219] In another embodiment, DNA encoding the recombinant protein of the present disclosure 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.

10 [00220] 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
15 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.

[00221] 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
20 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.

[00222] 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
25 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
30 bacterium auxotrophic for D-alanine synthesis will grow in the absence of D-alanine when transformed and expressing the plasmid of the present disclosure 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).

[00223] In another embodiment, once the auxotrophic bacteria comprising the plasmid of the present disclosure 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.

[00224] The skilled artisan will appreciate that, in another embodiment, other auxotroph strains and complementation systems are adopted for the use with this disclosure.

[00225] In one embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using homologous recombination. Techniques for homologous recombination are well known in the art, and are described, for example, in Baloglu S, Boyle SM, et al. (Immune responses of mice to vaccinia virus recombinants expressing either *Listeria monocytogenes* partial listeriolysin or *Brucella abortus* ribosomal L7/L12 protein. Vet Microbiol 2005, 109(1-2): 11-7); and Jiang LL, Song HH, et al., (Characterization of a mutant *Listeria monocytogenes* strain expressing green fluorescent protein. Acta Biochim Biophys Sin (Shanghai) 2005, 37(1): 19-24). In another embodiment, homologous recombination is performed as described in United States Patent No. 6,855,320. In this case, a recombinant *Lm* strain that expresses E7 was made by chromosomal integration of the E7 gene under the control of the *hly* promoter and with the inclusion of the *hly* signal sequence to ensure secretion of the gene product, yielding the recombinant referred to as Lm-AZ/E7. In another embodiment, a temperature sensitive plasmid is used to select the recombinants.

[00226] 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.

[00227] 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.

[00228] In another embodiment, one of various promoters is used to express protein containing same. In one embodiment, an *Lm* promoter is used, e.g. promoters for the genes *hly*, *actA*, *plcA*, *plcB* and *mpl*, which encode the *Listerial* proteins hemolysin, ActA, phosphatidylinositol-specific phospholipase, phospholipase C, and metalloprotease, respectively.

[00229] In another embodiment, methods and compositions of the present disclosure utilize a homologue of a heterologous antigen or LLO or ActA or PEST containing sequence of the present disclosure. 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.

[00230] 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.

[00231] 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.

[00232] In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-50 of greater than about 70%. In another embodiment, "homology" refers to identity to a sequence selected from SEQ ID No: 1-50 of greater than about 70%. In another embodiment, the identity is greater than about 75%. In another embodiment, the identity is greater than about 78%. In another embodiment, the identity is greater than about 80%. In another embodiment, the identity is greater than about 82%. In another embodiment, the identity is greater than about 83%. In another embodiment, the identity is greater than about 85%. In

another embodiment, the identity is greater than about 87%. In another embodiment, the identity is greater than about 88%. In another embodiment, the identity is greater than about 90%. In another embodiment, the identity is greater than about 92%. In another embodiment, the identity is greater than about 93%. In another embodiment, the identity is greater than about 95%. In another embodiment, the identity is greater than about 96%. In another embodiment, the identity is greater than about 97%. In another embodiment, the identity is greater than 98%. In another embodiment, the identity is greater than about 99%. In another embodiment, the identity is 100%.

[00233] 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.

[00234] In one embodiment of the present disclosure, "nucleic acids" refers to a string of at least two base-sugar-phosphate combinations. The term includes, in one embodiment, DNA and RNA. "Nucleotides" refers, in one embodiment, to the monomeric units of nucleic acid polymers. RNA may be, in one embodiment, in the form of a tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, small inhibitory RNA (siRNA), micro RNA (miRNA) and ribozymes. The use of siRNA and miRNA has been described (Caudy AA et al, Genes & Devel 16: 2491-96 and references cited therein). DNA may be in form of plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition, these forms of DNA and RNA may be single, double, triple, or quadruple stranded. The term also includes, in another embodiment, artificial nucleic acids that may contain other types of backbones but the same bases. In one embodiment, the artificial nucleic acid is a PNA (peptide nucleic acid). PNA contain peptide backbones and nucleotide bases and are able to bind, in one embodiment, to both DNA and RNA molecules. In another embodiment, the nucleotide is oxetane modified. In another embodiment, the nucleotide is modified by replacement of one or more phosphodiester bonds with a phosphorothioate bond.

In another embodiment, the artificial nucleic acid contains any other variant of the phosphate backbone of native nucleic acids known in the art. The use of phosphothiorate nucleic acids and DNA are known to those skilled in the art, and are described in, for example, Neilsen PE, Curr Opin Struct Biol 9:353-57; and Raz NK et al Biochem Biophys Res Commun. 297:1075-84. The production and use of nucleic acids is known to those skilled in art and is described, for example, in Molecular Cloning, (2001), Sambrook and Russell, eds. and Methods in Enzymology: Methods for molecular cloning in eukaryotic cells (2003) Purchio and G. C. Fareed.

[00235] In another embodiment, the terms “gene” and “recombinant gene” refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide of the disclosure. 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 disclosure.

[00236] 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.

Compositions

[00237] In one embodiment, a composition for use in the methods of the present disclosure comprises a recombinant *Listeria monocytogenes*, in any form or embodiment as described herein. In one embodiment, the composition for use in the present disclosure consists of a recombinant *Listeria monocytogenes* of the present disclosure, in any form or embodiment as described herein. In another embodiment, the composition for use in the methods of the present disclosure consists essentially of a recombinant *Listeria monocytogenes* of the present disclosure, in any form or embodiment as described herein. In one embodiment, the term “comprise” refers to the inclusion of a recombinant *Listeria monocytogenes* in the composition, as well as inclusion of other composition or treatments that may be known in the art. In another embodiment, the term “consisting essentially of” refers to a composition, whose functional component is the recombinant *Listeria monocytogenes*, however, other components of the composition may be

included that are not involved directly in the therapeutic effect of the composition and may, for example, refer to components which facilitate the effect of the recombinant *Listeria monocytogenes* (e.g. stabilizing, preserving, etc.). In another embodiment, the term “consisting” refers to a composition, which contains the recombinant *Listeria monocytogenes*.

5 [00238] In one embodiment, the immune response elicited by the compositions and methods disclosed herein is not antigen specific.

[00239] In one embodiment, the recombinant *Listeria monocytogenes* for use in the compositions, vaccines and methods of the present disclosure does not secrete a heterologous peptide. In another embodiment, the recombinant *Listeria monocytogenes* for use in the
10 compositions, vaccines and methods of the present disclosure does not express a heterologous peptide. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses and secretes a non-hemolytic LLO, as described herein. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses and secretes a truncated ActA polypeptide, as described herein. In another
15 embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses and secretes a PEST-containing polypeptide, as described herein.

[00240] In one embodiment, the recombinant *Listeria monocytogenes* for use in the compositions, vaccines and methods of the present disclosure secretes a heterologous peptide. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses a heterologous peptide. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses and secretes a non-hemolytic LLO, as
20 described herein. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses and secretes a truncated ActA polypeptide, as described herein. In another embodiment, the recombinant *Listeria monocytogenes* for use in the present disclosure expresses and secretes a PEST-containing polypeptide, as described herein.
25

[00241] In one embodiment, compositions of the present disclosure are immunogenic compositions. In one embodiment, compositions of the present disclosure induce a strong innate stimulation of interferon-gamma, which in one embodiment, has anti-angiogenic properties. In one embodiment, a *Listeria* of the present disclosure induces a strong innate stimulation of
30 interferon-gamma, which in one embodiment, has anti-angiogenic properties (Dominiecki et al., Cancer Immunol Immunother. 2005 May;54(5):477-88. Epub 2004 Oct 6, incorporated herein by reference in its entirety; Beatty and Paterson, J. Immunol. 2001 Feb 15;166(4):2276-82, incorporated herein by reference in its entirety). In one embodiment, anti-angiogenic properties of *Listeria* are mediated by CD4⁺ T cells (Beatty and Paterson, 2001). In another embodiment,

anti-angiogenic properties of *Listeria* are mediated by CD8⁺ T cells. In another embodiment, IFN-gamma secretion as a result of *Listeria* vaccination is mediated by NK cells, NKT cells, Th1 CD4⁺ T cells, TC1 CD8⁺ T cells, or a combination thereof.

[00242] In another embodiment, administration of compositions of the present disclosure induce production of one or more anti-angiogenic proteins or factors. In one embodiment, the anti-angiogenic protein is IFN-gamma. In another embodiment, the anti-angiogenic protein is pigment epithelium-derived factor (PEDF); angiostatin; endostatin; fms-like tyrosine kinase (sFlt)-1; or soluble endoglin (sEng). In one embodiment, a *Listeria* of the present disclosure is involved in the release of anti-angiogenic factors, and, therefore, in one embodiment, has a therapeutic role in addition to its role as a vector for introducing an antigen to a subject.

[00243] The immune response induced by methods and compositions as disclosed herein is, in another embodiment, a T cell response. In another embodiment, the immune response comprises a T cell response. In another embodiment, the response is a CD8⁺ T cell response. In another embodiment, the response comprises a CD8⁺ T cell response. Each possibility represents a separate embodiment as disclosed herein.

[00244] In another embodiment, administration of compositions of the present disclosure increase the number of T cells. In another embodiment, administration of compositions activates co-stimulatory receptors on T cells. In another embodiment, administration of compositions induces proliferation of memory and/or effector T cells. In another embodiment, administration of compositions increases proliferation of T cells. disclosed herein

[00245] As used throughout, the terms “composition” and “immunogenic composition” are interchangeable having all the same meanings and qualities. The term “pharmaceutical composition” refers, in some embodiments, to a composition suitable for pharmaceutical use, for example, to administer to a subject in need.

[00246] In one embodiment, the term “immunogenic composition” may encompass the recombinant *Listeria* disclosed herein, and an adjuvant, a chimeric antigen receptor engineered cells (CAR T cells), or Receptor engineered T cells, or any combination thereof. In another embodiment, an immunogenic composition comprises a recombinant *Listeria* disclosed herein. In another embodiment, an immunogenic composition comprises an adjuvant known in the art or as disclosed herein. In another embodiment, an immunogenic composition comprises a PEST-containing polypeptide disclosed herein. It is also to be understood that administration of such compositions enhance an immune response, or increase a T effector cell to regulatory T cell ratio or elicit an anti-tumor immune response, as further disclosed herein.

[00247] Compositions of this disclosure may be used in methods of this disclosure in order to

improve maturation of immunity in a subject, in order enhance engraftment of cells disclosed herein in a subject, or for decreasing time to immune-competence in a subject, or for accelerating immunogenic competence, or any combination thereof. disclosed herein

[00248] In another embodiment, a composition comprising a *Listeria* strain of the present disclosure further comprises an adjuvant. In one embodiment, a composition of the present disclosure further comprises an adjuvant. The adjuvant utilized in methods and compositions of the present disclosure 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.

[00249] Following the administration of the immunogenic compositions disclosed herein, the methods disclosed herein induce the expansion of T effector cells in peripheral lymphoid organs leading to an enhanced presence of T effector cells at the tumor site. In another embodiment, the methods disclosed herein induce the expansion of T effector cells in peripheral lymphoid organs leading to an enhanced presence of T effector cells at the periphery. Such expansion of T effector cells leads to an increased ratio of T effector cells to regulatory T cells in the periphery and at the tumor site without affecting the number of Tregs. It will be appreciated by the skilled artisan that peripheral lymphoid organs include, but are not limited to, the spleen, peyer's patches, the lymph nodes, the adenoids, etc. In one embodiment, the increased ratio of T effector cells to regulatory T cells occurs in the periphery without affecting the number of Tregs. In another embodiment,

the increased ratio of T effector cells to regulatory T cells occurs in the periphery, the lymphoid organs and at the tumor site without affecting the number of Tregs at these sites. In another embodiment, the increased ratio of T effector cells decrease the frequency of Tregs, but not the total number of Tregs at these sites.

5 [00250] In another embodiment, an “immunogenic fragment” is one that elicits an immune response when administered to a subject alone or in a vaccine or composition as disclosed herein. Such a fragment contains, in another embodiment, the necessary epitopes in order to elicit an adaptive immune response.

[00251] In one embodiment, a composition of this disclosure comprises a recombinant
10 *Listeria monocytogenes* (*Lm*) strain.

[00252] The compositions of this disclosure, in another embodiment, are administered to a subject by any method known to a person skilled in the art, such as parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intra-dermally, subcutaneously, intra-peritoneally, intra-ventricularly, intra-cranially, intra-vaginally or intra-tumorally.

15 [00253] In some embodiments, when the CAR T cells disclosed herein are administered separately from a composition comprising a recombinant *Lm* strain or *Lm*-LLO strain (comprising a tLLO) disclosed herein, the CAR T cells may be injected intravenously, subcutaneously, or directly into the tumor or tumor bed. In one embodiment, a composition comprising CAR T cells is injected into the space left after a tumor has been surgically removed,
20 e.g., the space in a prostate gland following removal of a prostate tumor.

[00254] In another embodiment, the 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
25 the like. In another embodiment of the present disclosure, the active ingredient is formulated in a capsule. In accordance with this embodiment, the compositions of the present disclosure comprise, in addition to the active compound and the inert carrier or diluent, a hard gelating capsule.

[00255] In another embodiment, compositions are administered by intravenous, intra-arterial,
30 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 intramuscularly and are thus formulated in a form suitable for intra-muscular administration.

[00256] In one embodiment, an immunogenic composition comprises a recombinant *Listeria* disclosed herein. In another embodiment, an immunogenic composition comprises an adjuvant known in the art or as disclosed herein. It is also to be understood that administration of such compositions improves maturation of immunity, enhance an immune response, or increase a T effector cell to regulatory T cell ratio, or enhances engraftment of cells disclosed herein (for a cell-based therapy of a subject suffering from a tumor or cancer), or decreases time to immune-competence or elicit an anti-tumor immune response, or any combination thereof.

[00257] In one embodiment, this disclosure provides methods of use which comprise administering a composition comprising the described *Listeria* strains.

[00258] In one embodiment, the term "pharmaceutical composition" encompasses a therapeutically effective amount of the active ingredient or ingredients including the *Listeria* strain, together with a pharmaceutically acceptable carrier or diluent. It is to be understood that the term a "therapeutically effective amount" refers to that amount which provides a therapeutic effect for a given condition and administration regimen.

[00259] It will be understood by the skilled artisan that the term "administering" encompasses bringing a subject in contact with a composition of the present disclosure. In one embodiment, administration can be accomplished *in vitro*, i.e. in a test tube, or *in vivo*, i.e. in cells or tissues of living organisms, for example humans. In one embodiment, the present disclosure encompasses administering the *Listeria* strains and compositions thereof of the present disclosure to a subject.

[00260] The term "about" as used herein means in quantitative terms plus or minus 5%, or in another embodiment plus or minus 10%, or in another embodiment plus or minus 15%, or in another embodiment plus or minus 20%.

[00261] 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 disclosure.

[00262] Various embodiments of dosage ranges are contemplated by this disclosure. In one embodiment, in the case of vaccine vectors, the dosage is in the range of 0.4 LD₅₀/dose. In another embodiment, the dosage is from about 0.4-4.9 LD₅₀/dose. In another embodiment the dosage is from about 0.5-0.59 LD₅₀/dose. In another embodiment the dosage is from about 0.6-0.69 LD₅₀/dose. In another embodiment the dosage is from about 0.7-0.79 LD₅₀/dose. In another embodiment the dosage is about 0.8 LD₅₀/dose. In another embodiment, the dosage is 0.4 LD₅₀/dose to 0.8 of the LD₅₀/dose.

[00263] In another embodiment, the dosage is 10^7 bacteria/dose. In another embodiment, the dosage is 1.5×10^7 bacteria/dose. In another embodiment, the dosage is 2×10^7 bacteria/dose. In another embodiment, the dosage is 3×10^7 bacteria/dose. In another embodiment, the dosage is 4×10^7 bacteria/dose. In another embodiment, the dosage is 6×10^7 bacteria/dose. In another embodiment, the dosage is 8×10^7 bacteria/dose. In another embodiment, the dosage is 1×10^8 bacteria/dose. In another embodiment, the dosage is 1.5×10^8 bacteria/dose. In another embodiment, the dosage is 2×10^8 bacteria/dose. In another embodiment, the dosage is 3×10^8 bacteria/dose. In another embodiment, the dosage is 4×10^8 bacteria/dose. In another embodiment, the dosage is 6×10^8 bacteria/dose. In another embodiment, the dosage is 8×10^8 bacteria/dose. In another embodiment, the dosage is 1×10^9 bacteria/dose. In another embodiment, the dosage is 1.5×10^9 bacteria/dose. In another embodiment, the dosage is 2×10^9 bacteria/dose. In another embodiment, the dosage is 3×10^9 bacteria/dose. In another embodiment, the dosage is 5×10^9 bacteria/dose. In another embodiment, the dosage is 6×10^9 bacteria/dose. In another embodiment, the dosage is 8×10^9 bacteria/dose. In another embodiment, the dosage is 1×10^{10} bacteria/dose. In another embodiment, the dosage is 1.5×10^{10} bacteria/dose. In another embodiment, the dosage is 2×10^{10} bacteria/dose. In another embodiment, the dosage is 3×10^{10} bacteria/dose. In another embodiment, the dosage is 5×10^{10} bacteria/dose. In another embodiment, the dosage is 6×10^{10} bacteria/dose. In another embodiment, the dosage is 8×10^{10} bacteria/dose. In another embodiment, the dosage is 8×10^9 bacteria/dose. In another embodiment, the dosage is 1×10^{11} bacteria/dose. In another embodiment, the dosage is 1.5×10^{11} bacteria/dose. In another embodiment, the dosage is 2×10^{11} bacteria/dose. In another embodiment, the dosage is 3×10^{11} bacteria/dose. In another embodiment, the dosage is 5×10^{11} bacteria/dose. In another embodiment, the dosage is 6×10^{11} bacteria/dose. In another embodiment, the dosage is 8×10^{11} bacteria/dose.

[00264] In one embodiment, the adjuvant vaccine of the present disclosure comprise a vaccine given in conjunction. In another embodiment, the adjuvant vaccine of the present disclosure is administered following administration of a vaccine regimen, wherein the vaccine regimen is a viral, bacteria, nucleic acid, or recombinant polypeptide vaccine formulation.

[00265] “Adjuvant” typically refers, in another embodiment, to compounds that, when administered to an individual or tested *in vitro*, increase the immune response to an antigen in the individual or test system to which the antigen is administered. In another embodiment, an immune adjuvant enhances an immune response to an antigen that is weakly immunogenic when administered alone, i.e., inducing no or weak antibody titers or cell-mediated immune response. In another embodiment, the adjuvant increases antibody titers to the antigen. In another

embodiment, the adjuvant lowers the dose of the antigen effective to achieve an immune response in the individual. However, in one embodiment, the adjuvant enhances an immune response in an antigen-unspecific manner in order to enable a heightened state of an immune response, as it applies to neonates, or in order to enable the recovery of the immune response following cytotoxic treatment, as it applies to older children and adults and also as further disclosed herein.

[00266] In another embodiment, the methods of the present disclosure 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.

[00267] In one embodiment, the prime and booster vaccinations are administered prior to a cell-based therapy disclosed herein.

[00268] In one embodiment, a vaccine or immunogenic composition of the present disclosure is administered alone to a subject prior to a cancer therapy. In another embodiment, the cancer therapy is a cell-based therapy, chemotherapy, immuno therapy, radiation, surgery or any other type of therapy available in the art as will be understood by a skilled artisan. Each possibility represents a separate embodiment of the present disclosure .

[00269] In another embodiment, the present disclosure provides a kit comprising a reagent utilized in performing a method of the present disclosure . In another embodiment, the present disclosure provides a kit comprising a composition, vaccine, tool, or instrument of the present disclosure .

[00270] The terms “contacting” or “administering,” in one embodiment, refer to directly contacting a cell or tissue of a subject with a composition of the present disclosure . In another embodiment, the terms refer to indirectly contacting a cell or tissue of a subject with a composition of the present disclosure.

Methods of Use

[00271] The ability of an adjuvant to increase the immune response to an antigen is typically

manifested by a significant increase in immune-mediated protection. For example, an increase in humoral immunity is typically manifested by a significant increase in the titer of antibodies raised to the antigen, and an increase in T-cell activity is typically manifested in increased cell proliferation, increased cytokine production and/or antigen specific cytolytic activity. An adjuvant may also alter an immune response, for example, by enabling a Th1 response against a background of a persistent Th2 phenotype. An adjuvant may also alter an immune response by relieving immunosuppression by regulatory T cells, myeloid-derived suppressor cells, either by reducing the levels of these cells in tissues or reducing the immunosuppressive molecules they secrete.

[00272] In one embodiment, this disclosure provides methods and compositions for subjects receiving an engraftment of immune cells in the form of a cell-based therapy. It will be understood by a skilled artisan that the term "engraftment" may encompass the transfer of autologous or recombinant cells into a subject. In another embodiment the cells are T-cells. In another embodiment, the T-cells are activated T-cells. In one embodiment, the autologous cells are extracted from the bone marrow of the subject as hematopoietic stem cells in order to differentiate them *in vitro* to recognize a specific antigen and are then transferred to or engrafted into the subject at a desired dose for the purposes of providing an anti-tumor therapy. In another embodiment, the recombinant cells are chimeric antigen receptor (CAR) T cells also for providing an anti-tumor therapy.

[00273] In one embodiment a subject is a human. In another embodiment, a subject is a non-human mammal. In another embodiment, a non-human mammal may be a dog, a cat, a pig, a cow, a sheep, a goat, a horse, a rat, a mouse. In another embodiment, a subject is immune-compromised. In another embodiment, a subject is immune-incompetent. The term "subject" does not exclude an individual that is normal in all respects.

[00274] In one embodiment, a subject is receiving an engraftment as a treatment for a solid tumor or solid cancer. In another embodiment, the solid tumor or solid cancer is any such tumor or cancer known in the art some of which are also disclosed herein.

[00275] In one embodiment, a subject is receiving an engraftment as a treatment for a cancer or a hematopoietic disease. In another embodiment, a hematopoietic disease is a hematopoietic malignancy. In one embodiment a hematopoietic malignancy comprises leukemia, myelodysplastic syndrome (MDS), lymphoma, multiple myeloma, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) or chronic myelogenous leukemia (CML), or any combination thereof.

[00276] In another embodiment, an engraftment is autologous, or allogeneic. In another

embodiment, the present disclosure is directed to enhancing immune response, or decreasing time to immunocompetence or improving maturation of immunity in an adult human, a human child, or a human neonate, or a non-human mammal that has received an engraftment as disclosed herein as a result of cancer.

5 [00277] In one embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing a truncated listeriolysin O in combination with other therapeutic modalities are useful for enhancing an immune response. In another embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing truncated listeriolysin O alone, or in combination with other therapeutics are useful for preventing, and treating infectious diseases in a subject.

10 [00278] In one embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing N-terminal ActA polypeptide in combination with other therapeutic modalities are useful for enhancing an immune response. In another embodiment, recombinant attenuated, antibiotic-free *Listerias* expressing N-terminal ActA polypeptide alone, or in combination with other therapeutics are useful for preventing, and treating infectious diseases in a subject.

15 [00279] In one embodiment, the immune response induced by the methods and compositions disclosed 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 disclosed herein. In another embodiment, the immune response leads to clearance of a disease disclosed
20 herein that is afflicting the subject.

[00280] It is to be understood that the methods of the present disclosure may be used to treat any infectious disease, which in one embodiment, is bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection. In another embodiment, the methods of the present disclosure are for inhibiting or suppressing a bacterial, viral, microbial, microorganism,
25 pathogenic, or combination thereof, infection in a subject. In another embodiment, the present disclosure provides a method of eliciting a cytotoxic T-cell response against a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof, infection in a subject. In another embodiment, the present disclosure provides a method of inducing a Th1 immune response against a bacterial, viral, microbial, microorganism, pathogenic, or combination thereof,
30 infection in a Th1 unresponsive subject. In one embodiment, the infection is viral, which in one embodiment, is HIV. In one embodiment, the infection is bacterial, which in one embodiment, is mycobacteria, which in one embodiment, is tuberculosis. In one embodiment, the infection is eukaryotic, which in one embodiment, is plasmodium, which in one embodiment, is malaria.

[00281] In another embodiment, disclosed herein is a method of improving the

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

[00282] In one embodiment, disclosed herein is a method of enhancing an immune response against a disease in an antigen-independent manner, the method comprising administering a *Listeria*-based adjuvant to a subject.

[00283] In another embodiment, the methods of the present disclosure 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 disclosure consist of the step of administering a recombinant *Listeria monocytogenes* of the present disclosure, in any form or embodiment as described herein. In another embodiment, the methods of the present disclosure consist essentially of the step of administering a recombinant *Listeria monocytogenes* of the present disclosure, 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.

[00284] In another embodiment, the immune response elicited by methods and compositions of the present disclosure comprises a CD8⁺ T cell-mediated response. In another embodiment, the immune response consists primarily of a CD8⁺ T cell-mediated response. In another embodiment, the only detectable component of the immune response is a CD8⁺ T cell-mediated response (see Examples 7-11).

[00285] In another embodiment, the immune response elicited by methods and compositions disclosed herein comprises a CD4⁺ T cell-mediated response. In another embodiment, the immune response consists primarily of a CD4⁺ T cell-mediated response. In another embodiment, the only detectable component of the immune response is a CD4⁺ T cell-mediated response. In another embodiment, the CD4⁺ T cell-mediated response is accompanied by a

measurable antibody response against the antigen. In another embodiment, the CD4⁺ T cell-mediated response is not accompanied by a measurable antibody response against the antigen (see Examples 7-11).

[00286] In another embodiment, the immune response elicited by methods and compositions disclosed herein comprises an innate immune response wherein M1 macrophages and dendritic cells (DCs) are activated.

[00287] In one embodiment, disclosed herein is a method of increasing intratumoral ratio of CD8+/T regulatory cells, whereby and in another embodiment, the method comprising the step of administering to the subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present disclosure (see Examples 7-11).

[00288] In another embodiment, disclosed herein is a method of increasing intratumoral ratio of CD8+/T regulatory cells, whereby and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant polypeptide, recombinant *Listeria*, or recombinant vector of the present disclosure (see Examples 7-11).

[00289] In one embodiment, disclosed herein is a method of increasing intratumoral ratio of CD8+/ myeloid-derived suppressor cells (MDSC), whereby and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria*, or recombinant vector of the present disclosure.

[00290] In another embodiment, disclosed herein is a method of increasing the ratio of CD8+/ myeloid-derived suppressor cells (MDSC) at sites of disease, whereby and in another embodiment, the method comprises the step of administering to the subject a composition comprising the recombinant *Listeria*, or recombinant vector of the present disclosure .

[00291] 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/or granulocytic as well.

[00292] In one embodiment, MDSCs at disease sites 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 7-11), the live attenuated *Listeria* disclosed herein reduces the amount or quantity of suppressor cells in a disease thereby allowing CD8 T cell replication and infiltration at the

disease site, for example, a tumor site.

[00293] In another embodiment, both monocytic and granulocytic MDSCs purified from the tumors of *Listeria*-treated mice are less able to suppress the division of CD8+ T cells than MDSCs purified from the tumors of untreated mice, whereas monocytic and granulocytic MDSCs purified from the spleens of these same tumor-bearing mice show no change in their function after vaccination with *Listeria* (see Examples 7-11 herein). In one embodiment, this effect is seen because splenic MDSCs are only suppressive in an antigen-specific manner. Hence, treatment with *Listeria* has the distinct advantage that it allows for tumor-specific inhibition of tumor suppressive cells such as Tregs and MDSCs (see Examples 7-11 herein).

Another unexpected advantage provided by the live attenuated *Listeria* of the methods and compositions disclosed herein is that there are lower amount of Tregs in the tumor, and the ones that persist lose the ability to suppress T cell replication (see Examples 7-11 herein).

[00294] In one embodiment, disclosed 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.

[00295] In another embodiment, disclosed 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.

[00296] 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.

[00297] In one embodiment, disclosed 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.

[00298] In another embodiment, disclosed 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.

[00299] In one embodiment, the term "percentage" is representative of the amount, quantity,

or numbers, etc., of either Tregs, MDSCs, or CD8/CD4 T cells measures in an assay or in an immune response. In another embodiment, it refers to the amount, quantity, percentage, etc. of any composition, cell, protein, bacteria or *Listeria* cell disclosed herein.

[00300] In another embodiment, disclosed herein is a method of eliciting an enhanced

immune response in a subject recovering from cytotoxic treatment to a tumor or a cancer, the method comprising administering to said subject a composition comprising the recombinant *Listeria* strain disclosed herein. In another embodiment, the recombinant *Listeria* strain comprises a mutation or deletion of the *inlB* gene, the *inlC* gene, an *actA* gene, a *prfA* gene, a *PlcA* gene, a *PLcB* gene, a *dal* gene, a *dal* and *dat*, a *dal* or *dat* gene, or any combination thereof.

In another embodiment, the recombinant *Listeria* strain comprises an *inlA* and *actA* mutation or deletion. In another embodiment, the recombinant *Listeria* strain comprises an *inB* or *actA* mutation or deletion. In another embodiment, the recombinant *Listeria* strain consists of an *inlB* and *actA* mutation or deletion.

[00301] In one embodiment, disclosed herein is a method of administering the composition of

the present disclosure . In another embodiment, disclosed herein is a method of administering the vaccine of the present disclosure . In another embodiment, disclosed herein is a method of administering the recombinant polypeptide or recombinant nucleotide of the present disclosure .

In another embodiment, the step of administering the composition, vaccine, recombinant polypeptide or recombinant nucleotide of the present disclosure is performed with an attenuated

recombinant form of *Listeria* comprising the composition, vaccine, recombinant nucleotide or expressing the recombinant polypeptide, each in its own discrete embodiment. In another embodiment, the administering is performed with a DNA vaccine (e.g. a naked DNA vaccine).

In another embodiment, administration of a recombinant polypeptide of the present disclosure is performed by producing the recombinant protein, then administering the recombinant protein to a subject.

[00302] Subjects for which methods of this disclosure may be helpful include those receiving a bone marrow transplant or an engraftment of cells as further disclosed herein. In one embodiment, administration of a composition of this disclosure , a recombinant *Listeria* of this disclosure , or a vaccine comprising a composition or recombinant *Listeria* of this disclosure is

carried out up to 3 days prior to engraftment of cells for the cell-based therapy. In another embodiment, In another embodiment, administration of a composition of this disclosure , a recombinant *Listeria* of this disclosure , or a vaccine comprising a composition or recombinant *Listeria* of this disclosure is carried out up to 24 hours prior to engraftment of cells for the cell-based therapy. In another embodiment, administration of a composition of this disclosure is

carried out from 1 to 5 hours, prior to engraftment of cells for the cell-based therapy. In another embodiment, administration of a composition of the present disclosure is carried out from 5-10 hours, 11-15 hours, 15-20 hours, 21-24 hours, 24-32 hours, 32-37 hours, 37-42 hours, 42-47 hours, 48-63 hours, 64-69 hours, or 70- 72 hours prior to engraftment of cells for the cell-based therapy. In another embodiment, administration of a composition of the present disclosure is carried out from 1, 2, or 3 days prior to engraftment of cells for the cell-based therapy. In another embodiment, administration is carried out at the same time as the engraftment.

[00303] In one embodiment, a composition, a recombinant *Listeria*, or a vaccine comprising a composition or recombinant *Listeria* disclosed herein is also administered 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days following the engraftment.

[00304] In another embodiment, the present disclosure provides a method of reducing an incidence of cancer or infectious disease, comprising administering a composition of the present disclosure. In another embodiment, the present disclosure provides a method of ameliorating cancer or infectious disease, comprising administering a composition of the present disclosure.

[00305] In one embodiment, the cancer treated by a method of the present disclosure 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 lymphoma. In another embodiment it is a brain tumor. 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.

[00306] In another embodiment of the methods of the present disclosure, the subject mounts an immune response against an antigen-expressing tumor or target antigen, thereby mediating anti-tumor effects.

[00307] In one embodiment, a treatment protocol of the present disclosure is therapeutic. In

another embodiment, the protocol is prophylactic. In another embodiment, the vaccines of the present disclosure 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 are used as a cancer immunotherapy in early stage disease, or after debulking of tumor growth by surgery, conventional chemotherapy or radiation treatment. Following such treatments, the vaccines of the present disclosure 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 disclosure are used to effect the growth of previously established tumors and to kill existing tumor cells.

EXAMPLES

MATERIALS AND EXPERIMENTAL METHODS

Bacterial strains, transformation and selection

[00308] *E. coli* strain MB2159 was used for transformations, using standard protocols.

Bacterial cells were prepared for electroporation by washing with H₂O.

[00309] *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

[00310] 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.

[00311] 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.

[00312] 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.

[00313] pDP1526 (pKSV7:: Δ plcA) was constructed by a single three-part ligation of pKSV7 restricted with BAMHI and XbaI, the 468 bp XbaI and NsiI-generated fragment from pAM401::plcA containing the 5' end of plcA (bases 882 to 1351; Mengaud et al., supra) and, the
5 501 bp PstI- and BamHI-generated fragment from pAM401::plcA prfA containing the 3' end of plcA (bases 77 to 429; Mengaud et al., supra).

[00314] 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
10 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

[00315] 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
15 (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
20 10 μ g of chloramphenicol per ml of media. In addition, maintenance of pKSV7 derivatives required growth at 30°C., a permissive temperature for plasmid replication in Gram-positive bacteria.

[00316] Integration of pKSV7 derivatives into the L. monocytogenes chromosome occurred by homologous recombination between L. monocytogenes DNA sequences on the plasmids and
25 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 μ g chloramphenicol per ml of media. Each integration strain was subsequently colony purified on BHI agar containing 10 μ g chloramphenicol per ml of media and incubated at 40°C. Southern blot analyses of chromosomal
30 DNA isolated from each integration strain confirmed the presence of the integrated plasmid.

[00317] Construction of DP-L1552 is achieved by integration of the pKSV7 derivative, pDP1526, to generate a merodiploid intermediate was done as described above. Spontaneous excision of the integrated plasmid, through intramolecular homologous recombination, occurred

at a low frequency. Bacteria in which the plasmid had excised from the chromosome were enriched by growth at 30°C. in BHI broth for approximately 50 generations. The nature of the selective pressure during this step was not known but may be due to a slight growth defect of strains containing integrated temperature-sensitive plasmids. Approximately 50% of excision events, i.e., those resulting from homologous recombination between sequences 3' of the deletion, resulted in allelic exchange of Δ plcA for the wild-type allele on the chromosome.

[00318] The excised plasmids were cured by growing the bacteria at 40°C in BHI for approximately 30 generations. Bacteria cured of the plasmid retaining the Δ plcA allele on the chromosome were identified by their failure to produce a zone of turbidity surrounding colonies after growth on BHI agar plates containing a 5 ml overlay of BHI agar/2.5% egg yolk/2.5% phosphate-buffered saline (PBS) (BHI/egg yolk agar). The turbid zones resulted from PI-PLC hydrolysis of PI in the egg yolk, giving an insoluble diacylglycerol precipitate. The correct plcA deletion on the *L. monocytogenes* chromosome was confirmed by amplifying the deleted allele using PCR and sequencing across the deletion.

[00319] Thus, PI-PLC negative mutants (plcA deletion mutants) may be used according to the present disclosure 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

[00320] 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.

[00321] 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.

5 ***Bacterial culture and in vivo passaging of Listeria***

[00322] *E. coli* were cultured following standard methods. *Listeria* were grown at 37° C, 250 rpm shaking in LB media (Difco, Detroit, MI)+ 50 µg streptomycin, and harvested during exponential growth phase. For Lm-LLO-E7, 37 µg chloramphenicol was added to the media. For growth kinetics determinations, bacteria were grown for 16 hours in 10 ml of LB +
10 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

[00323] 1 x 10⁸ CFU were injected intraperitoneally (ip.) into C57BL/6 mice. On day three, spleens were isolated and homogenized in PBS. An aliquot of the spleen suspension was plated
15 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

[00324] **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: 31) (reverse primer: 5'-GCT AGC CTA ATG GAT GTA TTT TCT AGG-3'; SEQ ID NO: 32) and a minimal p60 promoter sequence (forward primer: 5'-TTA ATT AAC AAA TAG TTG GTA TAG TCC-3'; SEQ ID No: 33) (reverse primer: 5'-GAC GAT GCC AGC CTG TCA CCA TGG AAA ACT CCT CTC-3'; SEQ ID No: 34) were isolated by PCR
25 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:

30 CAAATAGTTGGTATAGTCCTCTTTAGCCTTTGGAGTATTATCTCATCATTTGTTTTT
AGGTGAAAACCTGGGTAACTTAGTATTATCAATATAAAATTAATTCTCAAATACTT
AATTACGTACTGGGATTTTCTGAAAAAAGAGAGGAGTTTTCC (SEQ ID NO: 35
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).

[00325] **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: 36) and a 3'-primer that added a NheI site downstream of the sequence (GCTAGCCAGCAAAGAAAAACAAACACG, SEQ ID NO: 37). The PCR product was cloned into cloning vector pCR2.1 and sequence verified.

[00326] 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: 38); (primer 2, 5'-TTA ATT AAG CTA GCC AGC AAA GAA AAA CAA ACA CG-3'; SEQ ID No: 39) 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 1B**). 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

[00327] 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.

[00328] In order to obtain standard target DNA for real-time PCR, the LLO-E7 gene was PCR amplified from pGG55 (5'-ATGAAAAAATAATGCTAGTTTTTATTAC-3' (SEQ ID NO: 40); 5'-GCGGCCGCTTAATGATGATGATGATGATGTGGTTTCTG AGAACAGATG-3' (SEQ ID NO: 41)) 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

[00329] 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: 42); 5'-TGCCCATTAACAGGTCTTCCA-3' (SEQ ID NO: 43); 5'-FAM-TGCGTACAAAGCACACACGTAGACATTCGTAC-TAMRA-3' (SEQ ID NO: 44) and the one-copy gene *plcA* (TGACATCGTTTGTGTTTGAGCTAG -3' (SEQ ID NO: 45, 5'-GCAGCGCTCTCTATACCAGGTAC-3' (SEQ ID NO: 46); 5'-TET-TTAATGTCCATGTATGTCTCCGTTATAGCTCATCGTA-TAMRA-3'; SEQ ID NO: 47) as a *Listeria* genome target.

[00330] 0.4 μ M 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

[00331] Bacteria were grown at 37°C, 250 rpm shaking in Luria Bertani (LB) Medium +/- 100 micrograms (μ g)/ml D-alanine and/or 37 μ g/ml chloramphenicol. The starting inoculum was adjusted based on OD₆₀₀ nm measurements to be the same for all strains.

Hemolytic Lysis Assay

[00332] 4×10^9 CFU of *Listeria* were thawed, pelleted by centrifugation (1 minute, 14000 rpm) and resuspended in 100 μ l 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 μ l of 10% washed blood cells were mixed with 100 μ l 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 μ l 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

[00333] 10⁵ TC-1 (ATCC, Manassas, VA) were implanted subcutaneously in C57BL/6 mice (n=8) and allowed to grow for about 7 days, after which tumors were palpable. TC-1 is a C57BL/6 epithelial cell line that was immortalized with HPV E6 and E7 and transformed with activated ras, which forms tumors upon subcutaneous implantation. Mice were immunized with 0.1 LD₅₀ of the appropriate *Listeria* strain on days 7 and 14 following implantation of tumor cells. A non-immunized control group (naïve) was also included. Tumor growth was measured with electronic calipers.

Generation of an ActA deletion mutant

[00334] The strain *Lm dal dat* (Lmdd) was attenuated by the irreversible deletion of the virulence factor, ActA. An in frame deletion of *actA* in the *Lmdal dat* (Lmdd) background was constructed to avoid any polar effects on the expression of downstream genes. The *Lm dal dat* $\Delta actA$ contains the first 19 amino acids at the N-terminal and 28 amino acid residues of the C-terminal with a deletion of 591 amino acids of ActA. The deletion of the gene into the chromosomal spot was verified using primers that anneal external to the *actA* deletion region. These are primers 3 (Adv 305-tgggatggccaagaaattc) (SEQ ID NO: 48) and 4 (Adv304-ctaccatgtcttcggtgcttg) (SEQ ID NO: 49) as shown in the **Figure 2B**. The PCR analysis was performed on the chromosomal DNA isolated from Lmdd and Lm-dd $\Delta actA$. The sizes of the DNA fragments after amplification with two different set of primer pairs 1, 2 and 3, 4 in Lm-dd chromosomal DNA was expected to be 3.0 Kb and 3.4 Kb. However, for the Lm-dd $\Delta actA$ the expected sizes of PCR using the primer pairs 1, 2 and 3, 4 was 1.2 Kb and 1.6 Kb. Thus, PCR analysis in Fig. 3 confirms that 1.8 kb region of *actA* was deleted in the strain, Lm-dd $\Delta actA$. DNA sequencing was also performed on PCR products to confirm the deletion of *actA* containing region in the strain, Lm-dd $\Delta actA$ (**Figure 2C and 2D**).

[00335] gcgccaaatcattggtgattggtgaggatgtctgtgtcgtgggtcgcgagatggcggaataagaagcattaaagatcctg
acaaatataatcaagcggctcatatgaaagattacgaatcgtccactcacagaggaaggcgactggggcggagttcattataatagtgtg
atcccgaataaagcagcctataataactatcactaaactggaaaaaagaaacagaacagctttatcttcgcgccttaaagtactatttaacgaa
aaaatcccagtttaccgatgcgaaaaagcgcctcaacaagcagcgaaagatttatgtggaagatgcttctaaaaaagttgctgaagcttg
ggaagcagttgggggttaactgattaacaaatgtagagaaaaattaattctccaagtgtattcttaaaataattcatgaataattttcttatattag
ctaattaagaagataactaactgctaataccaatttttaacggaacaaattagtgaatgaaggccgaattttccttgttctaaaaagggtgtatta
gcgtatcacgaggagggtataagtgggattaaacagatttatgcgtgcgatgatgggtgtttcattactgccaaattgcattacgattaa

ccccgacgtcgacccatacgacgftaattcttgcaatgtagctattggcgtgttctctttagggcgfttatcaaaattattcaattaagaa
 aaaataattaaaaacacagaaacgaaagaaaaagtgaggtgaatgatatgaaattcaaaaagggtggttctaggtatgtgcttgatcgcaagt
 gttctagtcttccggtaacgataaaagcaaatgcctgttgatgaatacttacaacacccgcagctccgcatgatattgacagcaaattacc
 acataaacttagttgggtccgcgataacccgacaaatactgacgtaatacgcactattggcttttaacaagcggaataactagctaaa
 5 gatgtaaatcatatgcgagctaatttaagaatgaacttaaaaaattcgataaacaatagctcaaggaatatgatgcggatcataaaatcc
 atattatgatactagtagcatctttatctcattttataatcctgatagagataatacttattgccgggttttgctaatacgaaaataacaggagcaaa
 gtatttcaatcaatcggtgactgattaccgagaaggga (SEQ ID NO: 50).

***Listeria* immunization and *S. mansoni* infection**

[00336] Female (6–8 weeks old) BALB/c mice were maintained as naïve (un-infected) or
 10 infected with *S. mansoni*. For infection, mice were injected i.p. with 50 cercariae. Eight weeks
 later, both infected and un-infected mice were immunized i.p. (100 µg/injection) with 0.1 LD50
 Lm-gag, 0.2 LD50 Lm-gag, or 1 LD50 Lm-gag, or orally with 10 LD50 Lm-gag or 100 LD50
 Lm-gag. Two weeks later, some groups of mice were boosted i.p. with 0.1 LD50 Lm-gag or 0.2
 LD50 Lm-gag or orally with 10 LD50 Lm-gag or 100 LD50 Lm-gag in a similar manner. Lm-E7
 15 was used as a negative control. Two weeks after the final immunization, the T-cell immune
 response was analyzed as described below. Infection was confirmed at the time of sacrifice by
 examining the mice for the presence of worms, liver eggs and hepatosplenomegally.

MDSC and Treg Function

[00337] Tumors were implanted in mice on the flank or a physiological site depending on the
 20 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.

[00338] 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
 25 harvesting the tumor, non-tumor bearing mice were vaccinated to use for responder T cells.
 Splenocytes were prepared using standard methodology.

[00339] 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™ dissociator was used
 30 with the tumor dissociation kit.

[00340] MDSCs were purified from tumors and spleens using a Miltenyi kit and columns or
 the autoMACs separator. Cells were then counted.

[00341] Single cell suspension was prepared and the red blood cells were lysed. Responder T

cells were then labeled with CFSE.

[00342] Cells were plated together at a 2:1 ratio of responder T cells (from all division cycle stages) to MDSCs at a density of 1×10^5 T cells per well in 96 well plates. Responder T cells were then stimulated with either the appropriate peptide (PSA OR CA9) or non-specifically with PMA/ionomycin. Cells were incubated in the dark for 2 days at 37°C with 5% CO₂. Two days later, the cells were stained for FACS and analyzed on a FACS machine.

Analysis of T-cell responses

[00343] 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.

[00344] 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.

[00345] 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. The lower limit of detection for the assays was 30 pg/ml.

RESULTS

EXAMPLE 1: A PLASMID CONTAINING AN AMINO ACID METABOLISM ENZYME INSTEAD OF AN ANTIBIOTIC RESISTANCE GENE IS RETAINED IN E. COLI AND *Lm* BOTH IN VITRO AND IN VIVO

[00346] 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 (Figures 1A and 1B). DNA was purified from several colonies.

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

[00347] As virulence is linked to LLO function, the hemolytic lysis activity between *Lmdd*-TV3 and *Lm*-LLO-E7 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*-LLO-E7.

[00348] *In vivo* virulence was also measured by determining LD₅₀ values, a more direct, and therefore accurate, means of measuring virulence. The LD₅₀ of *Lmdd*-TV3 (0.75×10^9) was very close to that of *Lm*-LLOE7 (1×10^9), showing that plasmids containing a metabolic enzyme do not increase the virulence of bacteria.

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

[00349] 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 3) 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.

[00350] 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 4: SUPPRESSOR CELL FUNCTION AFTER *LISTERIA* VACCINE TREATMENT

[00351] At day 0 tumors were implanted in mice. At day 7 mice were vaccinated with Lmdda-E7 or LmddA-PSA. At day 14 tumors were harvested and MDSCs and Treg percentages and numbers were measured for vaccinated and naïve groups. It was found that there is a decrease in the percentages of both MDSC and Tregs in the tumors of *Listeria*-treated mice, whereas the same effect is not observed in the spleens or the draining lymph nodes (TLDN) (**Figures 4A and 4B**).

[00352] 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 (**Figures 5-17**) 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 **Figures 5-17**).

Analysis of cells in the blood of tumor-bearing mice

[00353] 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 5: MDSCs FROM TPSA23 TUMORS BUT NOT SPLEENS ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION

[00354] Suppressor assays were carried out using monocytic and granulocytic MDSCs isolated from TPSA23 tumors with non-specifically activated naïve murine cells, and specifically activated cells (PSA, CA9, PMA/ionomycin). Results demonstrated that the MDSCs isolated from tumors from the *Lm* vaccinated groups have a diminished capacity to suppress the division of activated T cells as compared to MDSC from the tumors of naïve mice. (see Lm-LLO-PSA and Lm-LLO-treated Groups in Figs. 8 & 10, right-hand panel in figures represents pooled cell division data from left-hand panel). In addition, T responder cells from untreated mice where no MDSCs were present and where the cells were unstimulated/activated, remained in their parental

(resting) state (**Figures 5C-5D and 7C and 7D**), whereas T cells stimulated with PMA or ionomycin were observed to replicate (**Figures 5A and 5B and 7A and 7B**). Further, it was observed that both, the Gr+Ly6G+ and the GrdimLy6G- 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.

[00355] 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 **Figures 5A-5D and 7A-7D**).

[00356] In addition, the observations discussed immediately above relating to **Figures 5A-5D and 7A-7D** 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 (**Figures 6A-6D and 8A-8D**). 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 6: TUMOR T REGULATORY CELLS' REDUCED SUPPRESSION BUT NOT THOSE FROM SPLEENS

[00357] 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 (**Figure 9A-9D**), however, it was found that splenic Tregs are still suppressive (**Figure 10A-10D**).

[00358] 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 (**Figure 11A-11D**).

EXAMPLE 7: MDSCs AND TREGS FROM 4T1 TUMORS BUT NOT SPLEENS ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[00359] 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 (**Figures 12A-12D and 14A-14D**), that *Listeria* has no specific effect on splenic monocytic MDSCs (**Figures 13A-13D and 15A-15D**), that there is a decrease in the suppressive

ability of Tregs from 4T1 tumors after *Listeria* vaccination (**Figure 16A-16D**), and that *Listeria* has no effect on the suppressive ability of splenic Tregs (**Figure 17A-17D**).

[00360] Finally, it was observed that *Listeria* has no effect on the suppressive ability of splenic Tregs.

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EXAMPLE 8: CHANGE IN THE SUPPRESSIVE ABILITY OF THE GRANULOCYTY AND MONOCYTYC MDSC IS DUE TO THE OVEREXPRESSION OF tLLO.

[00361] The LLO plasmid shows similar results as the *Listeria* vaccines with either the TAA or an irrelevant antigen (**Figures 18A-18D**). 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.

[00362] 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 (**Figures 19A-19D**).

[00363] 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 (**Figures 20A-20D**). 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 (**Figures 20A-20D**).

[00364] 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 (**Figures 21A-21D**).

[00365] 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 (**Figures 22A-22D**).

[00366] 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 (**Figures 23A-23D**).

[00367] 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 24A-24D and Figures 25A-25D**).

EXAMPLE 9: Immunization with the *LmddA*-142 strains induces regression of a tumor expressing PSA and infiltration of the tumor by PSA-specific CTLs.

Materials and methods

[00368] A recombinant *Lm* was developed that secretes PSA fused to tLLO (*Lm*-LLO-PSA), which elicits a potent PSA-specific immune response associated with regression of tumors in a mouse model for prostate cancer, wherein the expression of tLLO-PSA is derived from a plasmid based on pGG55 (Table 1), which confers antibiotic resistance to the vector. We recently developed a new strain for the PSA vaccine based on the pADV142 plasmid, which has no antibiotic resistance markers, and referred as *LmddA*-142 (Table 2). This new strain is 10 times more attenuated than *Lm*-LLO-PSA. In addition, *LmddA*-142 was slightly more immunogenic and significantly more efficacious in regressing PSA expressing tumors than the *Lm*-LLO-PSA.

[00369] **Table 1. Plasmids and strains**

Plasmids	Features
pGG55	pAM401/pGB354 shuttle plasmid with gram(-) and gram(+) <i>cm</i> resistance, LLO-E7 expression cassette and a copy of <i>Lm prfA</i> gene
pTV3	Derived from pGG55 by deleting <i>cm</i> genes and inserting the <i>Lm dal</i> gene
pADV119	Derived from pTV3 by deleting the <i>prfA</i> gene
pADV134	Derived from pADV119 by replacing the <i>Lm dal</i> gene by the <i>Bacillus dal</i> gene
pADV142	Derived from pADV134 by replacing HPV16 <i>e7</i> with <i>klk3</i>

pADV168	Derived from pADV134 by replacing HPV16 <i>e7</i> with <i>hmw-maa</i> ₂₁₆₀₋₂₂₅₈
Strains	Genotype
10403S	Wild-type <i>Listeria monocytogenes</i> :: <i>str</i>
XFL-7	10403S <i>prfA</i> ⁽⁻⁾
<i>Lmdd</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾
<i>LmddA</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾ <i>actA</i> ⁽⁻⁾
<i>LmddA-134</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾ <i>actA</i> ⁽⁻⁾ pADV134
<i>LmddA-142</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾ <i>actA</i> ⁽⁻⁾ pADV142
<i>Lmdd-143</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾ with <i>klk3</i> fused to the <i>hly</i> gene in the chromosome
<i>LmddA-143</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾ <i>actA</i> ⁽⁻⁾ with <i>klk3</i> fused to the <i>hly</i> gene in the chromosome
<i>LmddA-168</i>	10403S <i>dal</i> ⁽⁻⁾ <i>dat</i> ⁽⁻⁾ <i>actA</i> ⁽⁻⁾ pADV168
<i>Lmdd-143/134</i>	<i>Lmdd-143</i> pADV134
<i>LmddA-143/134</i>	<i>LmddA-143</i> pADV134
<i>Lmdd-143/168</i>	<i>Lmdd-143</i> pADV168
<i>LmddA-143/168</i>	<i>LmddA-143</i> pADV168

[00370] The sequence of the plasmid pAdv142 (6523 bp) was as follows:

[00371] cggagtgatactggcttactatgttggcactgatgaggggtgcagtgaagtgcctcatgtggcaggagaaaaaggctgca
 ccggtgctcagcagaatatgtgatacaggatatattccgttcctcgtcactgactcgctacgctcggtcgttcgactgccccgagcgg
 5 aaatggcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacaggaagtgagagggccgcccgaagccgttt
 ttccataggctccgccccctgacaagcatcacgaaatctgacgctcaaactcagtggtggcgaaacccgacaggactataaagatacca
 ggcgtttccccctggcggtccctcgtgcgtctcctgttcctgctttcggtttaccgggtgcatccgctgttatggccgcgtttgtctcattc
 cacgcctgacactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaacccccgttcagtcgaccgctgcgccttat
 ccggttaactatcgtcttgagtccaacccgaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagttagtct
 10 tgaagtcattgcgccggttaaggctaaactgaaaggacaagttttgggtgactgcgctcctccaagccagttacctcggttcaagagttggt
 agctcagagaaccttcgaaaaaccgcctgcaaggcggtttttcgttttcagagcaagagattacgcgcagacaaaacgatctcaaga
 agatcatcttattaatcagataaaatatttctagccctcctttgattagtagtattcctatcttaaagttacttttatgtggaggcattaacatttgtaaat
 gacgtcaaaaggatagcaagactagaataaagctataaagcaagcatataatattgcgtttcatctttagaagcgaatttcgccaatattata
 attatcaaaagagaggggtggcaaacgggtatttggcattattagggttaaaaaatgtagaaggagagtgaacccatgaaaaaataatgct
 15 agtttttattacacttatattagtttagtctaccaattgcgcaaaaactgaagcaaaaggatgcatctgcattcaataaagaaaattcaatttcac
 catggcaccaccagcatctccgctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcgataagtatatacaaggat

tggattacaataaaaacaatgtattagatataccacggagatgcagtgacaaatgtgccccaagaaaaggttacaagatggaatgaat
atattgttgggagaaaaagaagaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttcgagcctaacctatccaggtg
ctctcgtaaaagcgaattcggaaatagtagaaaatcaaccagatgtctccctgtaaaacgtgattcattaacactcagcattgattgccagg
tatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgftaacaacgcagtaatacattagtggaagatggaatg
5 aaaaatatgctcaagcttatccaaatgtaagtgcaaaaatgattatgatgacgaaatggcttacagtgaatcacaatfaattgcgaaattgg
tacagcatttaagctgtaataatagcttgaatgtaacttcggcgcaatcagtgagggaagaatgcaagaagaagtcattagtttaaca
aatttactataacgtgaatgtaatgaacctacaagacctccagatttttcggcaaagctgttactaaagagcagttgcaagcgcttggagt
aatgcagaaaatcctcctgcatatctcaagtgtggcgatggcgtaagtttattgaaattatcaactaattcccatagttactaaagtaaa
agctgctttgatgctgccgtaagcggaaaatctgtctcaggtgatgtagaactaacaatatcatcaaaaattcttccctcaaagccgtaattt
10 acggaggttccgcaaaagatgaagttcaaatcatcgacggcaacctcggagacttacgcgatattttgaaaaagcgctacttttaacg
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aactcaaaagcttatacagatggaaaaatfaacatcgatcactctggaggatagcttgcattcaacatttcttgggatgaagtaattat
gatctcgagattgtggaggctgggagtgcgagaagcattcccaacctggcaggtgcttggcctctcgtggcagggcagctcgcgg
cggtgttctggtgcacccccagtggtgtctcacagctgccactgcatcagggaacaaaagcgtgatcttctgggtcggcacagcctgtt
15 tcatctgaagacacagccaggtatttcaggtcagccacagcttccacacccgctctacgatatgagcctcctgaagaatcgaattctc
aggccaggtgatgactccagccacgacctcatgctgctccgctgtcagagcctgccgagctcacggatgctgtgaaggatcatggacct
gcccaccagggagccagcactggggaccacctgctacgcctcaggtggggcagcattgaaccagaggagtcttgaccccaaagaa
acttcagtggttgacctccatgttatttccaatgacgtgtgtgcgaagttcacctcagaagggtgaccaagttcatgctgtgtgctggacg
ctggacagggggcaaaagcacctgctcgggtgattctgggggccacttctctgttatggtgtgcttcaaggtatcacgtcatggggcag
20 tgaaccatgtgccctgcccgaagggccttccctgtacaccaagggtgtgcattaccggaagtgatcaaggacaccatcgtggccaacc
ccTAAccggggccactaactcaacgctagtagtgatttaatcccaaatgagccaacagaaccagaaccagaacagaacaagtaa
cattggagttgaaatggaagaagaaaaagcaatgatttcgtgtgaataatgcacgaaatcattgcttatttttaaaaagcgatatactag
atataacgaaacaacgaactgaataaagaatacaaaaaagagccacgaccagttaaagcctgagaactttaactgcgagccttaattg
attaccaccaatcaattaaagaagtcgagacccaaaatttggtaaagtatttaattactttattaatcagatacttaaatatctgtaaacccattat
25 atcgggtttttgaggggatttcaagcttttaagaagataccaggcaatcaatfaagaaaaacttagttgattgcctttttgtgtgattcaacttt
gatcgtagcttctaactaattaattttcgtaaagaaaggagaacagctgaatgaatatccctttgttgtagaaactgtgcttcatgacggcttgt
aaagtacaaatftaaaaatagtaaaatcgtcaatcactaccaagccaggtaaaagtaaaaggggctattttgcgtatcgtcaaaaaaaa
gcatgattggcggacgtggcggtgttctgacttccgaagaagcgattcacgaaaatcaagatacatttacgcattggacaccaaacgtttat
cgttatggtacgtatgcagacgaaaaccgttcataactaaaggacattctgaaaacaatttaagacaaatcaataccttctttattgattttgat
30 attcacacggaaaaagaaactatttcagcaagcgatattttaacaacagctattgatttaggtttatgcctacgttaattatcaaatctgataaa
ggttatcaagcatattttgttttagaaacgccagctctatgtgacttcaaaatcagaatttaaatctgtcaaagcagccaaaataatctcgaaaa
tatccgagaatattttgaaagctttgccagttgatctaactgcaatcattttgggattgctcgtataccaagaacggacaatgtagaattttt
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ggcgcaatagcgttatgtttaccctctcttttagcctacttttagttcaggctattcaatcgaacgtgcgaatataatgtttgagtttaataatc
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accattctttgcaaagcttgggtatcaagtgtttaaccagtaaaagatttattgtccgtcaaggggtggtttaaattcaagaaaaaagaagcg
aacgtcaacgtgttcatttgcagaatggaaagaagatttaattggcttatatttagcgaaaaagcgatgtatacaagccttatttagcgacga
5 ccaaaaaagagattagagaagtgttaggcattcctgaacggacattagataaattgctgaaggtactgaaggcgaatcaggaaattttcttt
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cccacaactcgatttgttagctacgatacaggctgaaaataaaacccgcactatgccattacatttatatctatgatacgtgtttgttttctttg
ctggctagcttaattgcttatatttacctgcaataaaggatttcttacttccattatactccattttccaaaaacatacggggaacacgggaact
10 tattgtacaggccacctcatagtttaattggtttcgagccttctgcaatctcatccatggaaatatattcatccccctgccggcctattaatgtga
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15 ggcgctgttcgcgcagtgaccattagattctttaacggcagcggagcaatcagctctttaaaagcgctcaaaactgcattaagaaatagcct
ctttcttttcatccgctgtcgcataaagggttaataccctttgcactttaaacgaggggttcgggtcaagaattgccatcacgttctgaacttct
tcctctgttttacaccaagtctgttcatccccgtatcgaccttcagatgaaaatgaagagaacctttttcgtgtggcggtgcctcctgaag
ccattcaacagaataacctgttaaggtcacgtcatactcagcagcgattgccacatactccgggggaaccgcgccaagcaccaatatag
gcgccttcaatccctttttgcgcagtgaatcgcttcatccaaaatggccacggccaagcatgaagcacctcgctcaagagcagcctttgc
20 tgtttctgcatcaccatgcccgtaggcggttgccttcacaactgccatcaagtgacatgttcaccgatattgttttcatattgctgacatttct
ttatcgcggaagcaatttccgcccacgtatctctgtaaaaagggtttgtgctcatggaaaactcctctctttttcagaaaatcccagtagct
aattaagtatttgagaattaattttatattgattaataactaagtttaccagttttcacctaaaaaataaatgatgagataatagctccaaaggcta
aagaggactataccaactatttgtaattaa (SEQ ID NO: 51). This plasmid was sequenced at Genewiz
facility from the *E. coli* strain on 2-20-08.

[00372] The therapeutic efficacy of the construct *LmddA*-142 (*LmddA*-LLO-PSA) was
determined using a prostate adenocarcinoma cell line engineered to express PSA (Tramp-C1-
PSA (TPSA); Shahabi et al., 2008). Mice were subcutaneously implanted with 2×10^6 TPSA
cells. When tumors reached the palpable size of 4-6 mm, on day 6 after tumor inoculation, mice
were immunized three times at one week intervals with 10^8 CFU *LmddA*-142, 10^7 CFU *Lm*-
LLO-PSA (positive control) or left untreated. The naïve mice developed tumors gradually
30 (Figure 26A). The mice immunized with *LmddA*-142 were all tumor-free until day 35 and
gradually 3 out of 8 mice developed tumors, which grew at a much slower rate as compared to
the naïve mice (Figure 26B). Five out of eight mice remained tumor free through day 70. As
expected, *Lm*-LLO-PSA-vaccinated mice had fewer tumors than naïve controls and tumors

developed more slowly than in controls (**Figure 26C**). Thus, the construct LmddA-LLO-PSA could regress 60 % of the tumors established by TPSA cell line and slow the growth of tumors in other mice. Cured mice that remained tumor free were rechallenged with TPSA tumors on day 68.

[00373] Immunization of mice with the *LmddA*-142 can control the growth and induce regression of 7-day established Tramp-C1 tumors that were engineered to express PSA in more than 60% of the experimental animals (**Figure 26B**), compared to none in the untreated group (**Figure 26A**). The *LmddA*-142 was constructed using a highly attenuated vector (*LmddA*) and the plasmid pADV142 (**Table 1**).

[00374] Further, the ability of PSA-specific CD8 lymphocytes generated by the LmddA-LLO-PSA construct to infiltrate tumors was investigated. Mice were subcutaneously implanted with a mixture of tumors and matrigel followed by two immunizations at seven day intervals with naïve or control (Lm-LLO-E7) *Listeria*, or with LmddA-LLO-PSA. Tumors were excised on day 21 and were analyzed for the population of CD8⁺CD62L^{low} PSA^{tetramer+} and CD4⁺CD25⁺FoxP3⁺ regulatory T cells infiltrating in the tumors.

[00375] A very low number of CD8⁺CD62L^{low} PSA^{tetramer+} tumor infiltrating lymphocytes (TILs) specific for PSA that were present in the both naïve and Lm-LLO-E7 control immunized mice was observed. However, there was a 10-30-fold increase in the percentage of PSA-specific CD8⁺CD62L^{low} PSA^{tetramer+} TILs in the mice immunized with LmddA-LLO-PSA (**Figure 4A**). Interestingly, the population of CD8⁺CD62L^{low} PSA^{tetramer+} cells in spleen was 7.5 fold less than in tumor (**Figure 27A**).

[00376] In addition, the presence of CD4⁺/CD25⁺/Foxp3⁺ T regulatory cells (Tregs) in the tumors of untreated mice and *Listeria* immunized mice was determined. Interestingly, immunization with *Listeria* resulted in a considerable decrease in the number of CD4⁺CD25⁺FoxP3⁺ Tregs in tumor but not in spleen (**Figure 27B**). However, the construct LmddA-LLO-PSA had a stronger impact in decreasing the frequency of CD4⁺CD25⁺FoxP3⁺ Tregs in tumors when compared to the naïve and Lm-LLO-E7 immunized group (**Figure 27B**).

[00377] Thus, the *LmddA*-142 vaccine can induce PSA-specific CD8⁺ T cells that are able to infiltrate the tumor site (**Figure 27A**). Interestingly, immunization with *LmddA*-142 was associated with a decreased number of regulatory T cells in the tumor (**Figure 27B**), probably creating a more favorable environment for an efficient anti-tumor CTL activity.

EXAMPLE 10: CD4⁺CD25⁺ REGULATORY T CELLS THAT SECRETE TGFAND IL-10 ARE PREFERENTIALLY INDUCED BY A VACCINE VECTOR

[00378] **Mice:** Six- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA).

[00379] **Cell Lines-** The C57BL/6 syngeneic TC-1 tumor was immortalized with PV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. TC-1 expresses low levels of E6 and E7 and is highly tumorigenic.

[00380] TC-1 was grown in RPMI 1640, 10% FCS, 2 mmol/LL-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 50 µmol/L 2-ME, 400 µg/mL G418, and 10% National Collection Type Culture-109 medium at 37°C with 10% CO₂. Mink lung epithelial cells (MLEC) for the TGFassay were stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene.

[00381] ***L. monocytogenes Strains and Propagation***

[00382] The Listeria strains Lm-LLO-E7 (hly-E7 fusion gene in an episomal expression system) and Lm-E7 (single-copy E7 gene cassette integrated into Listeria genome) were described in detailed previously.

[00383] Bacteria were grown in brain heart infusion medium with (Lm-LLO-E7) or without (Lm-E7) chloramphenicol (20 µg/mL). Bacteria were frozen in aliquots at -80°C for injection.

[00384] **Tumor Inoculation and Vaccination of Mice**

[00385] Six- to 8-week-old C57BL/6 mice (Charles River) received 2×10^5 TC-1 cells subcutaneously on the left flank. One week after tumor inoculation, when the tumors were palpable, 4 to 5 mm in diameter, mice were treated with 0.1 LD₅₀ intraperitoneal Lm-LLO-E7 (10^7 CFU) or Lm- E7 (10^6 CFU) on days 7 and 14.

[00386] ***Immunomagnetic Bead Separation Assay*** CD4⁺ cells were isolated from C57BL/6 mice spleens using immunomagnetic depletion with anti-CD8 and anti-I-Ab monoclonal antibodies followed by anti-rat IgG-, anti-mouse IgG-, and anti-mouse IgM-coupled magnetic beads (Perseptive Biosystems, Cambridge, MA). The resultant population was more than 90% CD4⁺ cells by FACS analysis. CD4⁺T cells were fractionated into CD4⁺CD25⁺ and CD4⁺CD25⁻ subsets by positive and negative selection using MACS separation. Briefly, CD4⁺

[00387] T cells were labeled with anti-CD25-PE monoclonal antibody (PharMingen, San Diego, CA) followed by anti-PE MACS magnetic beads (Miltenyi Biotec, Sunnyvale, CA) and subsequently separated into CD4⁺ CD25⁺ and CD4⁺CD25⁻ populations through a magnetic separation column fitted between a MACS magnet (Miltenyi Biotec).

[00388] ***Suppression Assay*** CD4⁺CD25⁻ cells were labeled with CFSE (Molecular Probes, Eugene, OR) and established in culture at 5×10^4 /well in flat-bottom 96-well plates with 10^5

mitomycin C-treated antigen-presenting cells and 10 µg/mL of anti-CD3 antibody.

[00389] Cell Tracker (Molecular Probes)-labeled CD4+CD25+ cells were titrated in at amounts of 0 to 50,000 cells. After 72 hours in culture the cells were washed and labeled with anti-CD4–antigen-presenting cells and analyzed by flow cytometry for suppression of cell division in the CD4+ CD25– population.

[00390] *Flow Cytometric Analysis*

[00391] Three-color flow cytometry for all cell surface markers (all fluorescent conjugated markers were obtained from BD/Pharmingen) and cell sorting was performed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA).

[00392] *Cytokine Assays*

[00393] Cells were activated or left unstimulated in culture for 3 days and supernatants were collected. TGF α was detected using a highly sensitive MLEC assay that detects all three forms of TGF.

[00394] Briefly, supernatants were added to MLEC expressing PAI-1 with firefly luciferase. TGF α activates PAI-1, and the amount of TGF α present is quantitated by the amount of luciferase activity. IL-10 was detected using the commercially available enzyme-linked immunosorbent assay (ELISA) kit from Pharmingen according to protocols provided by the manufacturer.

[00395] *Statistics*

[00396] Statistical significance was determined by the Student *t*-test. In all experiments, $P \leq 0.05$ was considered significant.

RESULTS

[00397] Lm-E7 Vaccination Results in an Increase in CD4+CD25+ T Cells in Tumor-Bearing and Non-Tumor-Bearing Mice

[00398] Splenocytes from mice were analyzed by flow cytometry after labeling for co-expression of CD4 and CD25 cell surface molecular markers as well as the marker CD152 (CTLA-4), all commonly used to identify CD4+CD25+regulatory T cells (**Figure 28A**). We consistently observed increased numbers of CD4+ T cells induced by the Lm-LLO-E7 vaccine in the spleen (gate R1, **Figure 28B**), of which fewer numbers were CD4+CD25+ compared with the Lm-E7-vaccinated group (gate R2, **Figure 28B**). **Figure 28B** shows that a significant proportion of the CD4+CD25+ T cells from all the groups were CTLA-4 hi, consistent with the recently described phenotype profile of regulatory T cells.

[00399] To determine whether the numbers of CD4+CD25+ T cells in mice were affected by

tumor burden alone or by the vaccination regimen alone, splenocytes were analyzed from mice in different groups, including those inoculated with TC-1 tumor alone compared with control naive mice and mice that received the vaccination regimen alone in the absence of any endogenous tumor (**Figures 29A-29C**). **Figure 29A** shows that there was no significant difference in numbers of CD4+CD25+ T cells in the spleens of mice that received the tumor alone compared with naive mice, whether analyzed at day 5 or day 10 after tumor implantation. However, when mice received only the vaccinations alone in the absence of tumor (see **Figure 29B**), there were significant differences in the numbers of CD4+CD25+ T cells in the spleen (12% vs. 9%) of Lm-E7-vaccinated mice versus Lm-LLO-E7-vaccinated mice.

[00400] Increased CD4+CD25+ T Lymphocytes Are Seen Infiltrating the Tumor and Spleens of Lm-E7-Vaccinated Tumor-Bearing Mice Compared With Lm-LLO-E7 Tumor-Bearing Mice

[00401] We next examined the prevalence of CD4+ CD25+ T cells in mice that were tumor-bearing followed by challenge with the respective vaccines. To follow the site of induction as well as circulation of the cells, we analyzed different samples, including the lymphoid organs at the site of inoculation (mesenteric lymph node), the spleen, the tumor, the tumor-draining lymph nodes (left brachial lymph node), peripheral blood, and the liver. Interestingly, we found significant differences in the numbers of CD4+CD25+ T cells in the spleen and especially in the tumor of the vaccinated mice, with Lm-E7 mice having significantly more of these cells than the Lm-LLO-E7 mice (13.5% vs. 9.6% and 30% vs. 13% in the spleen and tumor, respectively) (see **Figure 29C**). However, we found no significant differences in any of the other organs. These results suggest that more of these cells are recruited in the spleen and home to the tumor as tumor-infiltrating lymphocytes in Lm-E7 vaccinated mice.

[00402] CD4+CD25+ T Cells Isolated From Vaccinated Animals Can Suppress Effector T-Cell Function.

[00403] There is a significant concern that by using a live vaccine vector, we might not be detecting CD4+ CD25+ regulatory T cells but instead activated CD4+CD25+ effector T cells. To address this issue we isolated the CD4+CD25+ population from each of the vaccine systems as well as from control nonvaccinated mice and analyzed them for suppressor function. The cells were separated into CD4+CD25+ cells as well as CD4+CD25- cells and were set up in a suppressor assay (**Figures 30A-30-C**). The CD4+CD25+ cells isolated from each of the groups

were all able to significantly suppress proliferation of the CD4+CD25⁺ cells upon activation and were clearly suppressor T cells and not effector T cells.

[00404] Lm-E7-Vaccinated Mice Show Greater Production of Suppressive Cytokines TGF and IL-10.

[00405] To further characterize the mechanism of action of these CD4+CD25⁺ regulatory T cells, we looked at whether they could secrete suppressor cytokines. We stimulated T cells isolated from the tumors and spleens of vaccinated, as well as control, mice via the T cell receptor (TCR), collected the supernatants, and tested for the presence of suppressor cytokines. We found that while there was no significant difference in the amount of these cytokines produced in the spleen of the different groups, there was a significant increase in both TGF and IL-10 in the tumor-infiltrating T cells produced by the Lm-E7-vaccinated group versus the Lm-LLO-E7-vaccinated group (**Figure 31A-31C**). To confirm whether the T cells producing these cytokines in the tumor were indeed CD4⁺ T cells, we depleted the CD4⁺ T-cell population from the tumor cells and analyzed the levels of TGF produced compared with undepleted samples.

[00406] While there was still a significant amount of TGF produced by cells activated by anti-CD3 Ab in the tumor, there was a dramatic drop in the production of TGF when treated with anti-CD4 antibody, confirming that it is the CD4⁺ T cells that are producing the suppressor cytokines.

EXAMPLE 11: SYNERGY OF LM-LLO AND CHIMERIC ANTIGEN RECEPTOR (CAR) T CELL THERAPY.

[00407] *hHER-2 transgenic mice*. These mice are profoundly tolerant to hHER-2 but do not form autochthonous tumors. The transgene is expressed by the whey acidic protein promoter and is found in the mammary gland and in the brain (cerebellum). Usually 8 mice per group are used for transplantable tumor studies. They are on a C57BL/6 background. These mice were obtained from the Peter MacCallum Cancer Centre.

[00408] *hHER-2 expressing tumor cells syngeneic with C57BL/6: 24JK-hHER-2*. These cells were derived from a mouse sarcoma and are MHC class I negative. They have been used SQ to generate solid, measurable tumors (1 x 10⁶ grows to 10mm diameter in 15 to 20 days) and IV to generate experimental metastases in the lung; the end point for these experiments was death.

[00409] *EO771 (LMB variant) breast cancer line*. These have been used to generate breast tumors by injection into the fourth mammary fat pad (5 x 10⁵ grows to 10mm diameter in 10

days). They metastasize to the lung. These cells were obtained from the Peter MacCallum Cancer Centre.

[00410] MC57-HER/2, a mouse methylcholanthrene-induced fibrosarcoma cell line virally transduced with human HER-2/neu and GFP. These are good targets for CTL assays and are high expressers of MHC class I. However, MC does form solid tumors when injected SQ in the flank (8×10^6 cells per site will form measurable tumors in 4 days that reach 10mm in 16 days). These cells were obtained from the Peter MacCallum Cancer Centre.

[00411] All of these lines were retrovirally transduced with human HER-2/neu.

[00412] CART cells specific for human HER-2/neu: scFv-anti-Her-2–transduced T cells and control LXS–transduced T cells. These cells were obtained from the Peter MacCallum Cancer Centre.

[00413] Bispecific CAR T cells: scFv-anti-Her-2–CAR T cells and control LXS–control CAR T cells, transduced with a conventional TCR specific for the SIINFEKL epitope of chicken ovalbumin (OVA). These cells were obtained from the Peter MacCallum Cancer Centre.

Listeria strains

[00414] All strains were obtained from Advaxis, Inc. These have been received intact and stored at -80°C .

Test strains

- i. *LmddA* – LLO (pADV-274)
- ii. *LmddA* – LLO-ChHER2/neu-SIINFEKL
- iii. *LmddA* – LLO-ChHER2/neu (pADV-164)
- iv. *LmddA*-LLO-SIINFEKL

Control strains

- i. *LmddA* – empty plasmid (pADV-275)
- ii. *LmddA*-LLO-PSA-PSMA-SIINFEKL

[00417] The purpose of this study is to determine the synergy between LM-LLO therapy and CAR-T cells, particularly:

1) The ability of Lm-LLO, without antigen, to recondition the tumor microenvironment enhance CAR-T cell therapy of a solid tumor.

2) Synergism of antigen-specific LM therapy with CAR-T cells directed to the same target cell-surface antigen.

3) Whether addition of a conventional antigen (TAG) specific T cell receptor to CAR-T cells could direct Lm-LLO-TAG therapy to improve CAR-T cell therapy by activation through this receptor by Lm-LLO-TAG immunization.

[00418] *Choice of transplantable tumor model:* A cell line with good MHC I expression.

[00419] *Choice of mice:* The use of the HER-2 Tg mouse allows testing these strategies in the face of tolerance to human HER-2/neu.

[00420] *Protocol for point 1 above:* 40 hHER-2 Tg mice were divided into 4 groups of 10 mice. 2×10^5 EO771 are implanted into the fat pad (day 0) of all 40 mice.

Group 1 – No treatment 10 mice.

Group 2 – Receive *LmddA* – empty plasmid (pADV-275) and CAR-T cells.

Group 3 – Receive *LmddA* –LLO (pADV-274) and CAR T cells.

Group 4 – Receive “standard therapy” irradiation and CAR T cells.

[00421] The timing and application of these treatments can be seen in **Figure 32A**.

[00422] CAR T cell prep is “bulk” made on the same day as tumor injection (day 0) they are then ready by day 7. The syngeneic mouse that is used as a source is PTP (CD45) RC^a. (The Tg mouse is RC^b). 10 donor mice are required for the CAR T cells. IL-2, 250,000 units once a day is given for five days after T cell transfer.

[00423] On day 14, three mice from each group are sacrificed and tumors are removed for FACS and immunohistochemistry to detect MDSCs, CD4 T cells both foxp893 negative and positive, CD8 T cells and memory markers including the tissue resident (CD103⁺) T cell marker. The analysis of these tissue-infiltrating cells (TIC) can be found **Figure 32B**.

[00424] Remaining mice, 7 per group, are monitored for tumor growth and survival. Mice must be sacrificed at tumor size of 150mm², usually about day 21. This experiment is followed up with a second experiment (see **Figures 33A-33-B**) in which a post CART cell boost of Lm-LLO is included.

[00425] *Protocol for point 2:* 40 hHER-2 Tg mice are divided into 4 groups of 10 mice. 5×10^5 EO771 are implanted into the fat pad of all 40 mice.

Group 1 – No treatment.

Group 2 – receive *LmddA* – empty plasmid (pADV-275) or *LmddA*-LLO (depending on the results obtained in Protocol 1) with CAR-T cells.

Group 3 – receive *LmddA* - ChHER2/neu (pADV-164) with CAR T cells.

Group 4 – “standard therapy” irradiated on day 7 (AM) transfer CAR T cells 2hours later (control for efficacy of the CAR T cell prep).

[00426] On day 21, two mice from each group are sacrificed and tumors are removed for immunohistochemistry to detect MDSCs, CD4 T cells both fox893 negative and positive , CD8 T cells, as described above. Remaining mice, 8 per group, are monitored for tumor growth and survival.

[00427] *Protocol for point 3:* 40 hHER-2 Tg mice are divided into 3 groups of 10 mice. 5×10^5 EO771 are implanted into the fat pad of all 30 mice.

Group 1 – No treatment.

Group 2 – receive *Lmdda* – empty plasmid (pADV-275) on day 3 and a booster on day 10 with OVA-TCR/CAR-T cells on day 10.

Group 3 receive *Lmdda*-chHER-2/neu-SIINFEKL on day 3 and a booster on day 10 with OVA-TCR/CAR T cells on day 10.

Group 4 receive *Lmdda*-chHER-2/neu (pADV 164) on day 3 and a booster on day 10 with OVA-TCR/CAR T cells on day 10.

[00428] On day 21, two mice from each group are sacrificed and tumors are removed for immunohistochemistry to detect MDSCs, CD4 T cells both fox903 negative and positive, CD8 T cells. The idea is to look for expansion of the CAR T cells and survival as memory T cells (perhaps tissue resident cells). Remaining mice, 8 per group, are monitored for tumor growth and survival.

[00429] The preceding examples are presented in order to more fully illustrate the embodiments of the disclosure. They should in no way be construed, however, as limiting the broad scope of the disclosure.

[00430] While certain features of the disclosure have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the disclosure.

CLAIMS

What is claimed is:

1. A method of pre-conditioning a tumor microenvironment prior to administration of a cell based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), wherein said administration enhances an engraftment of cells from said administration of said cell based therapy, thereby pre-conditioning a tumor microenvironment.
2. The method of claim 1, wherein said *Listeria* expresses and secretes said LLO.
3. The method of any one of claims 1-2, wherein said polypeptide comprises a tumor associated antigen or a fragment thereof.
4. The method of claim 3, wherein said fragment comprises one or more peptides comprising one or more neo-epitopes.
5. The method of claim 3, wherein said tumor associated antigen is a self-antigen.
6. The method of claim 3, wherein said tumor associated antigen is CD20, EGFR-VIII, Tyrosinase related protein-2 (TRP-2), mesothelin, PSA, HER2/neu.
7. The method of claim 3, wherein said tumor associated antigen is an angiogenic antigen associated with a tumor vasculature.
8. The method of claim 7, wherein said angiogenic antigen is HMW-MAA or a fragment thereof.
9. The method of any one of claims 1-8, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements a mutation in the chromosome of said recombinant *Listeria* strain.
10. The method of claim 9, wherein said mutation comprises a mutation in the *dal* or *dat* gene, or any combination thereof.
11. The method of claim 10, wherein said *Listeria* further comprises a mutation in the *actA* gene.
12. The method of any one of claims 9-11, wherein said metabolic enzyme comprises an alanine racemase enzyme or a D-amino acid transferase enzyme.
13. The method of any one of claims 9-12, wherein said metabolic enzyme complements a *dal/dat* gene mutation in said *Listeria*.
14. The method of any one of claims 1-13, wherein said nucleic acid molecule is

- integrated into the *Listeria* genome.
15. The method of any one of claims 1-13, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
 16. The method of any one of claims 1-15, wherein said recombinant *Listeria* comprises a mutation, an inactivation or a deletion of a genomic *internalin* (*inlC*) gene, an *inlB* gene, an *actA* gene, a *plcA* gene, a *prfA* gene, a *plcB* gene or a combination thereof.
 17. The method of any one of claims 1-16, wherein said subject is an adult human or a child.
 18. The method of any one of claims 1-17, wherein said method accelerates immunogenic competence in said subject.
 19. The method of any one of claims 1-18, wherein said method decreases the time to full immunogenic engraftment.
 20. The method of any one of claims 1-19, wherein said subject is receiving said engraftment as a treatment for a tumor, a cancer or a hematopoietic disease.
 21. The method of claim 20, wherein said hematopoietic disease is a hematopoietic malignancy.
 22. The method of claim 21, wherein said hematopoietic malignancy is selected from the group consisting of leukemia, myelodysplastic syndrome (MDS), lymphoma, multiple myeloma, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML).
 23. The method of claim 20, wherein said tumor or said cancer is a solid tumor or solid cancer.
 24. The method of any one of claims 1-23, wherein said cells from said cell-based therapy comprise autologous T cells, allogeneic T cells or chimeric antigen receptor engineered T-cells (CAR T cells) specific for said tumor.
 25. The method of any one of claims 1-24, wherein said engraftment comprises a transfer of said cells from said cell-based therapy into a tumor or cancerous tissue.
 26. The method of any one of claims 1-25, wherein said administering of said recombinant *Listeria* is carried out up to 7 days prior to said engraftment of said cells.
 27. The method of any one of claims 1-25, wherein said administering of said recombinant *Listeria* is carried out on day 3 and on day 10 as a booster dose.
 28. The method of any one of claims 1-25, wherein the administering is carried out at the

- same time as the engraftment of said cells.
29. The method of any one of claims 1-25, wherein the *Listeria* strain is administered until 7 days following said engraftment.
 30. A method of enhancing an engraftment of cells for providing a cell-based therapy in a subject, the method of enhancing an engraftment of cells for providing a cell-based therapy in a subject, the method comprising the step of administering a live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), thereby enhancing an engraftment of said cells.
 31. The method of claim 30, wherein said *Listeria* expresses and secretes said LLO.
 32. The method of any one of claims 29-30, wherein said polypeptide comprises a tumor associated antigen or a fragment thereof.
 33. The method of claim 31, wherein said fragment comprises one or more peptides comprising one or more neo-epitopes.
 34. The method of claim 31, wherein said tumor associated antigen is a self-antigen associated with a host cell antigen.
 35. The method of claim 31, wherein said tumor associated antigen is CD20, EGFR-VIII, mesothelin, HMW-MAA, PSA, Her2/neu, Tyrosinase related protein-2 (TRP-2).
 36. The method of claim 31, wherein said tumor associated antigen is expressed on the surface of the tumor cell.
 37. The method of claim 31, wherein said tumor associated antigen is an angiogenic antigen associated with a tumor vasculature.
 38. The method of claim 37, wherein said angiogenic antigen is HMW-MAA or a fragment thereof.
 39. The method of any one of claims 30-38, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements a mutation in the chromosome of said recombinant *Listeria* strain.
 40. The method of claim 39, wherein said mutation comprises a mutation in the *dal* or *dat* gene, or any combination thereof.
 41. The method of claim 40, wherein said *Listeria* further comprises a mutation in the *actA* gene.
 42. The method of any one of claims 39-41, wherein said metabolic enzyme comprises an

- alanine racemase enzyme or a D-amino acid transferase enzyme.
43. The method of any one of claims 39-42, wherein said metabolic enzyme complements a *dal/dat* gene mutation in said *Listeria*.
 44. The method of any one of claims 30-43, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
 45. The method of any one of claims 30-43, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
 46. The method of any one of claims 30-45, wherein said recombinant *Listeria* comprises a mutation, an inactivation or a deletion of a genomic *internalin C (inlC)* gene, *inlB*, an *actA* gene, a *plcA* gene, a *prfA* gene, a *plcB* gene, or a combination thereof.
 47. The method of any one of claims 30-46, wherein said subject is an adult human, a child.
 48. The method of any one of claims 30-47, wherein said method accelerates immunogenic competence in said subject.
 49. The method of any one of claims 30-47, wherein said method decreases the time to full immunogenic engraftment.
 50. The method of any one of claims 30-47, wherein said subject is receiving said engraftment as a treatment for a tumor, a cancer or a hematopoietic disease.
 51. The method of claim 50, wherein said hematopoietic disease is a hematopoietic malignancy.
 52. The method of claim 50, wherein said hematopoietic malignancy is selected from the group consisting of leukemia, myelodysplastic syndrome (MDS), lymphoma, multiple myeloma, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML).
 53. The method of claim 50, wherein said tumor or said cancer is a solid tumor or solid cancer.
 54. The method of any one of claims 30-53, wherein said cells from said cell-based therapy comprise autologous T cells, allogeneic T cells or chimeric antigen receptor engineered T-cells (CAR T cells) specific for said tumor.
 55. The method of any one of claims 30-54, wherein said engraftment comprises a transfer of said cells from said cell-based therapy into a tumor or cancerous tissue.
 56. The method of any one of claims 30-55, wherein said administering is carried out up to 3 days prior to said engraftment of said cells.

57. The method of any one of claims 29-55, wherein the *Listeria* strain is administered until 7 days following said engraftment.
58. The method of any one of claims 30-55, wherein said administering of said recombinant *Listeria* is carried out on day 3 and on day 10 as a booster dose.
59. The method of any one of claims 30-55, wherein the administering is carried out at the same time as the engraftment of said cells.
60. Use of a live attenuated recombinant *Listeria strain* for pre-conditioning a tumor microenvironment prior to administration of a cell based therapy in a subject, the use comprising the step of administering said recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), wherein said administration enhances an engraftment of cells from said administration of said cell based therapy, thereby pre-conditioning a tumor microenvironment.
61. The use of claim 60, wherein said *Listeria* expresses and secretes said LLO.
62. The use of any one of claims 60-61, wherein said polypeptide comprises a tumor associated antigen or a fragment thereof.
63. The use of claim 62, wherein said fragment comprises one or more peptides comprising one or more neo-epitopes.
64. The use of claim 62, wherein said tumor associated antigen is a self-antigen.
65. The use of claim 62, wherein said tumor associated antigen is CD20, EGFR-VIII, Tyrosinase related protein-2 (TRP-2), mesothelin, PSA, HER2/neu.
66. The use of claim 62, wherein said tumor associated antigen is an angiogenic antigen associated with a tumor vasculature.
67. The use of claim 66, wherein said angiogenic antigen is HMW-MAA or a fragment thereof.
68. The use of any one of claims 60-67, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements a mutation in the chromosome of said recombinant *Listeria* strain.
69. The use of claim 68, wherein said mutation comprises a mutation in the *dal* or *dat* gene, or any combination thereof.
70. The use of claim 69, wherein said *Listeria* further comprises a mutation in the *actA* gene.
71. The use of any one of claims 68-70, wherein said metabolic enzyme comprises an

alanine racemase enzyme or a D-amino acid transferase enzyme.

72. The use of any one of claims 68-71, wherein said metabolic enzyme complements a *dal/dat* gene mutation in said *Listeria*.
73. The use of any one of claims 60-72, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
74. The use of any one of claims 60-72, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
75. The use of any one of claims 60-74, wherein said recombinant *Listeria* comprises a mutation, an inactivation or a deletion of a genomic *internalin* (*inlC*) gene, an *inlB* gene, an *actA* gene, a *plcA* gene, a *prfA* gene, a *plcB* gene or a combination thereof.
76. The use of any one of claims 60-75, wherein said subject is an adult human or a child.
77. The use of any one of claims 60-76, wherein said use accelerates immunogenic competence in said subject.
78. The use of any one of claims 60-77, wherein said use decreases the time to full immunogenic engraftment.
79. The use of any one of claims 60-78, wherein said subject is receiving said engraftment as a treatment for a tumor, a cancer or a hematopoietic disease.
80. The use of claim 79, wherein said hematopoietic disease is a hematopoietic malignancy.
81. The use of claim 80, wherein said hematopoietic malignancy is selected from the group consisting of leukemia, myelodysplastic syndrome (MDS), lymphoma, multiple myeloma, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML).
82. The use of claim 79, wherein said tumor or said cancer is a solid tumor or solid cancer.
83. The use of any one of claims 60-82, wherein said cells from said cell-based therapy comprise autologous T cells, allogeneic T cells or chimeric antigen receptor engineered T-cells (CAR T cells) specific for said tumor.
84. The use of any one of claims 60-83, wherein said engraftment comprises a transfer of said cells from said cell-based therapy into a tumor or cancerous tissue.
85. The use of any one of claims 60-84, wherein said administering of said recombinant *Listeria* is carried out up to 7 days prior to said engraftment of said cells.
86. The use of any one of claims 60-85, wherein said administering of said recombinant

Listeria is carried out on day 3 and on day 10 as a booster dose.

87. The use of any one of claims 60-85, wherein the administering is carried out at the same time as the engraftment of said cells.
88. The use of any one of claims 60-85, wherein the *Listeria* strain is administered until 7 days following said engraftment.
89. Use of a live attenuated recombinant *Listeria* strain for enhancing an engraftment of cells for providing a cell-based therapy in a subject, the use comprising the step of administering said live attenuated recombinant *Listeria* strain to said subject, wherein said *Listeria* comprises a nucleic acid molecule comprising a first open reading frame encoding a polypeptide comprising a truncated listeriolysin O (LLO), thereby enhancing an engraftment of said cells.
90. The use of claim 89, wherein said *Listeria* expresses and secretes said LLO.
91. The use of any one of claims 89-90, wherein said polypeptide comprises a tumor associated antigen or a fragment thereof.
92. The use of claim 89, wherein said fragment comprises one or more peptides comprising one or more neo-epitopes.
93. The use of claim 89, wherein said tumor associated antigen is a self-antigen associated with a host cell antigen.
94. The use of claim 89, wherein said tumor associated antigen is CD20, EGFR-VIII, mesothelin, HMW-MAA, PSA, Her2/neu, Tyrosinase related protein-2 (TRP-2).
95. The use of claim 89, wherein said tumor associated antigen is expressed on the surface of the tumor cell.
96. The use of claim 89, wherein said tumor associated antigen is an angiogenic antigen associated with a tumor vasculature.
97. The use of claim 96, wherein said angiogenic antigen is HMW-MAA or a fragment thereof.
98. The use of any one of claims 89-97, wherein said nucleic acid molecule further comprises a second open reading frame encoding a metabolic enzyme, wherein said metabolic enzyme complements a mutation in the chromosome of said recombinant *Listeria* strain.
99. The use of claim 98, wherein said mutation comprises a mutation in the *dal* or *dat* gene, or any combination thereof.
100. The use of claim 99, wherein said *Listeria* further comprises a mutation in the *actA* gene.

101. The use of any one of claims 98-100, wherein said metabolic enzyme comprises an alanine racemase enzyme or a D-amino acid transferase enzyme.
102. The use of any one of claims 98-101, wherein said metabolic enzyme complements a *dal/dat* gene mutation in said *Listeria*.
103. The use of any one of claims 89-102, wherein said nucleic acid molecule is integrated into the *Listeria* genome.
104. The use of any one of claims 89-102, wherein said nucleic acid molecule is in a plasmid that is stably maintained in said recombinant *Listeria* vaccine strain in the absence of antibiotic selection.
105. The use of any one of claims 89-104, wherein said recombinant *Listeria* comprises a mutation, an inactivation or a deletion of a genomic *internalin C (inlC)* gene, *inlB*, an *actA* gene, a *plcA* gene, a *prfA* gene, a *plcB* gene, or a combination thereof.
106. The use of any one of claims 89-105, wherein said subject is an adult human, a child.
107. The use of any one of claims 89-106, wherein said use accelerates immunogenic competence in said subject.
108. The use of any one of claims 89-107, wherein said use decreases the time to full immunogenic engraftment.
109. The use of any one of claims 89-108, wherein said subject is receiving said engraftment as a treatment for a tumor, a cancer or a hematopoietic disease.
110. The use of claim 109, wherein said hematopoietic disease is a hematopoietic malignancy.
111. The use of claim 109, wherein said hematopoietic malignancy is selected from the group consisting of leukemia, myelodysplastic syndrome (MDS), lymphoma, multiple myeloma, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML).
112. The use of claim 109, wherein said tumor or said cancer is a solid tumor or solid cancer.
113. The use of any one of claims 89-112, wherein said cells from said cell-based therapy comprise autologous T cells, allogeneic T cells or chimeric antigen receptor engineered T-cells (CAR T cells) specific for said tumor.
114. The use of any one of claims 89-113, wherein said engraftment comprises a transfer of said cells from said cell-based therapy into a tumor or cancerous tissue.
115. The use of any one of claims 89-114, wherein said administering is carried out up to 3 days prior to said engraftment of said cells.

116. The use of any one of claims 89-114, wherein the *Listeria* strain is administered until 7 days following said engraftment.
117. The use of any one of claims 89-114, wherein said administering of said recombinant *Listeria* is carried out on day 3 and on day 10 as a booster dose.
118. The use of any one of claims 89-114, wherein the administering is carried out at the same time as the engraftment of said cells.

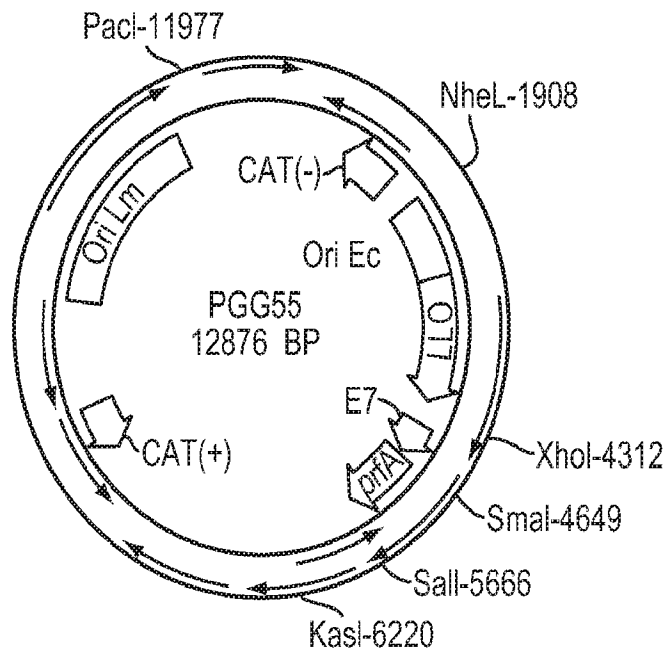


FIG. 1A

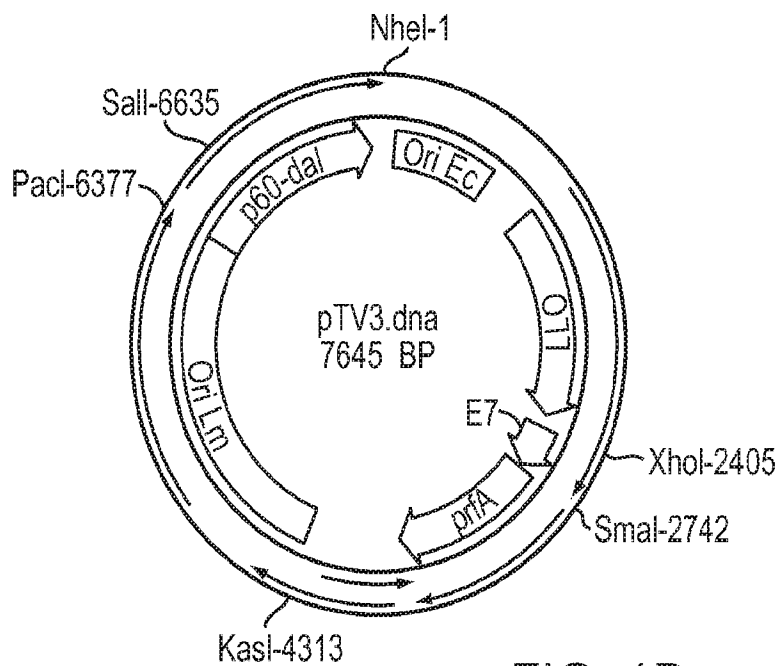


FIG. 1B

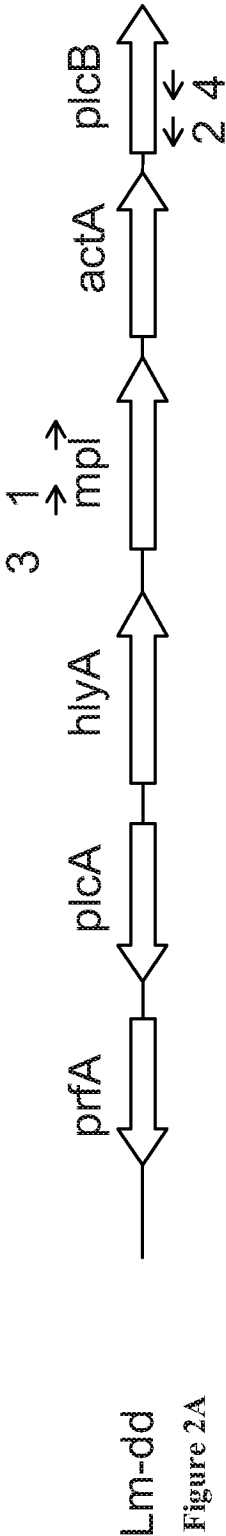


Figure 2A

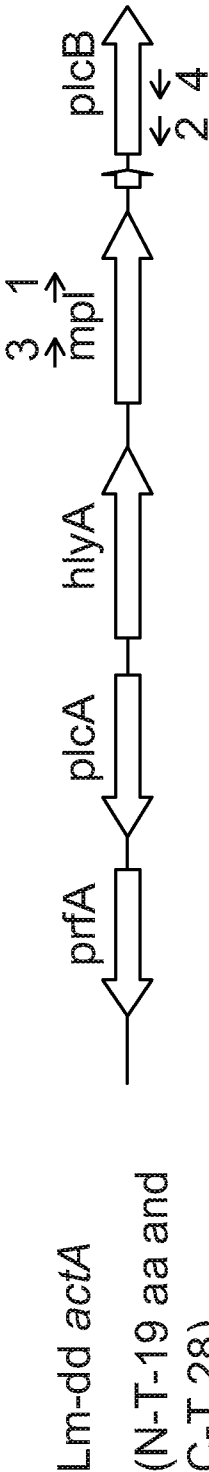
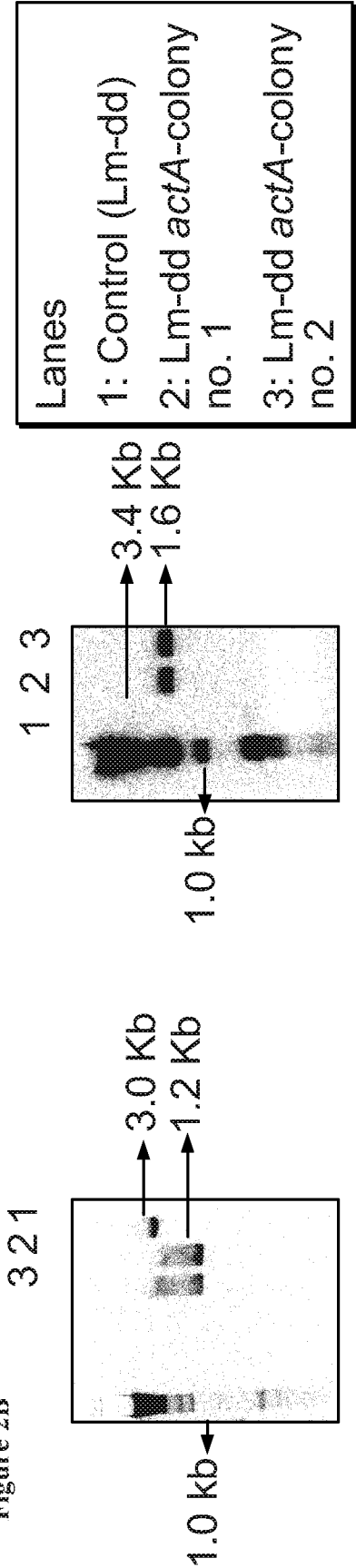


Figure 2B



PCR using oligo's 1/2

PCR using oligo's 3/4

Figure 2C

Figure 2D

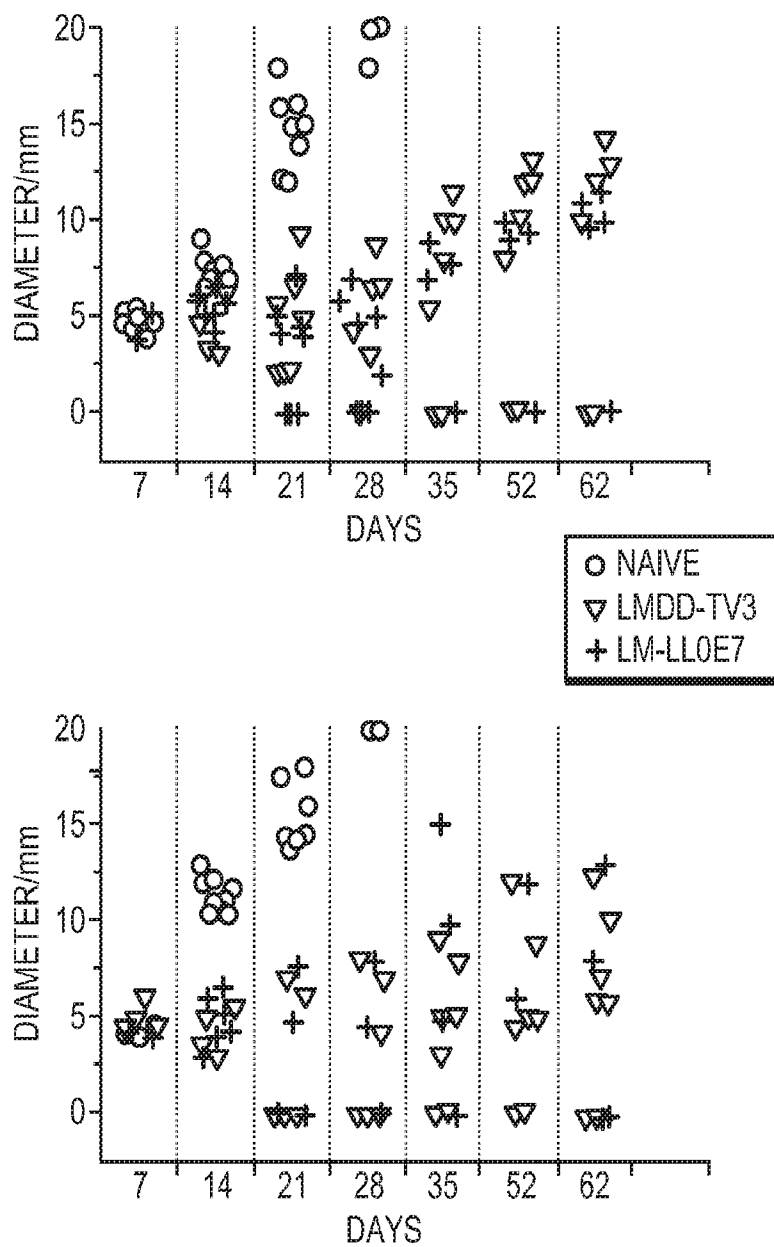


FIG. 3

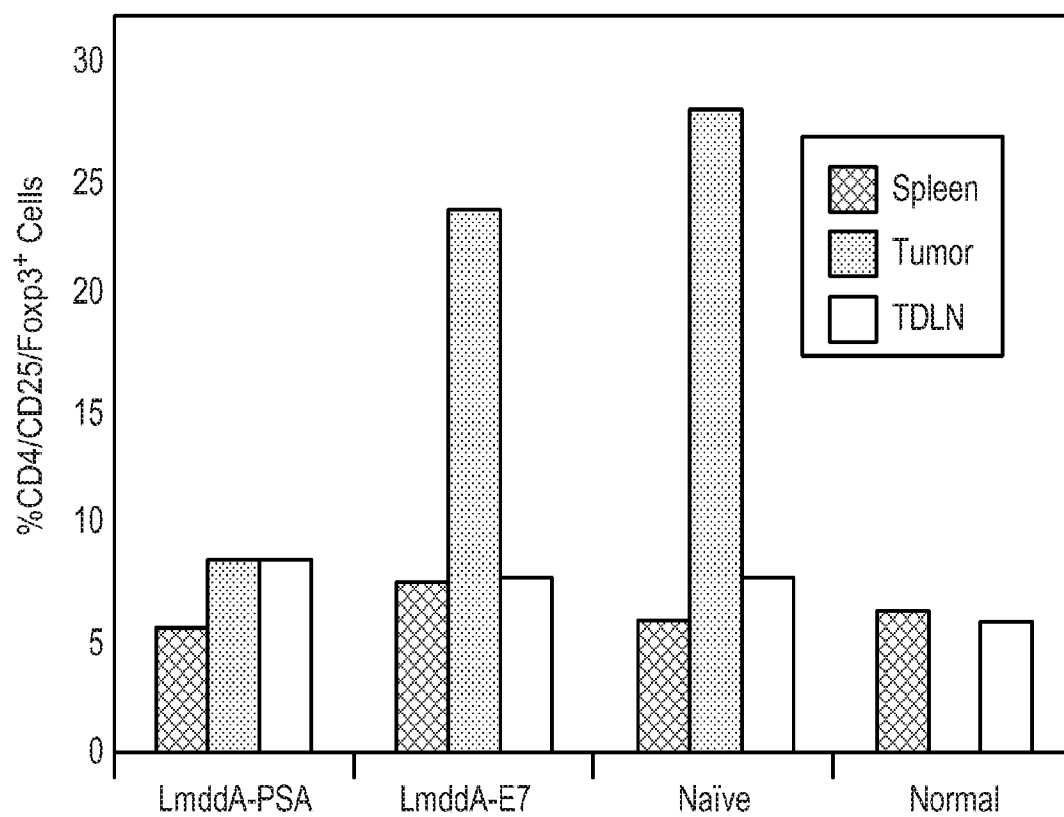


FIG. 4A

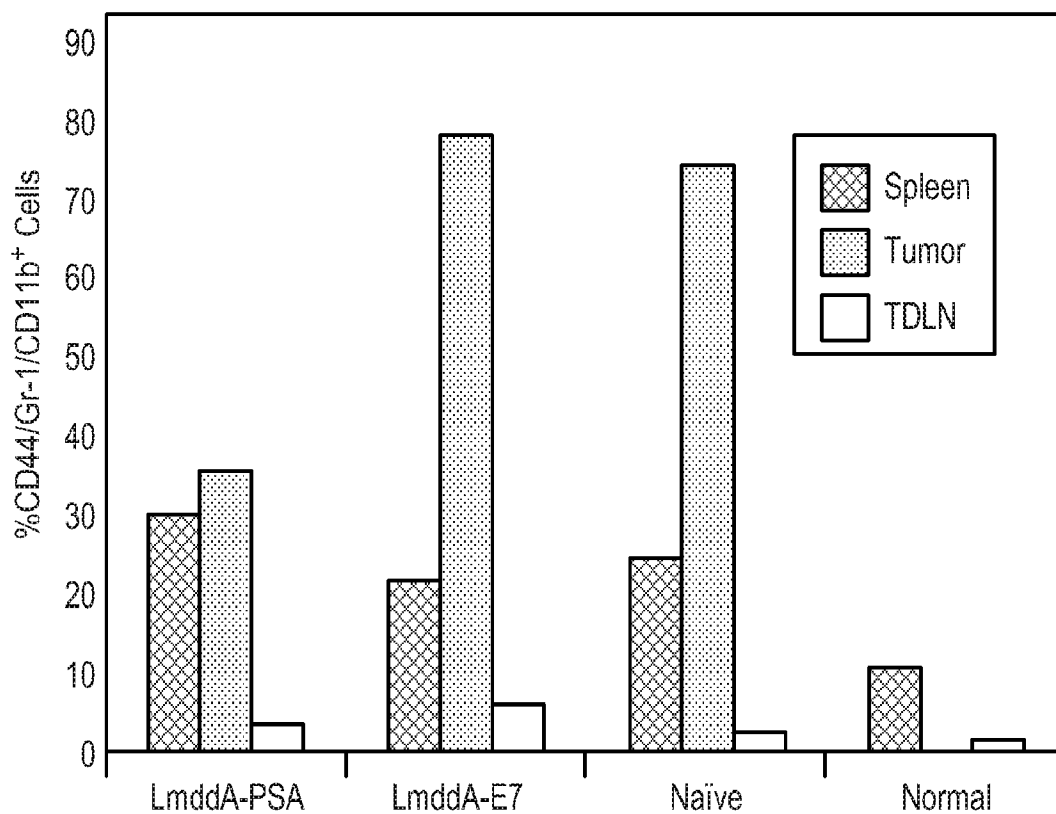


FIG. 4B

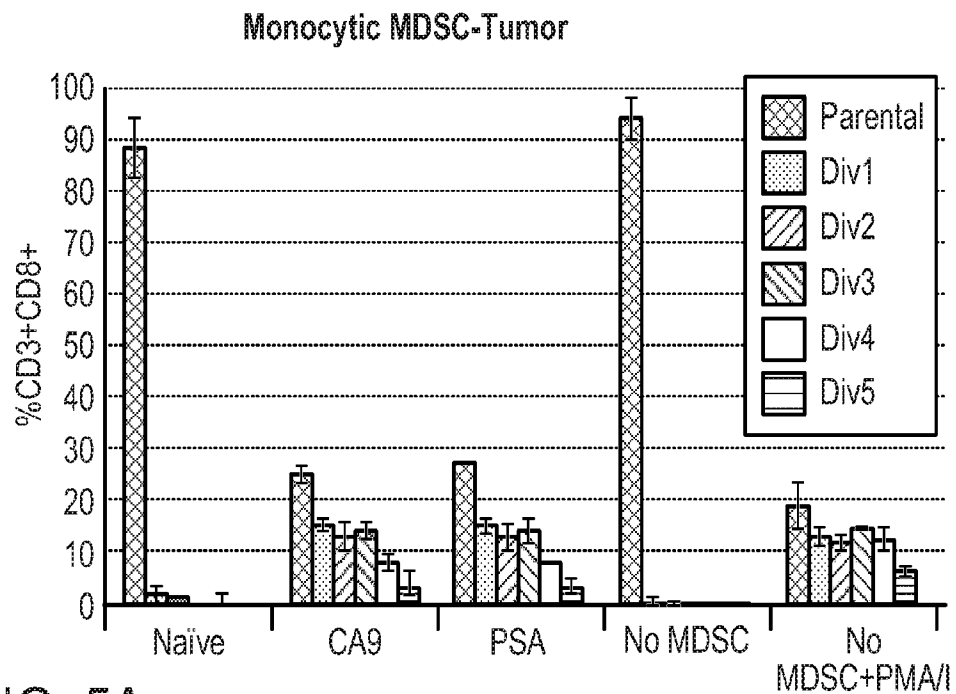


FIG. 5A

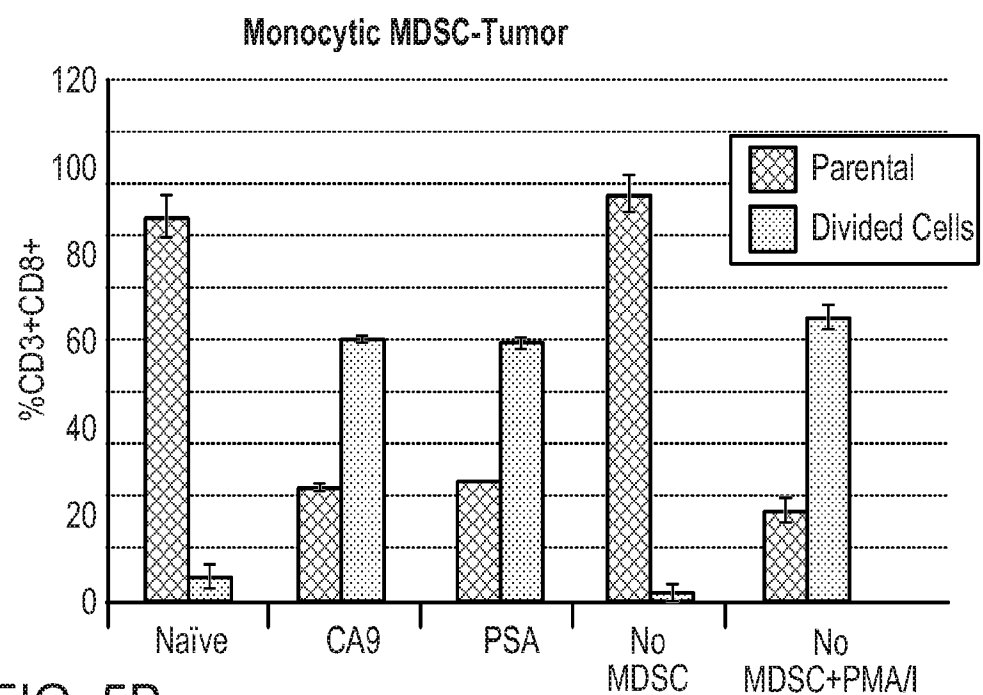


FIG. 5B

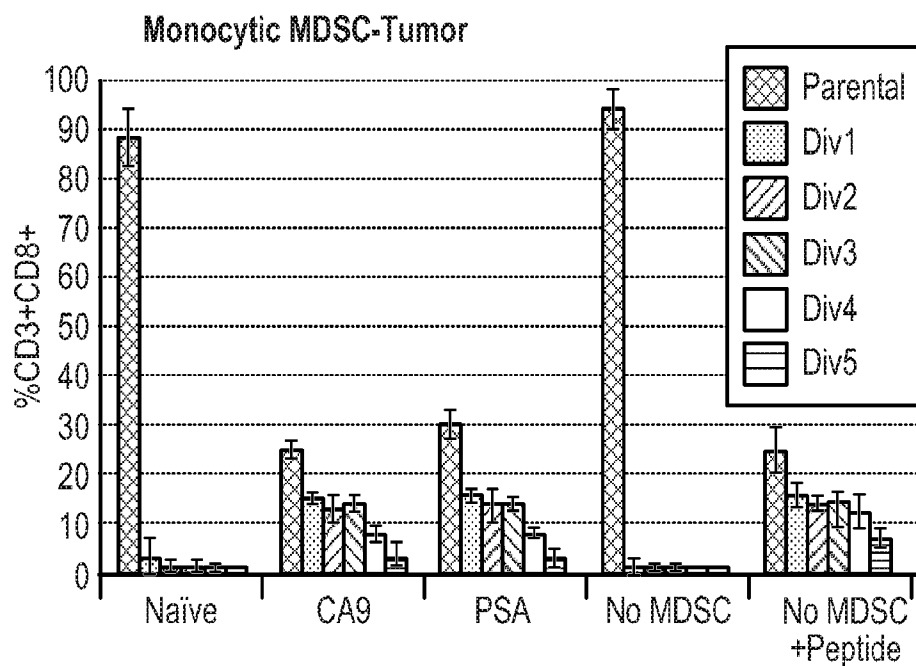


FIG. 5C

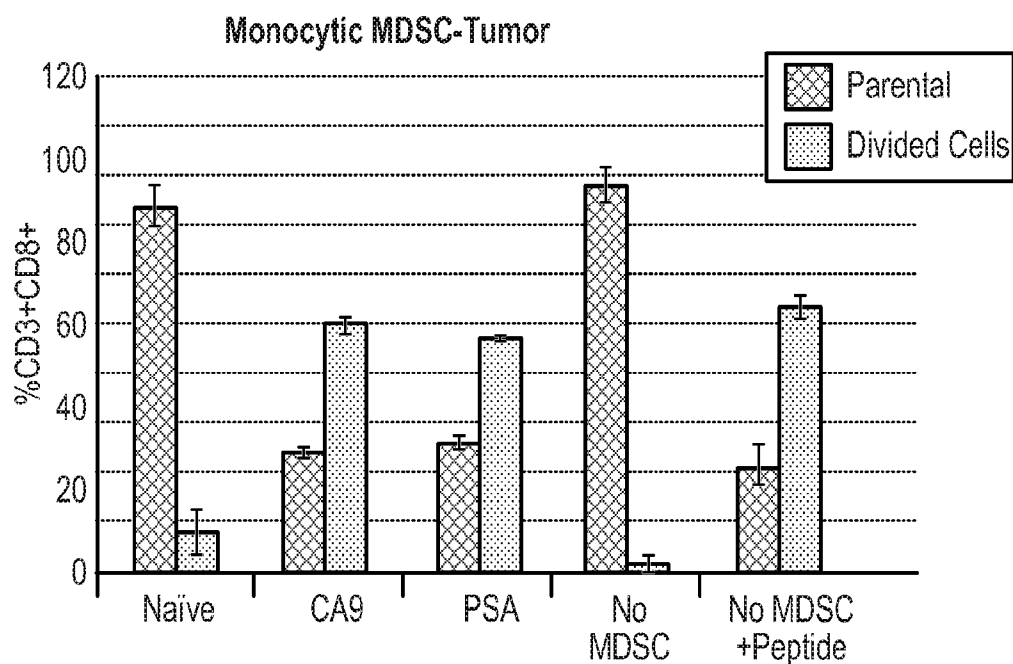


FIG. 5D

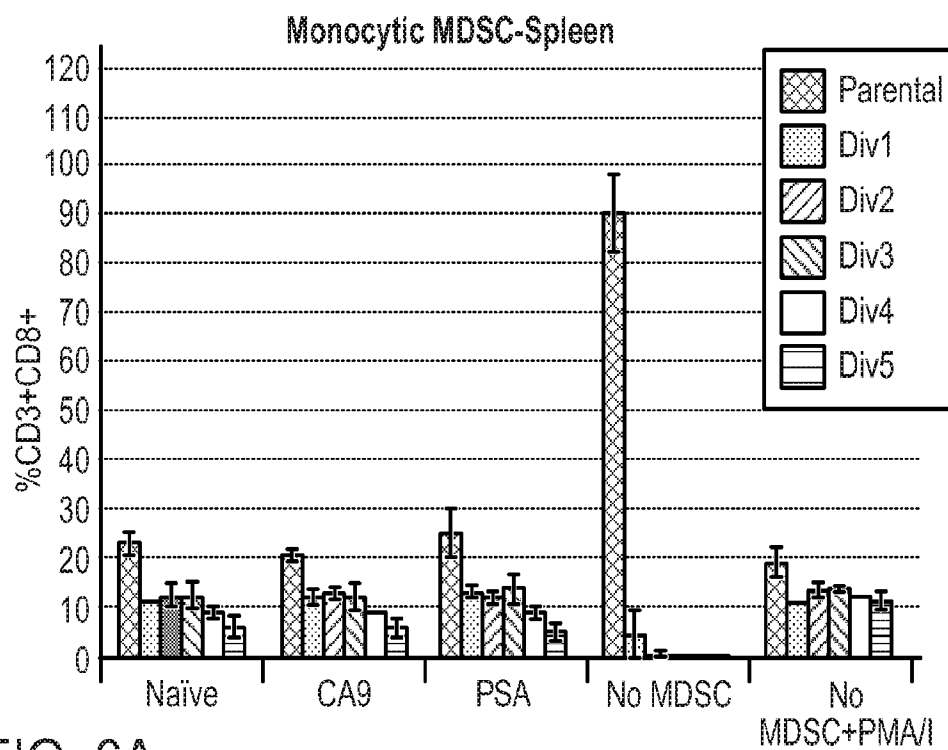


FIG. 6A

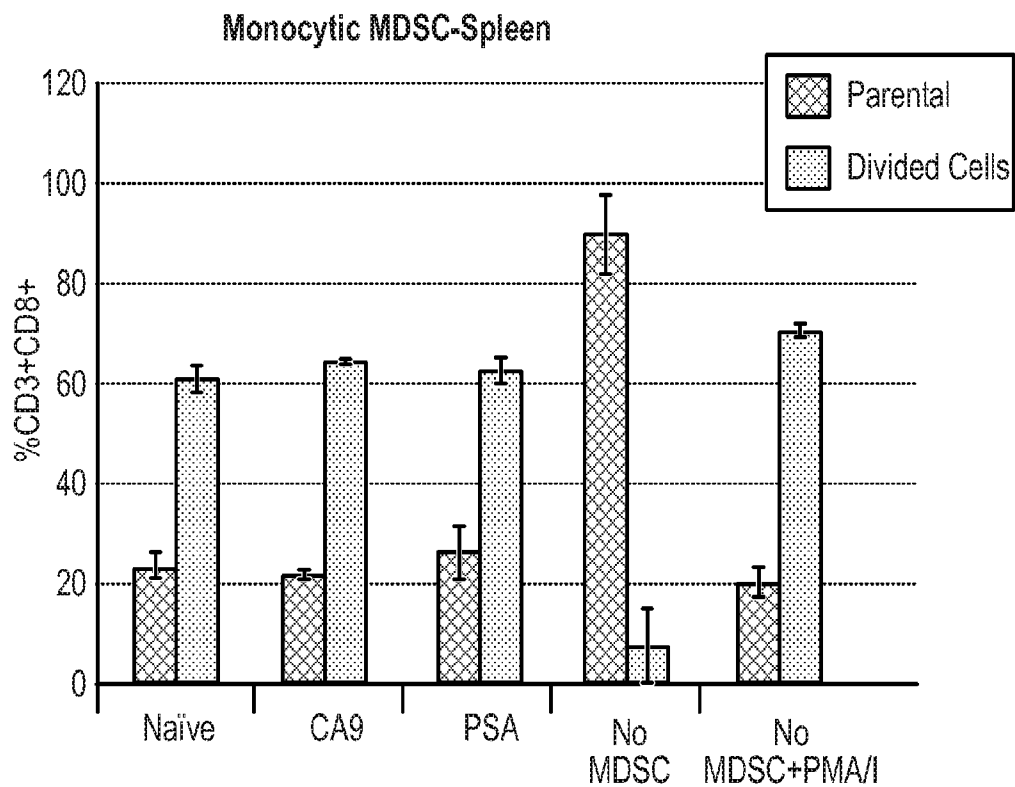


FIG. 6B

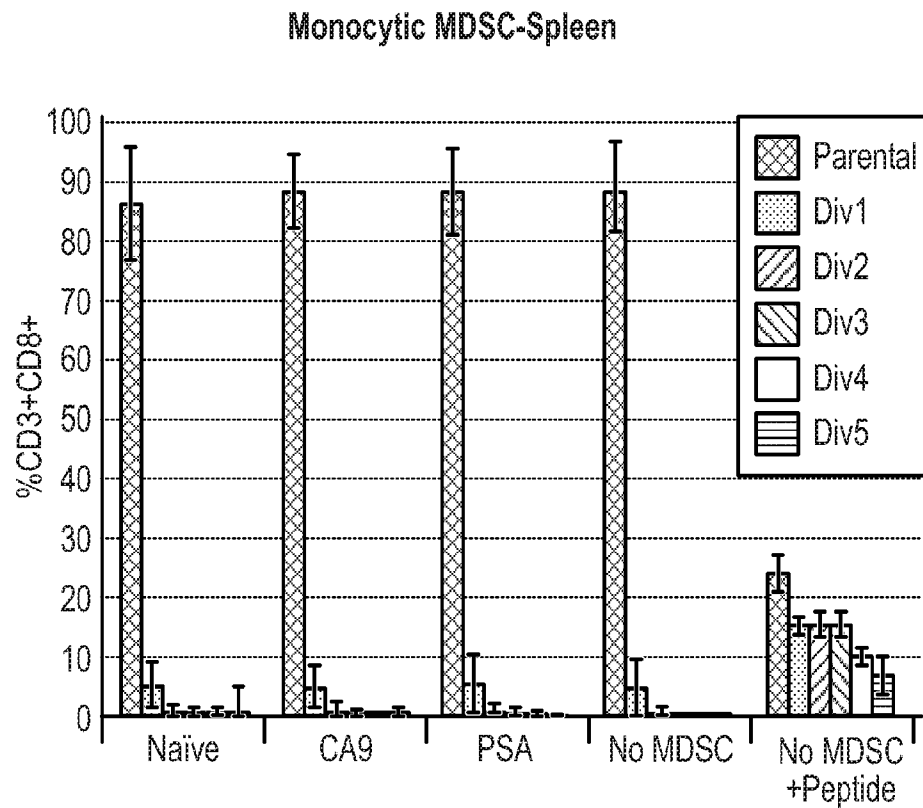


FIG. 6C

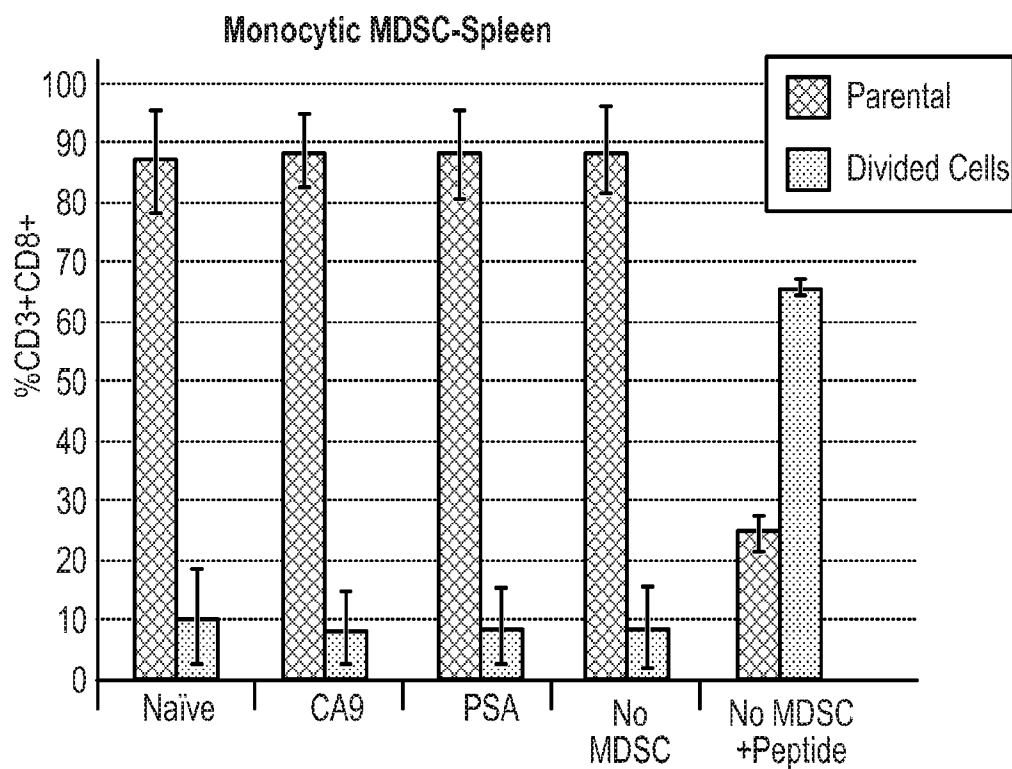


FIG. 6D

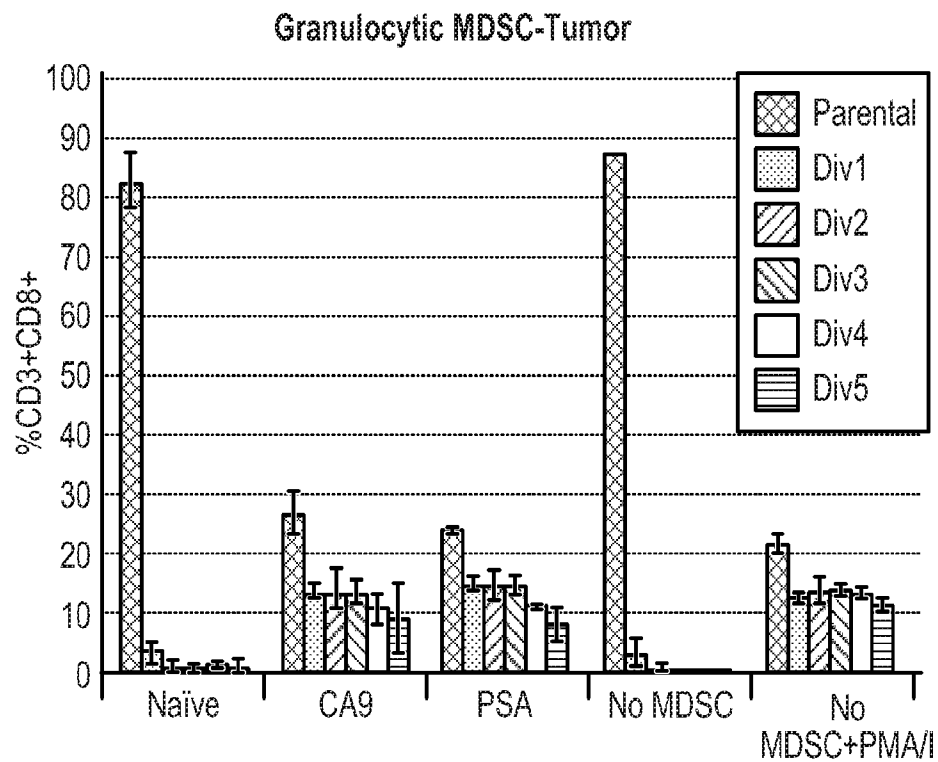


FIG. 7A

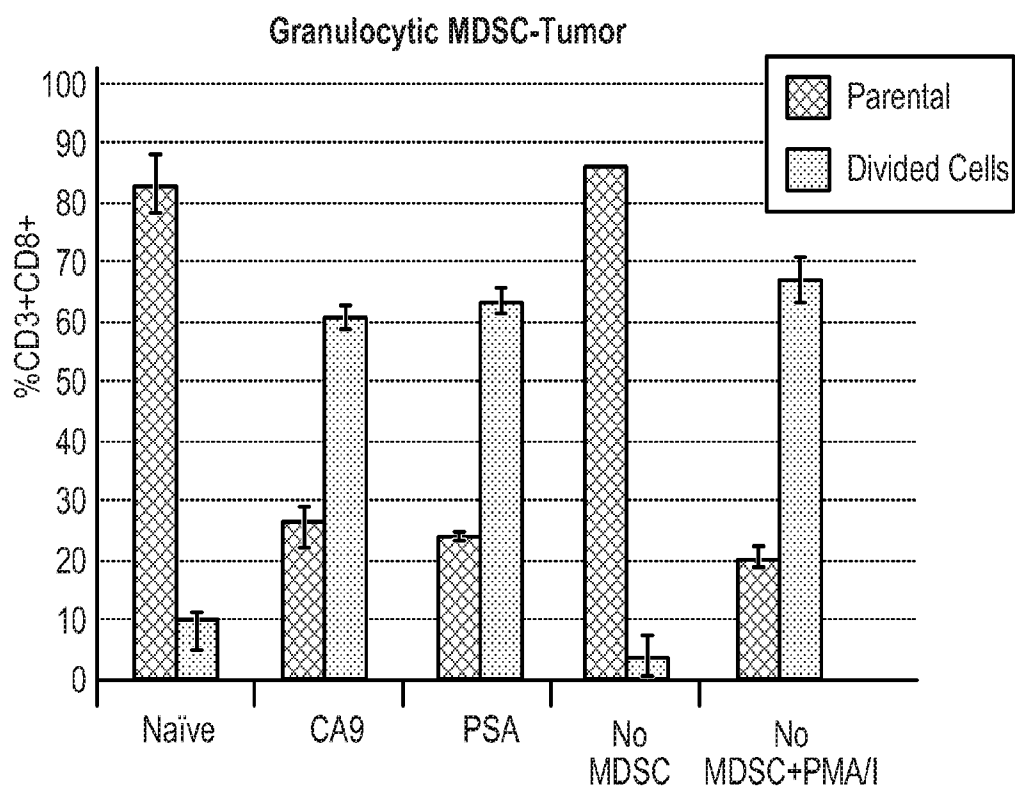


FIG. 7B

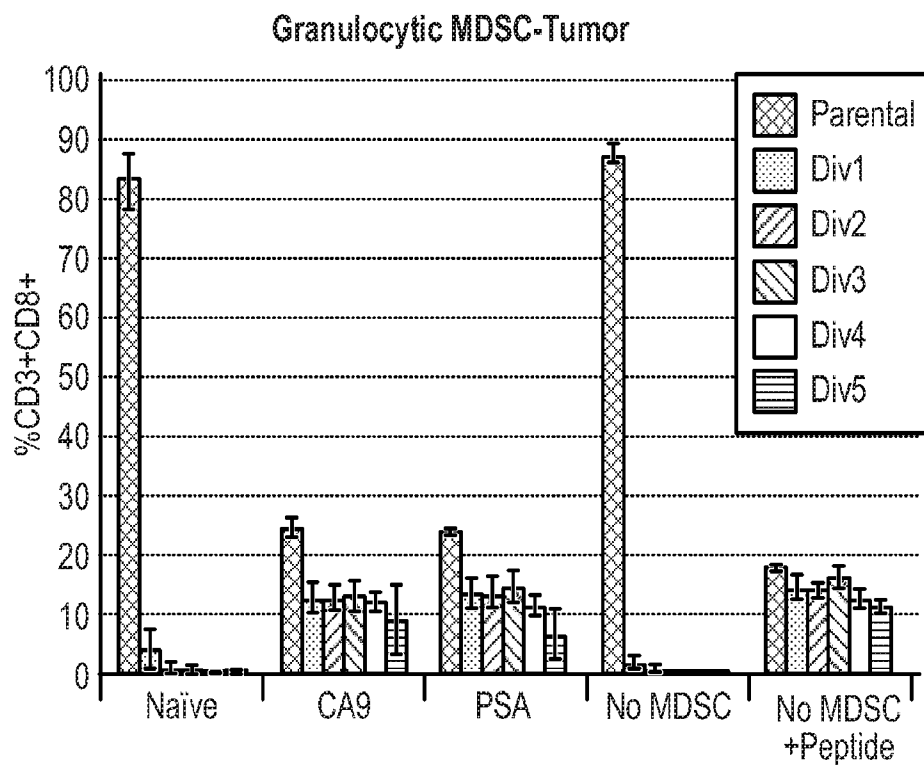


FIG. 7C

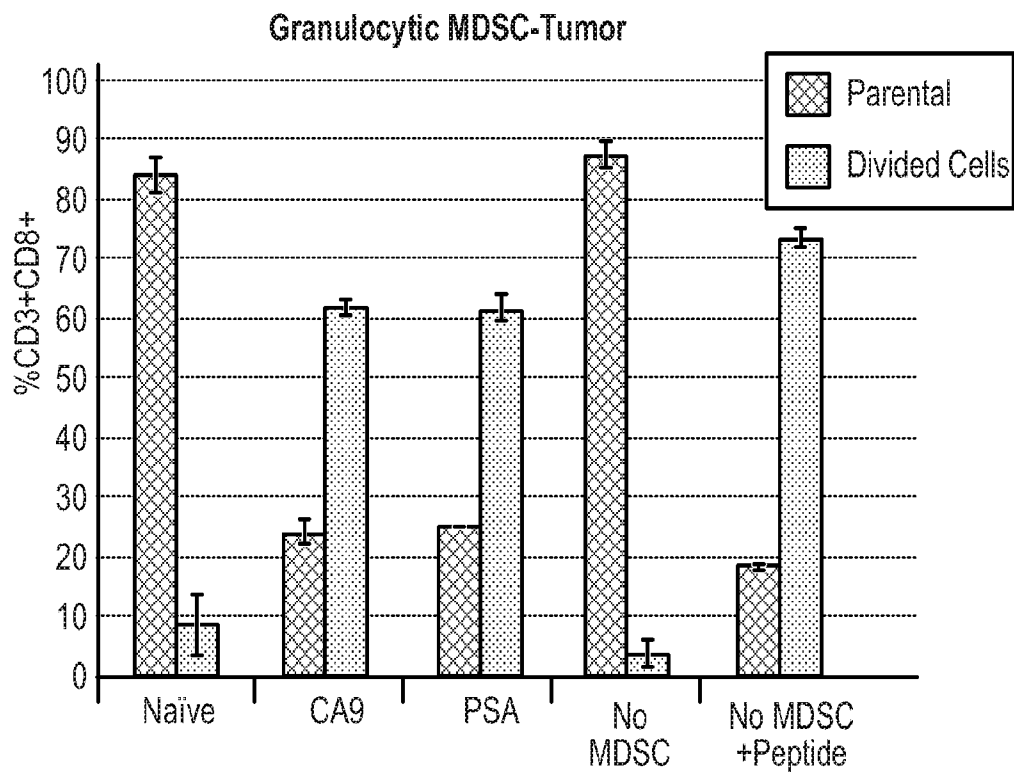


FIG. 7D

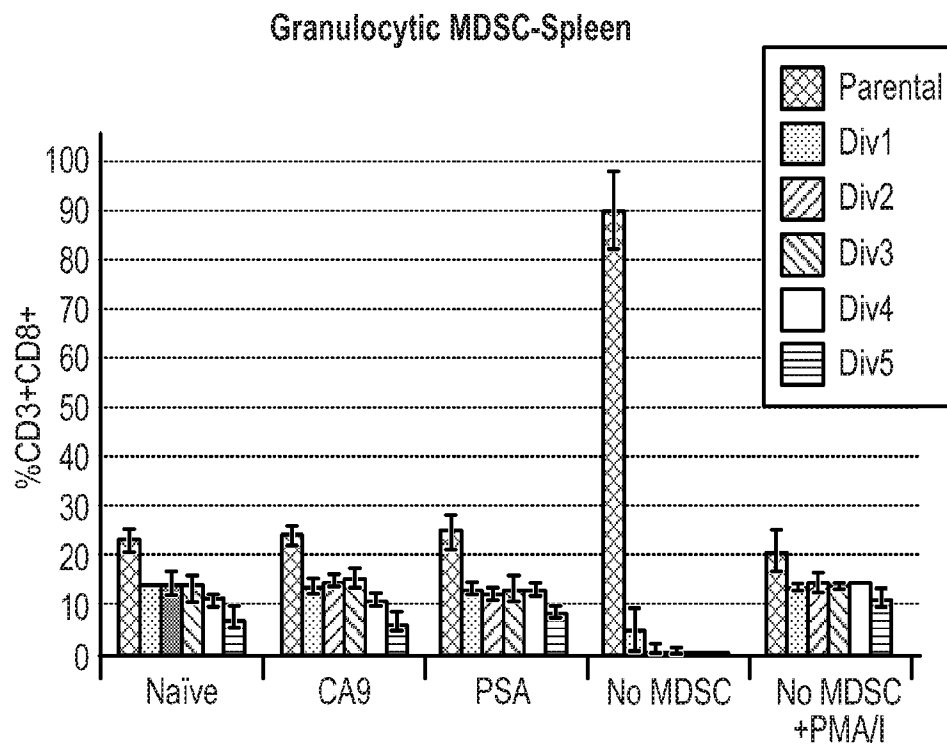


FIG. 8A

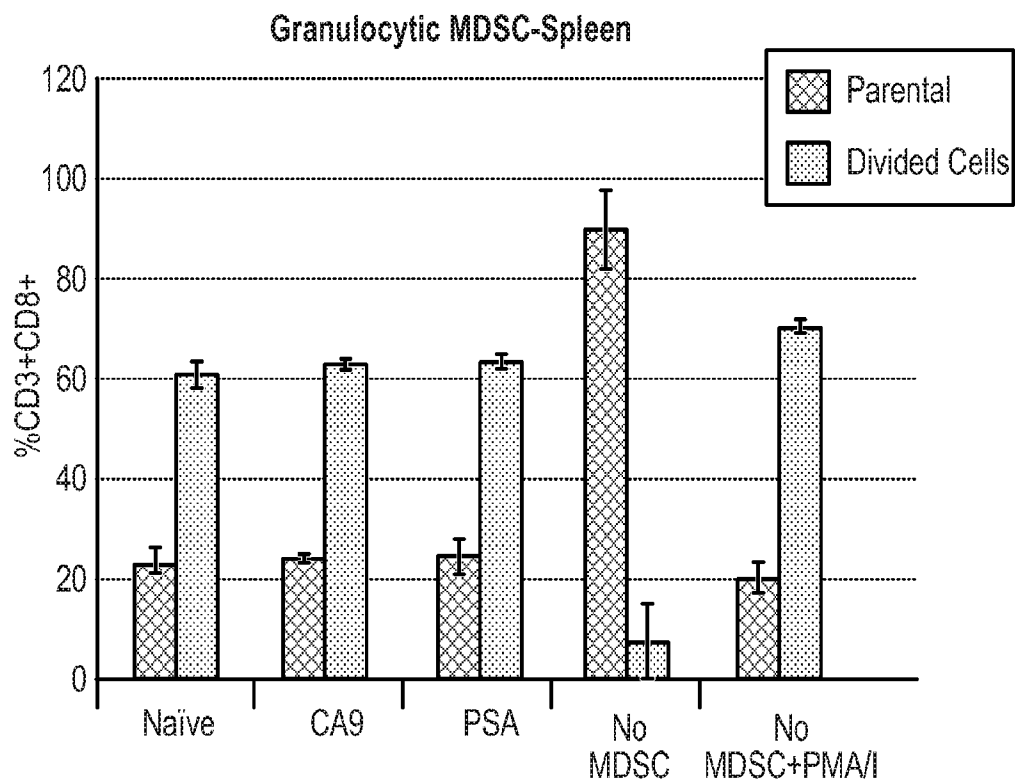


FIG. 8B

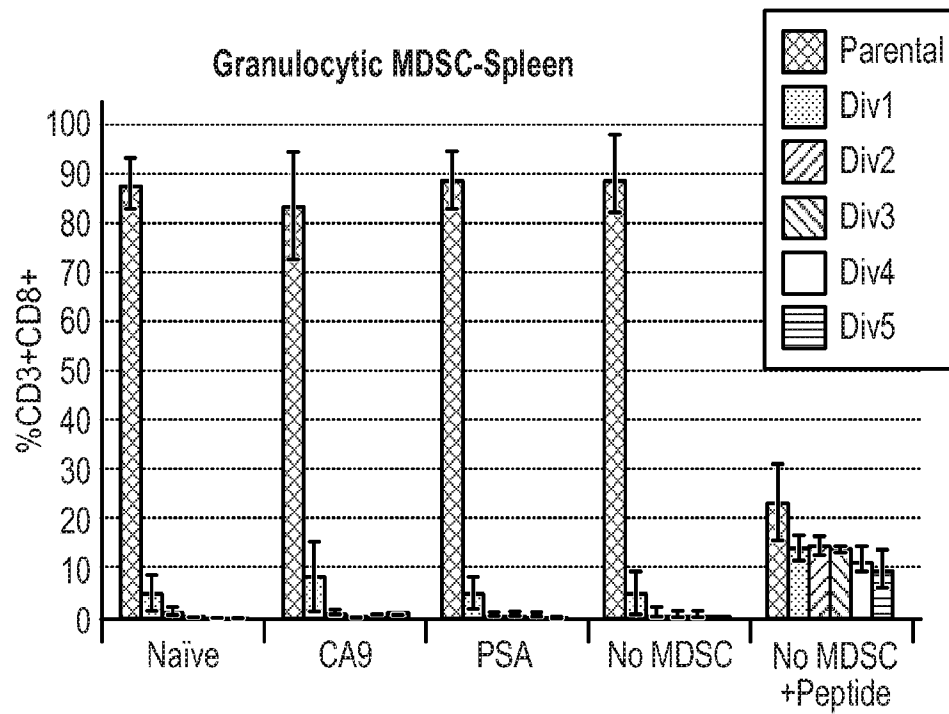


FIG. 8C

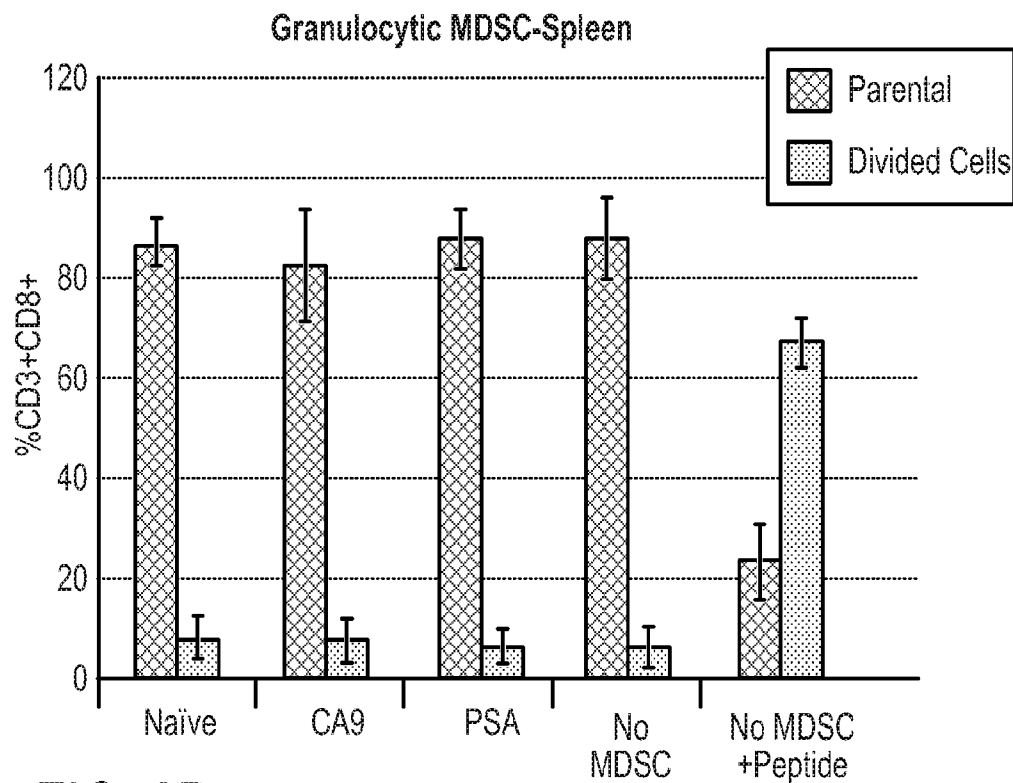


FIG. 8D

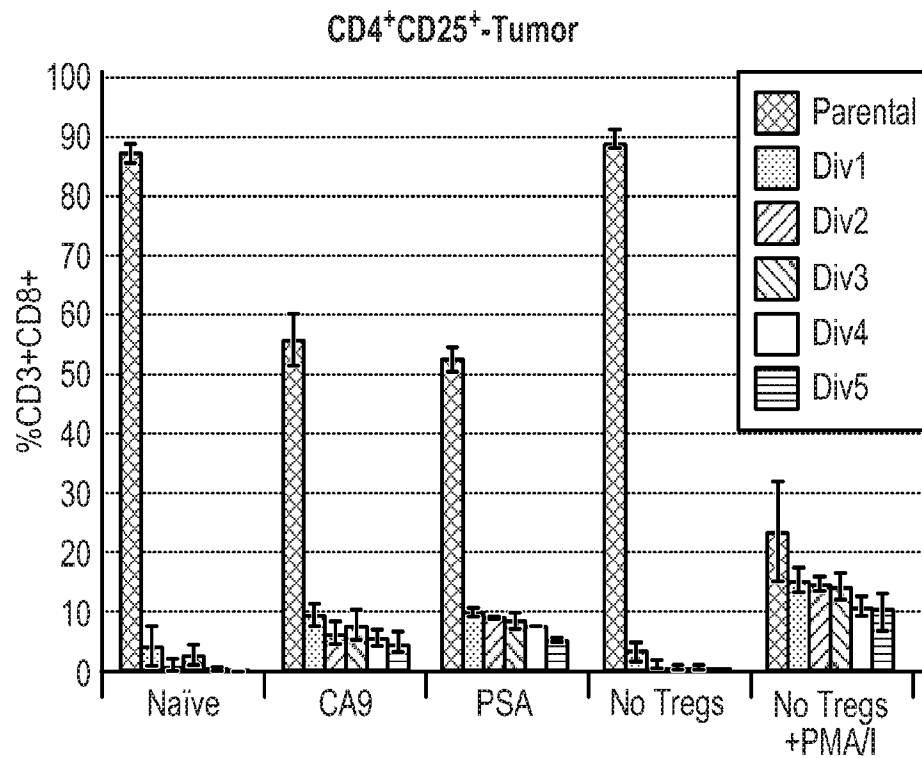


FIG. 9A

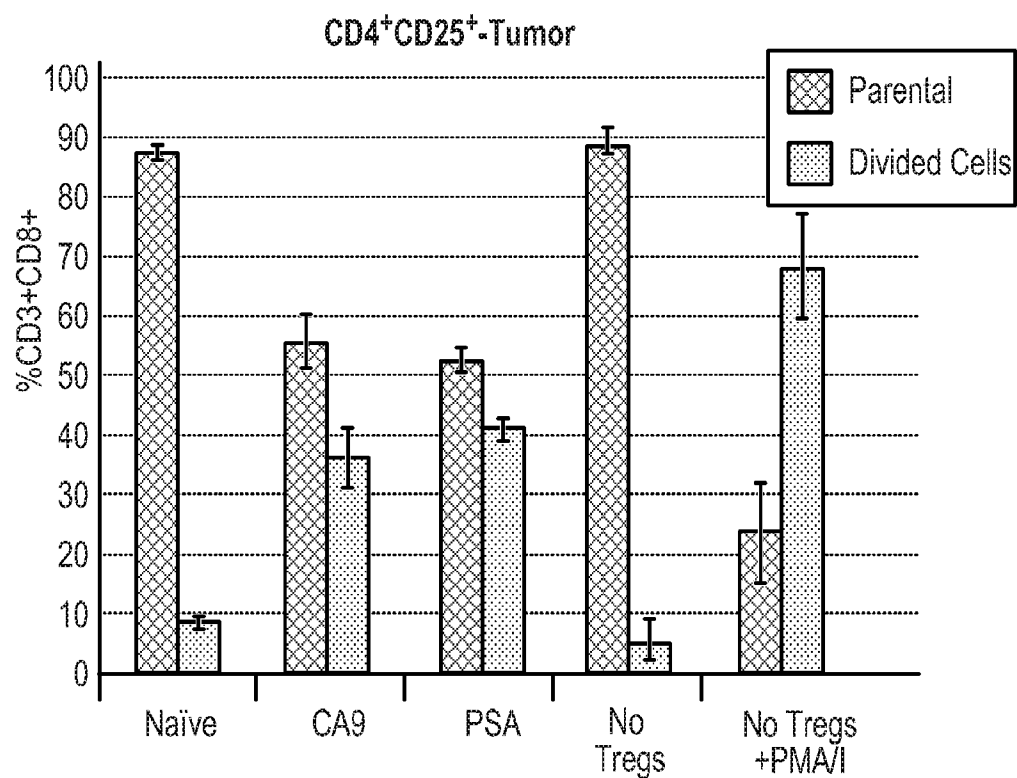


FIG. 9B

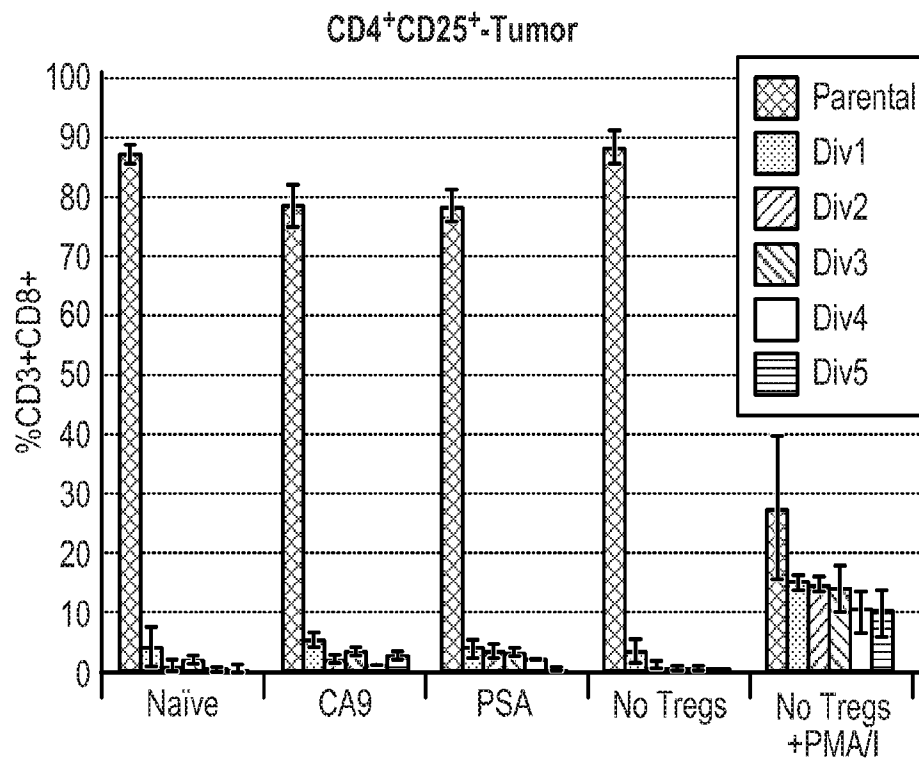


FIG. 9C

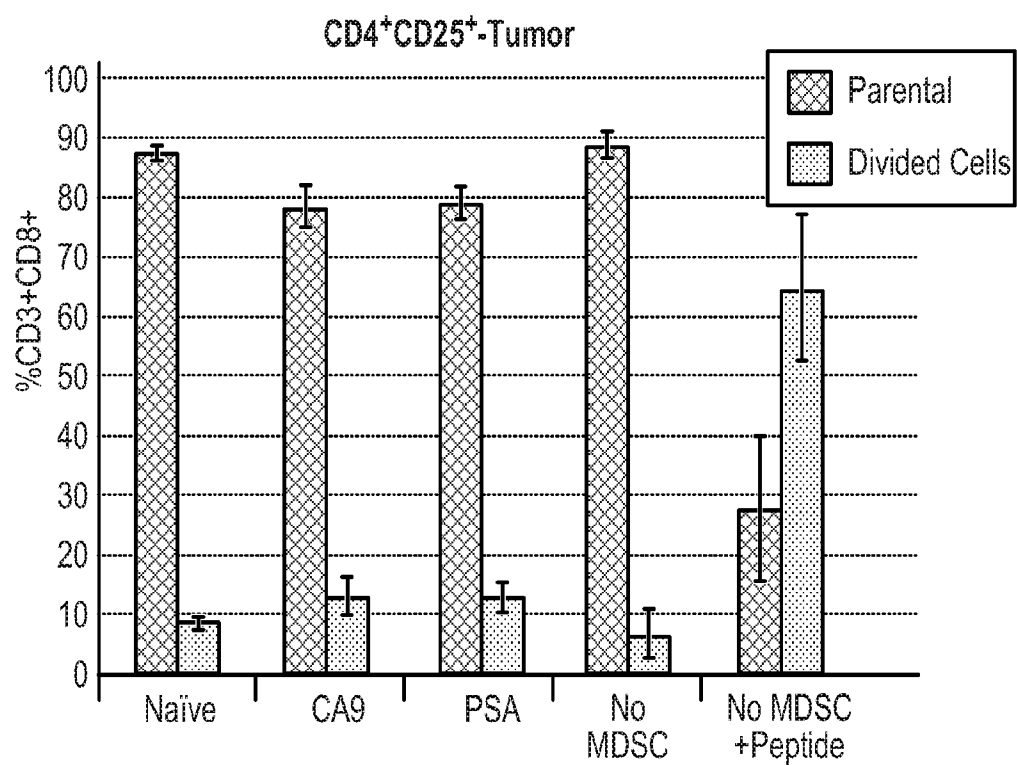


FIG. 9D

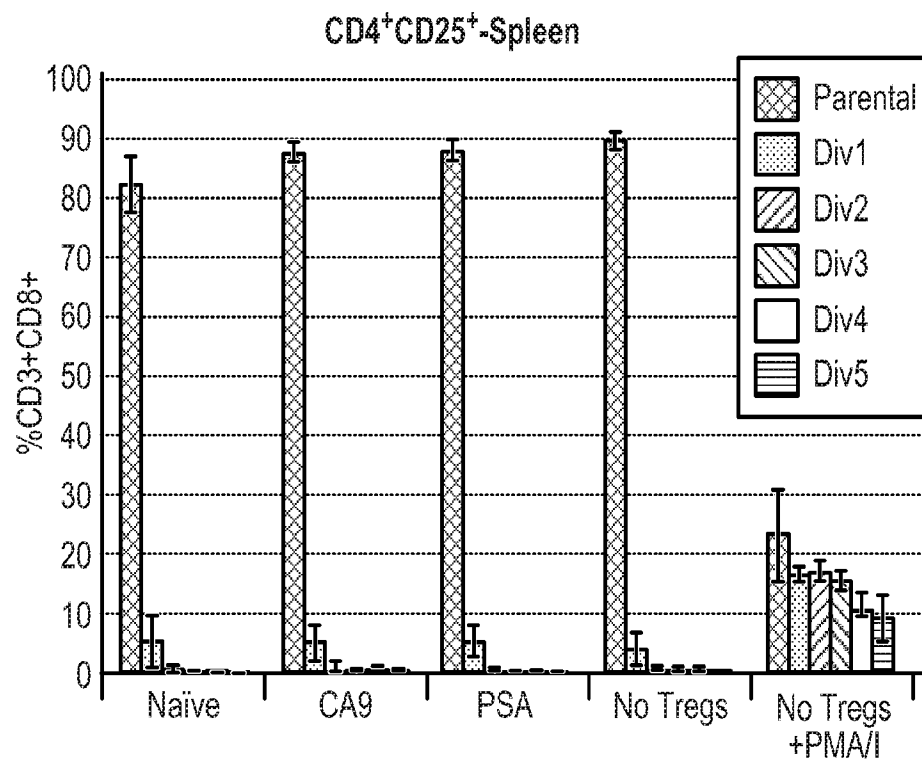


FIG. 10A

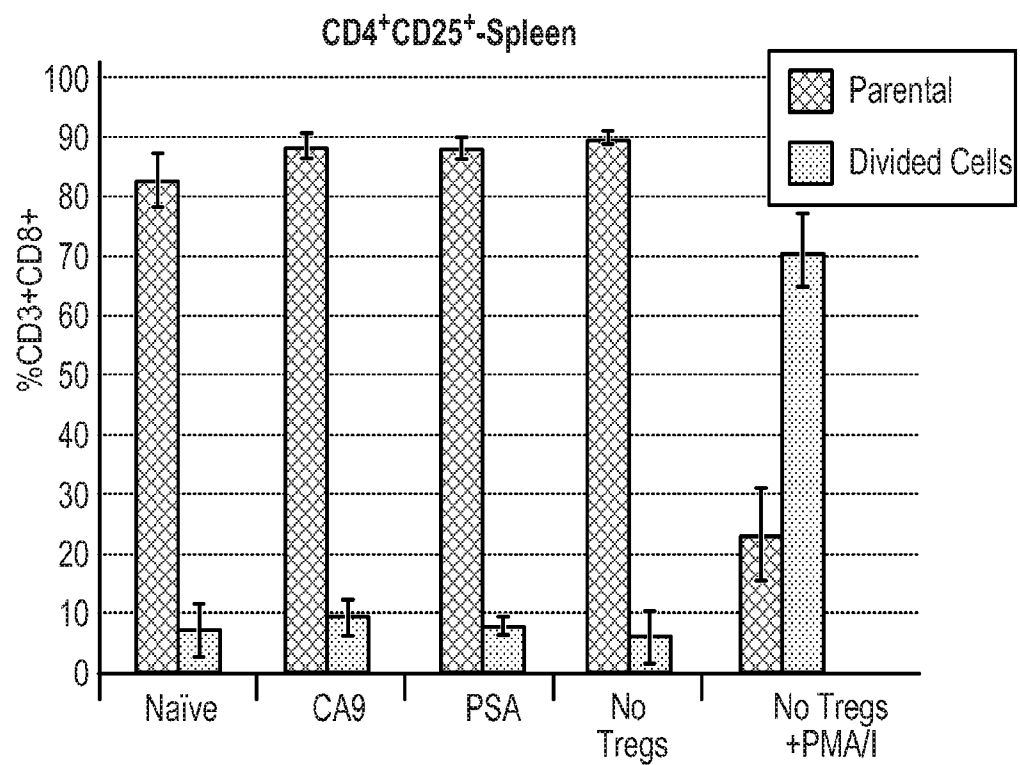


FIG. 10B

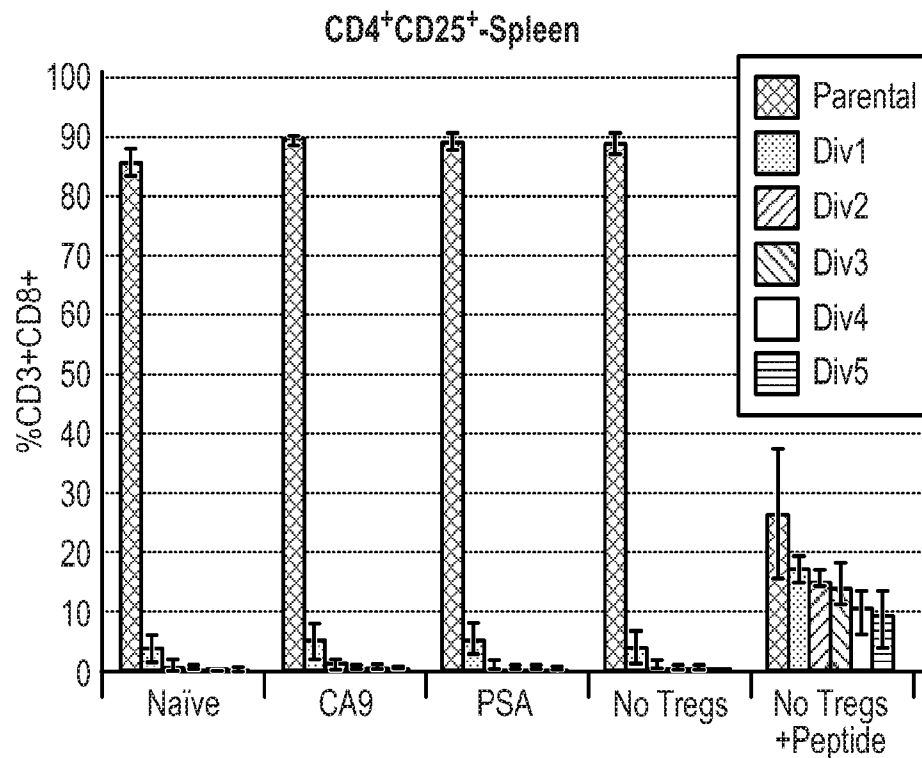


FIG. 10C

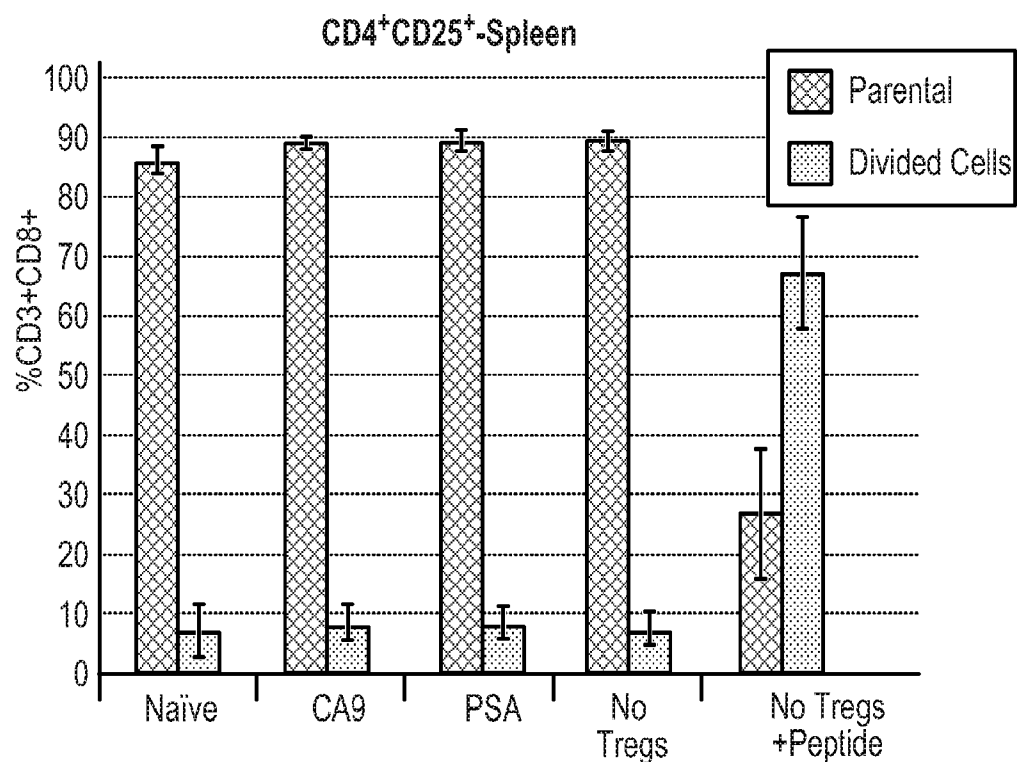


FIG. 10D

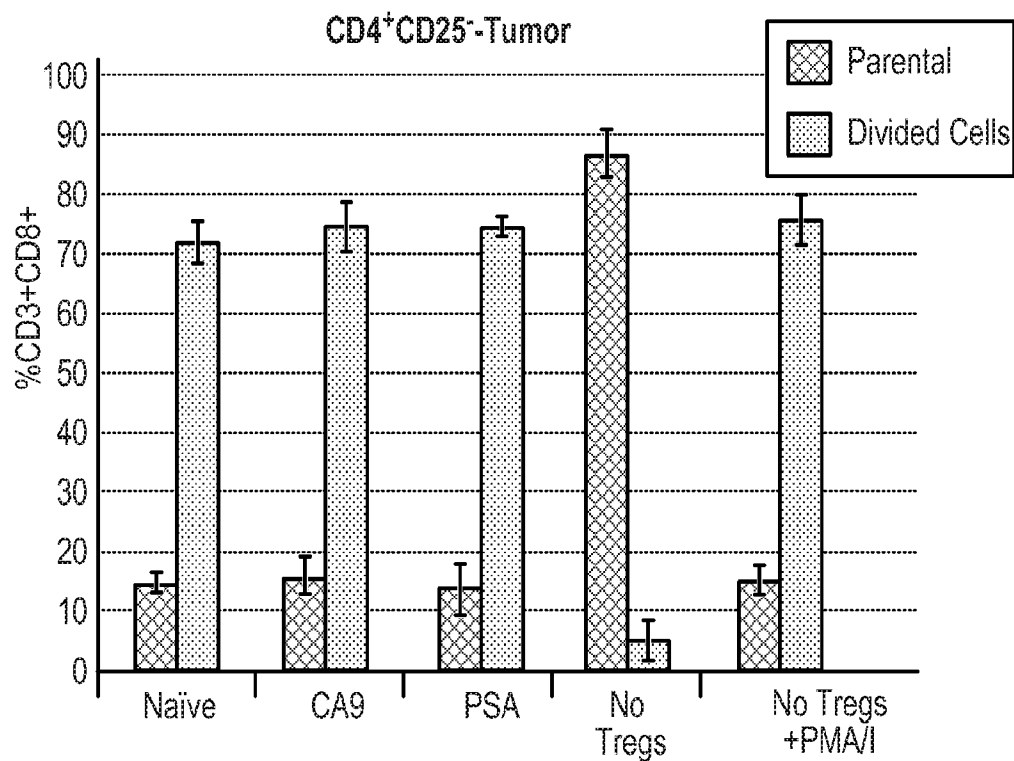


FIG. 11A

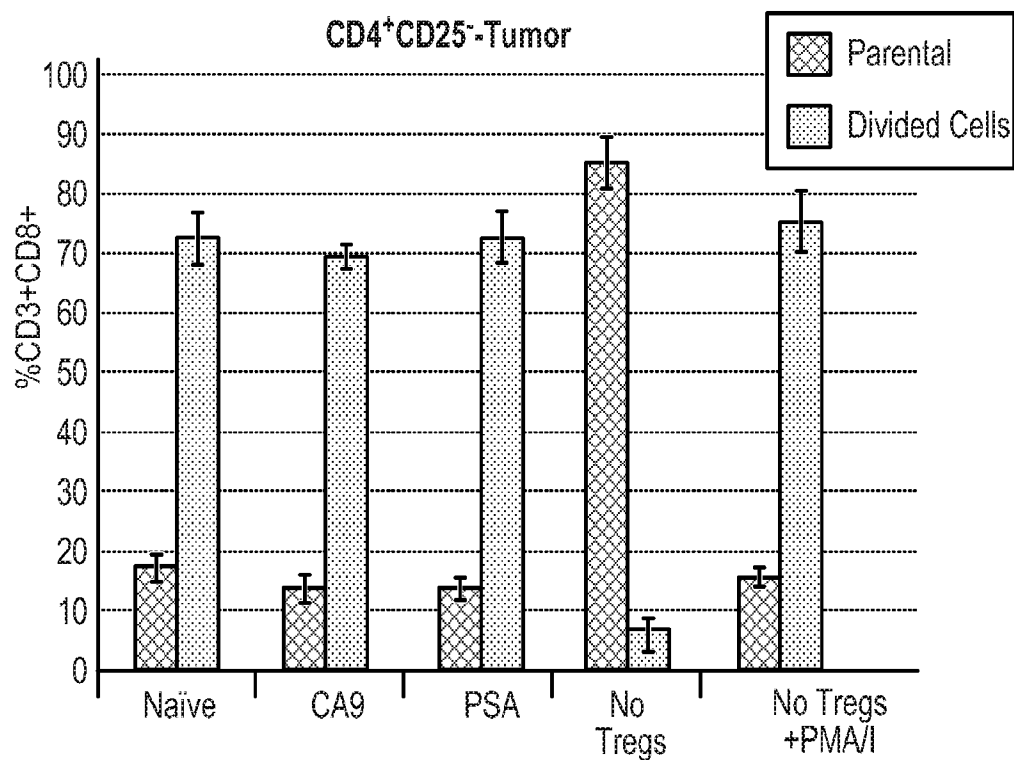


FIG. 11B

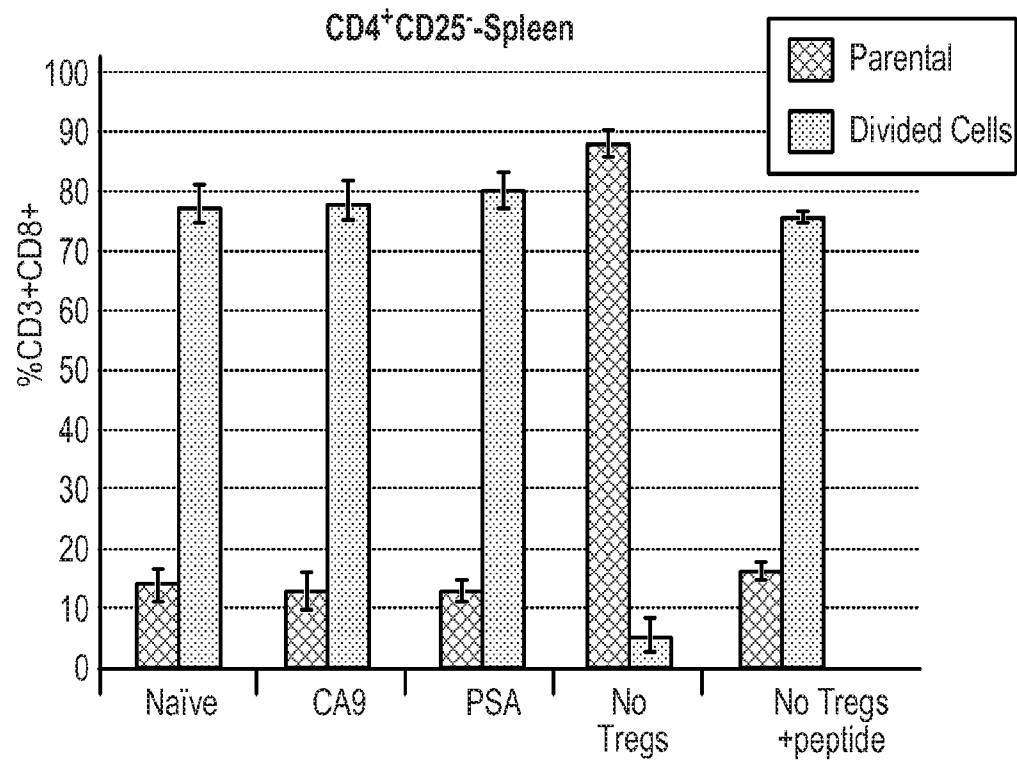


FIG. 11C

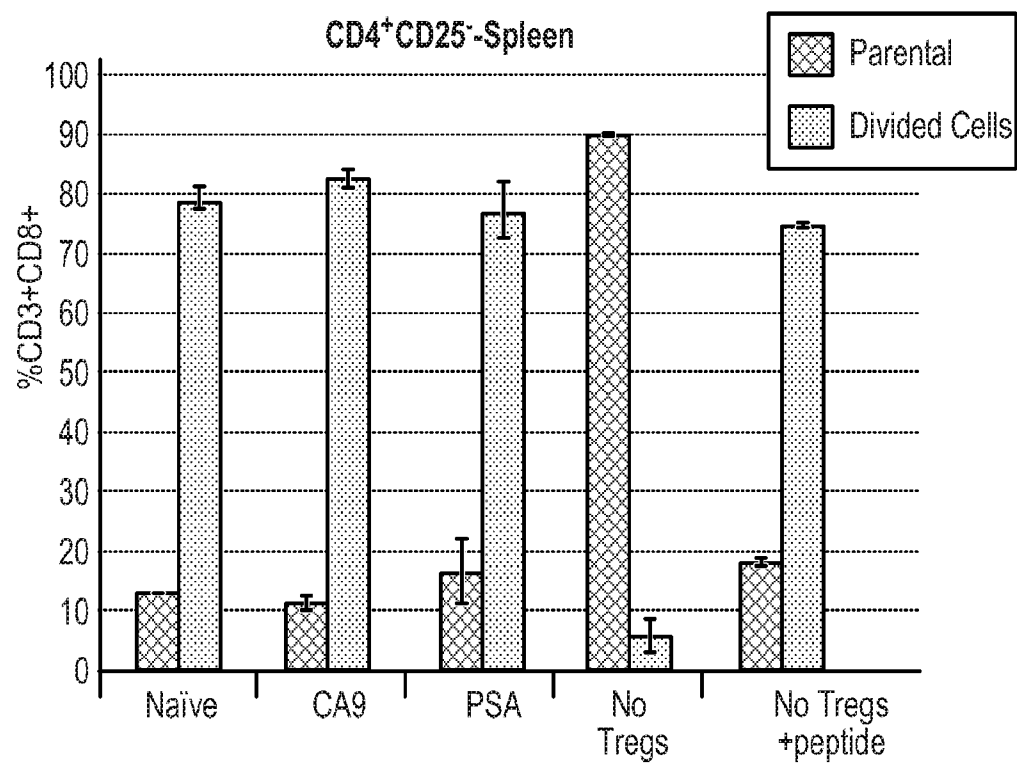


FIG. 11D

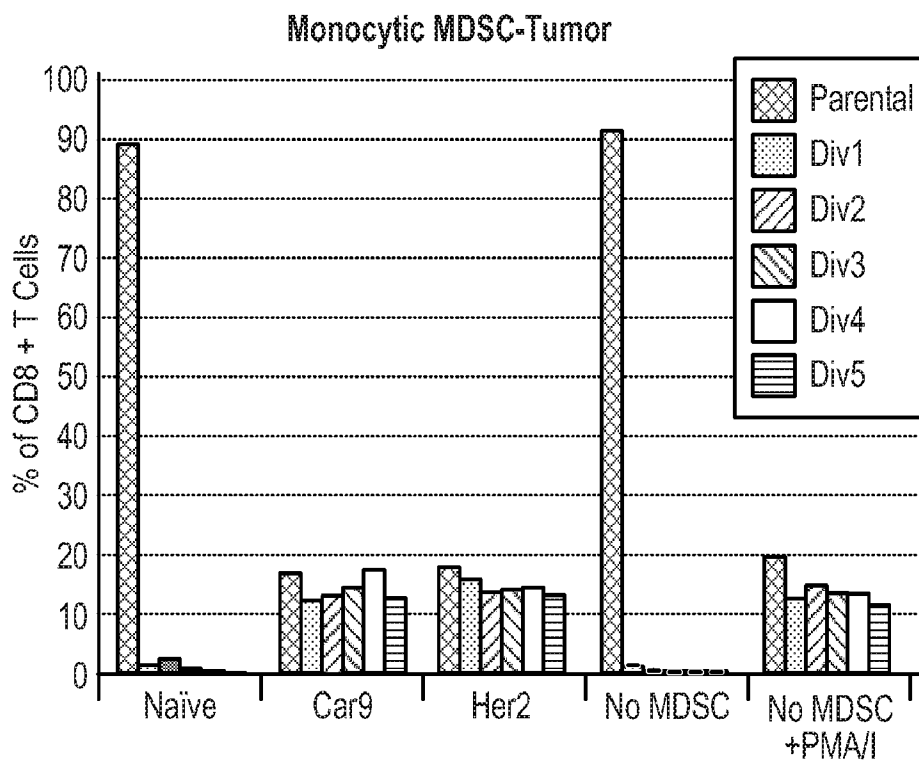


FIG. 12A

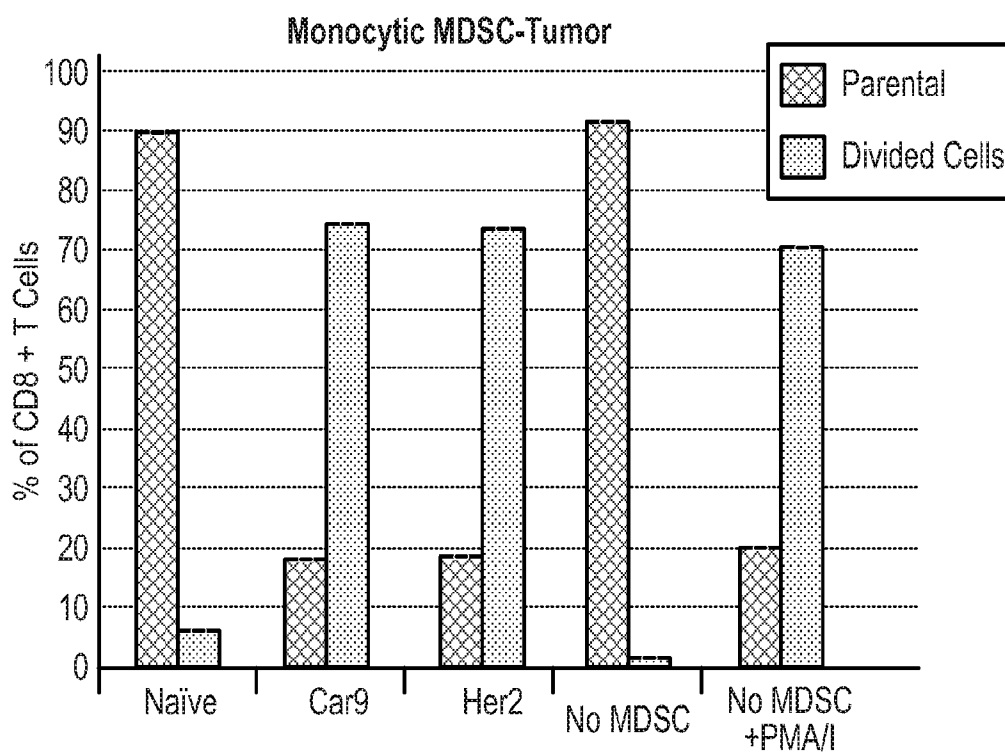


FIG. 12B

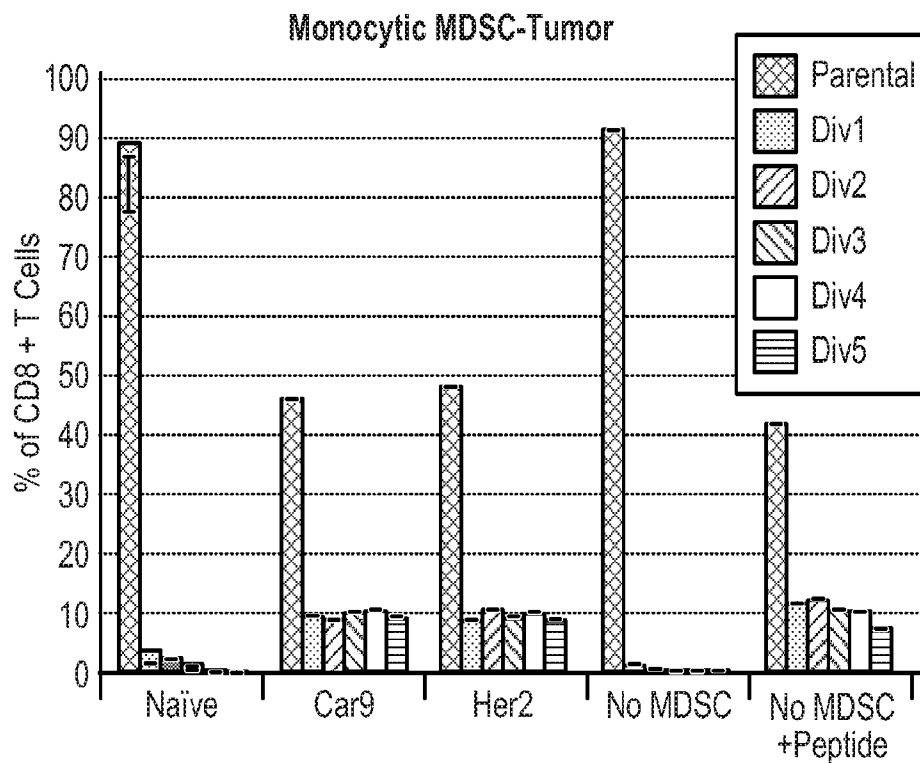


FIG. 12C

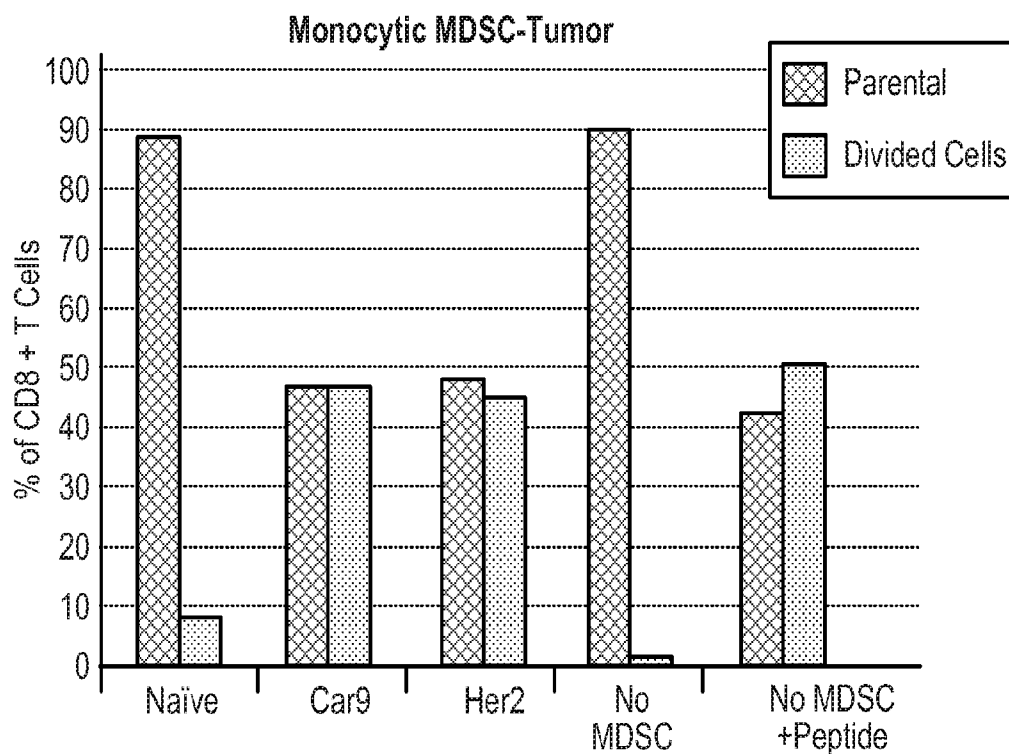


FIG. 12D

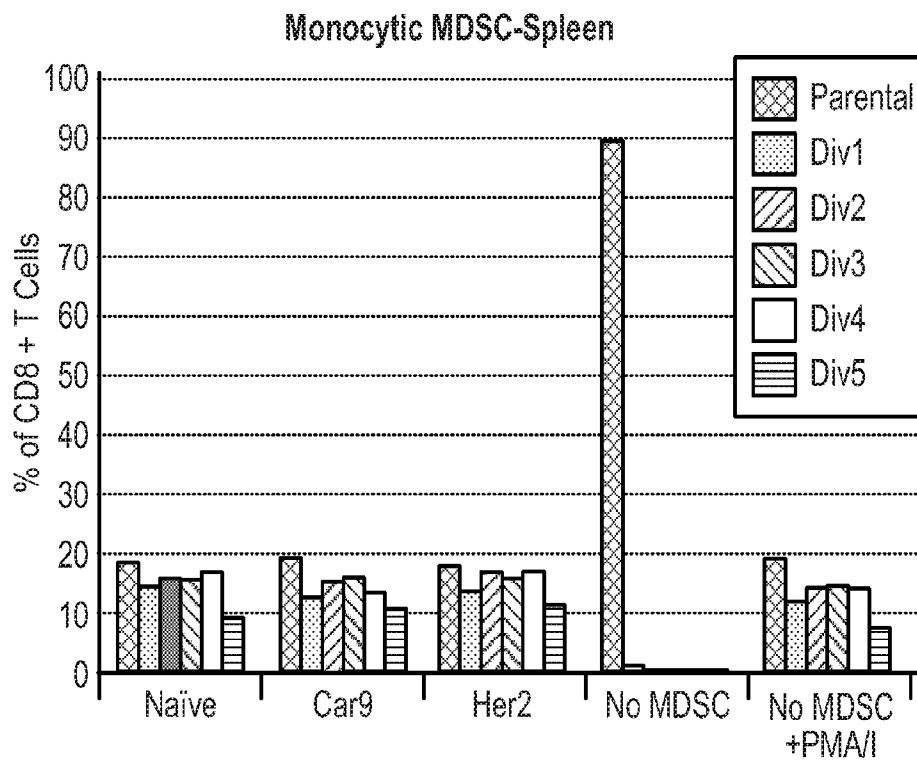


FIG. 13A

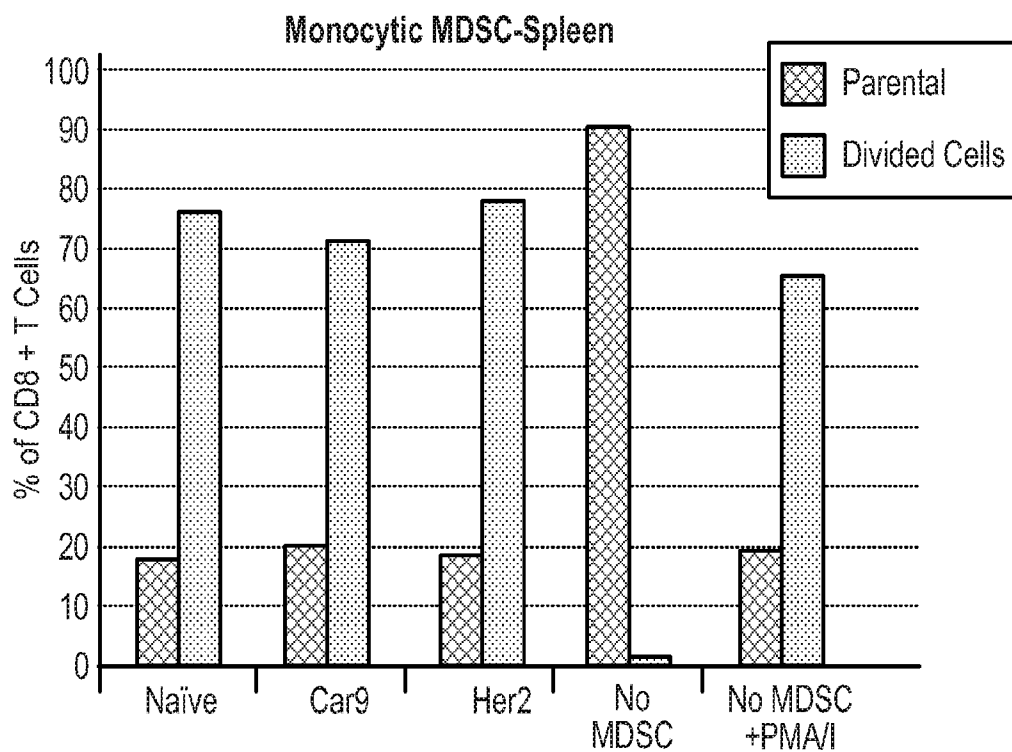


FIG. 13B

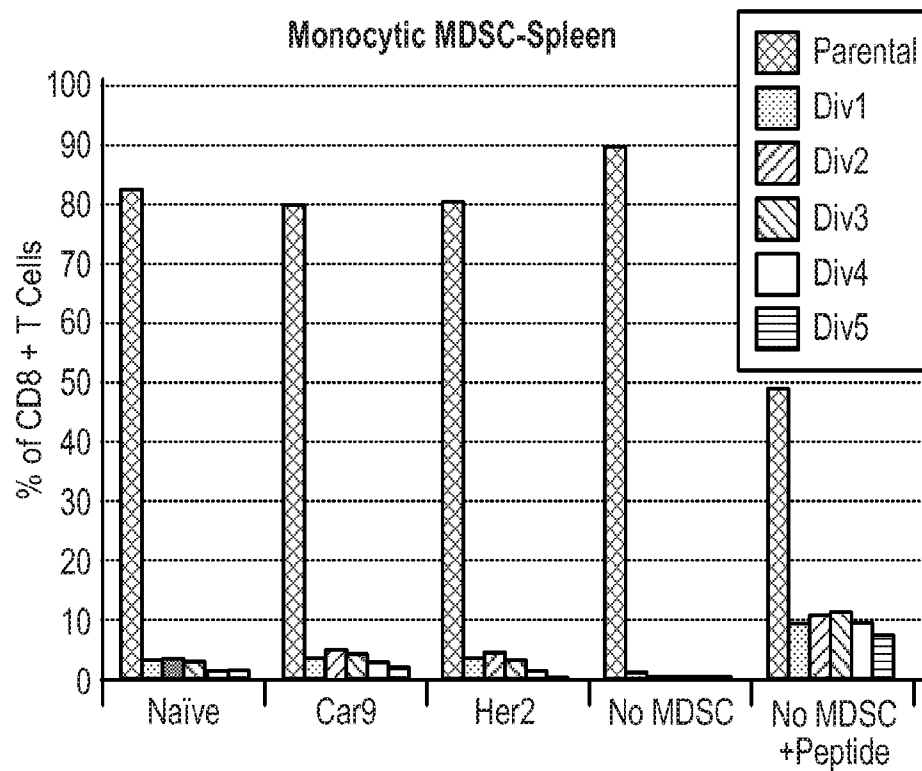


FIG. 13C

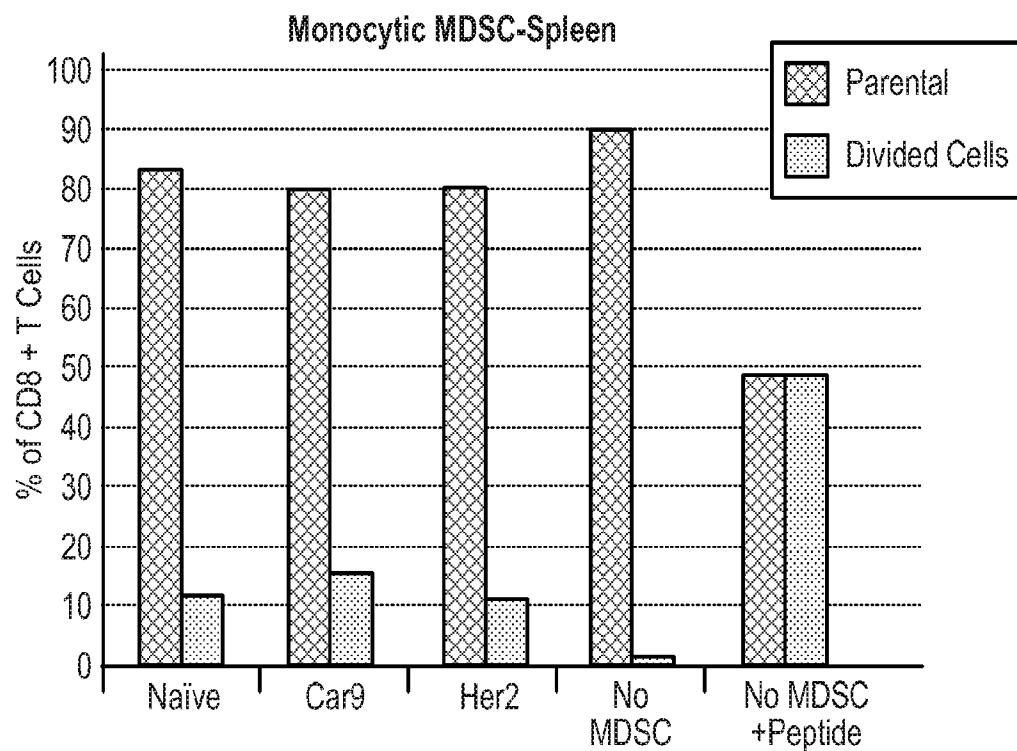


FIG. 13D

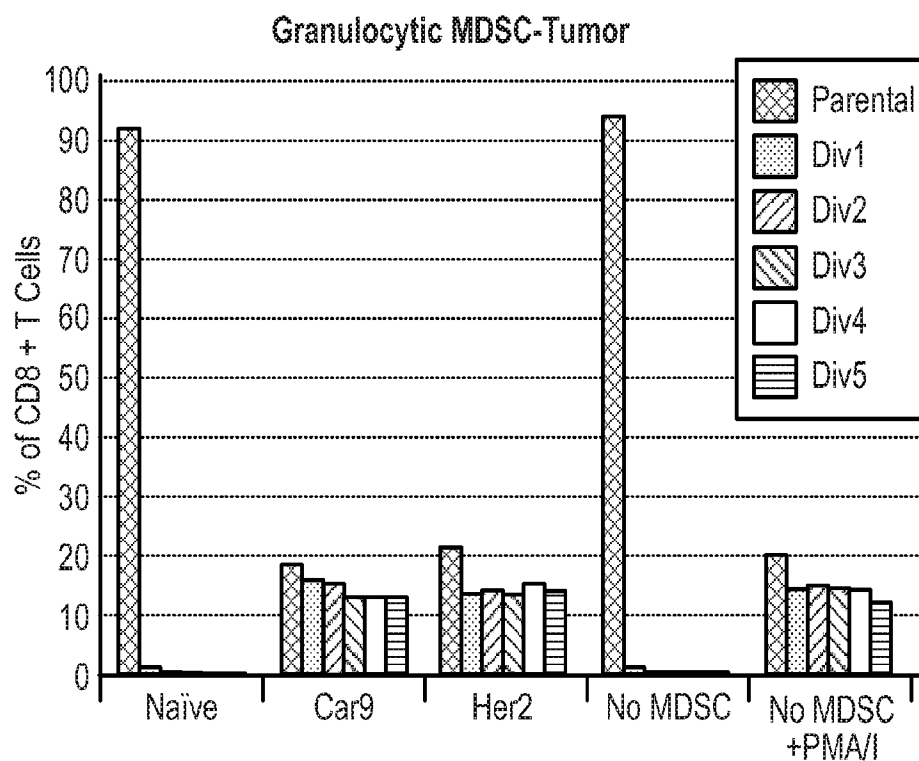


FIG. 14A

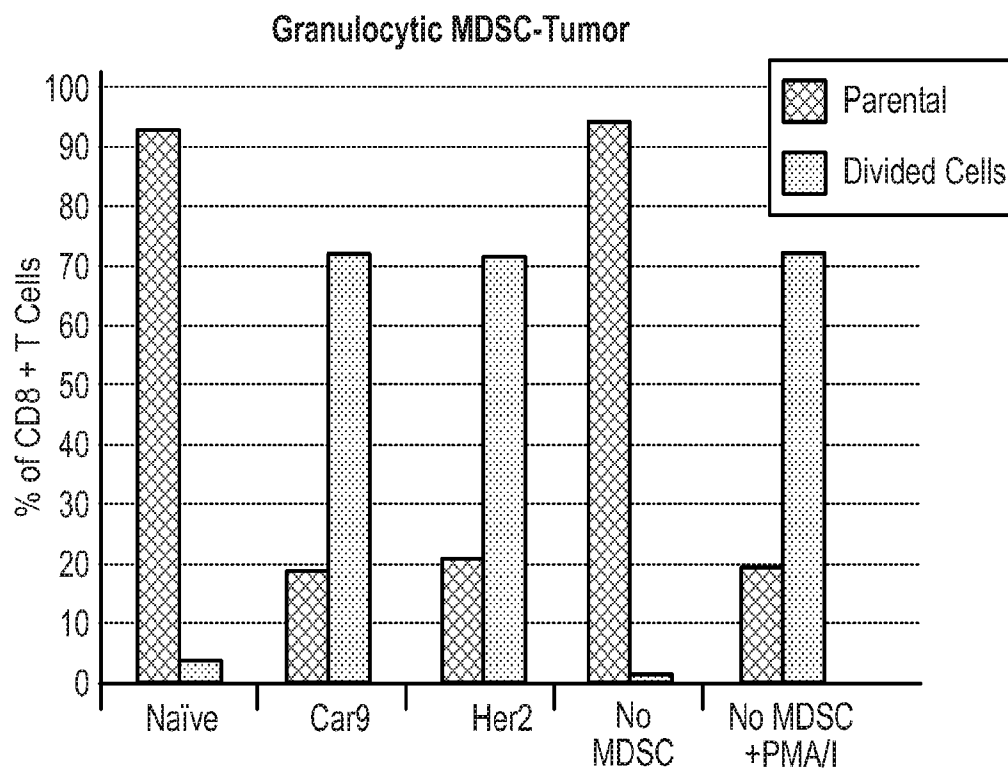


FIG. 14B

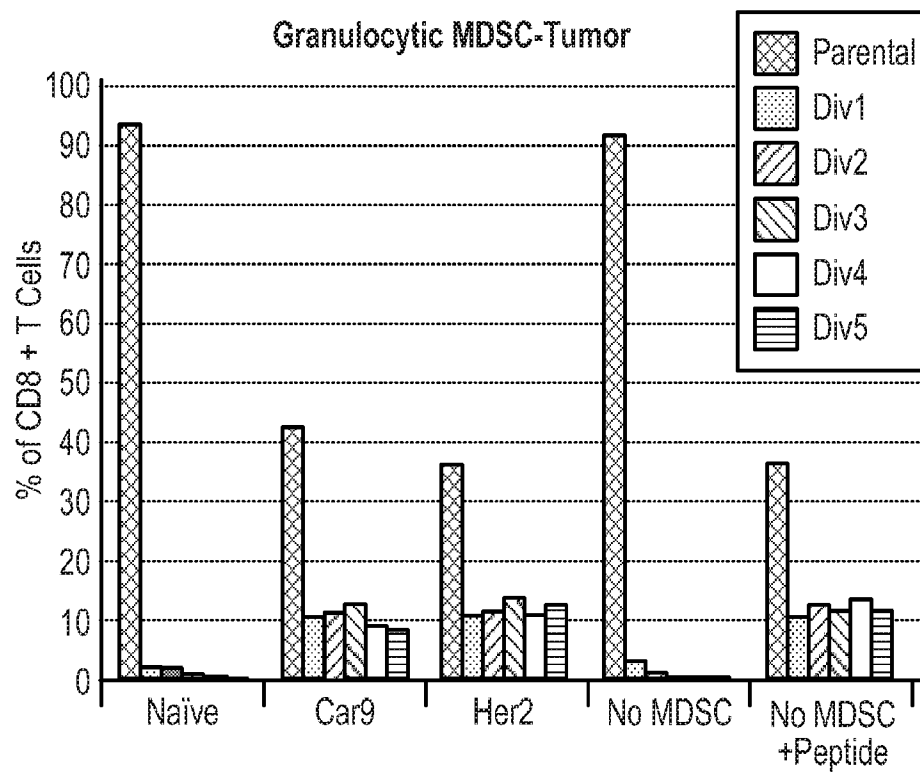


FIG. 14C

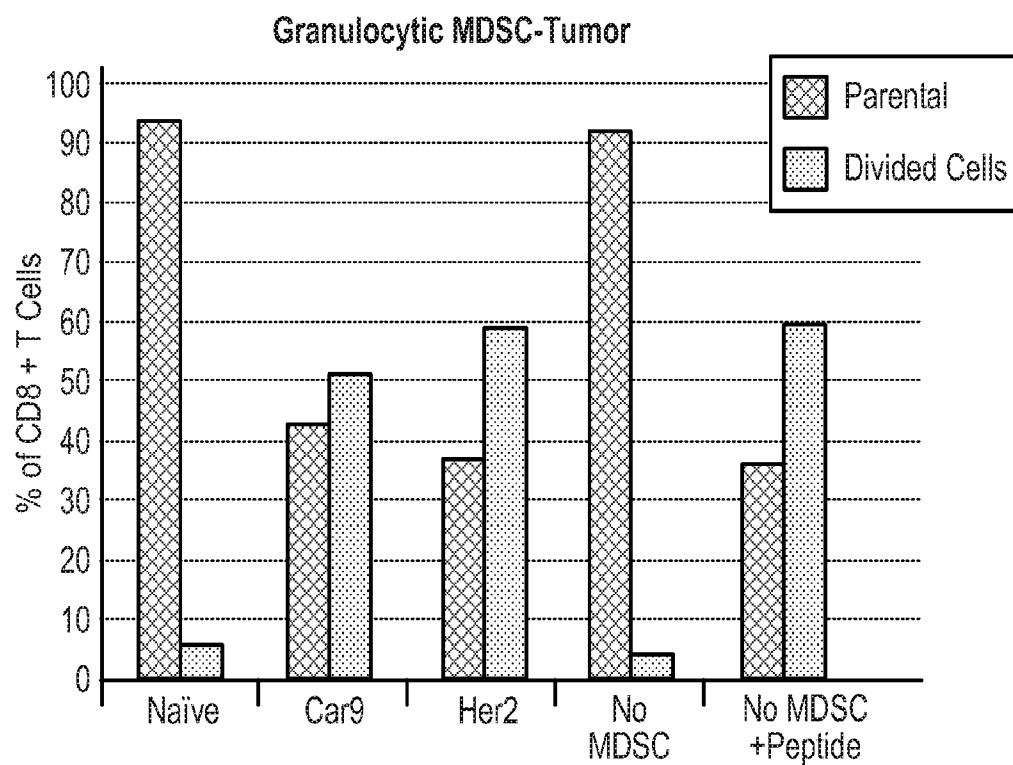


FIG. 14D

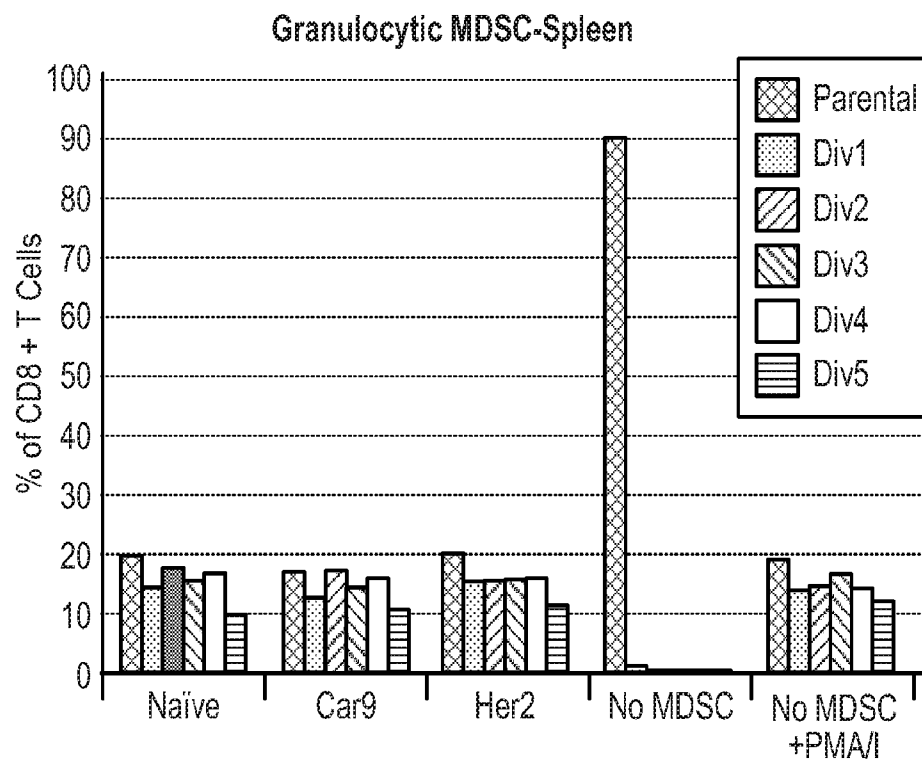


FIG. 15A

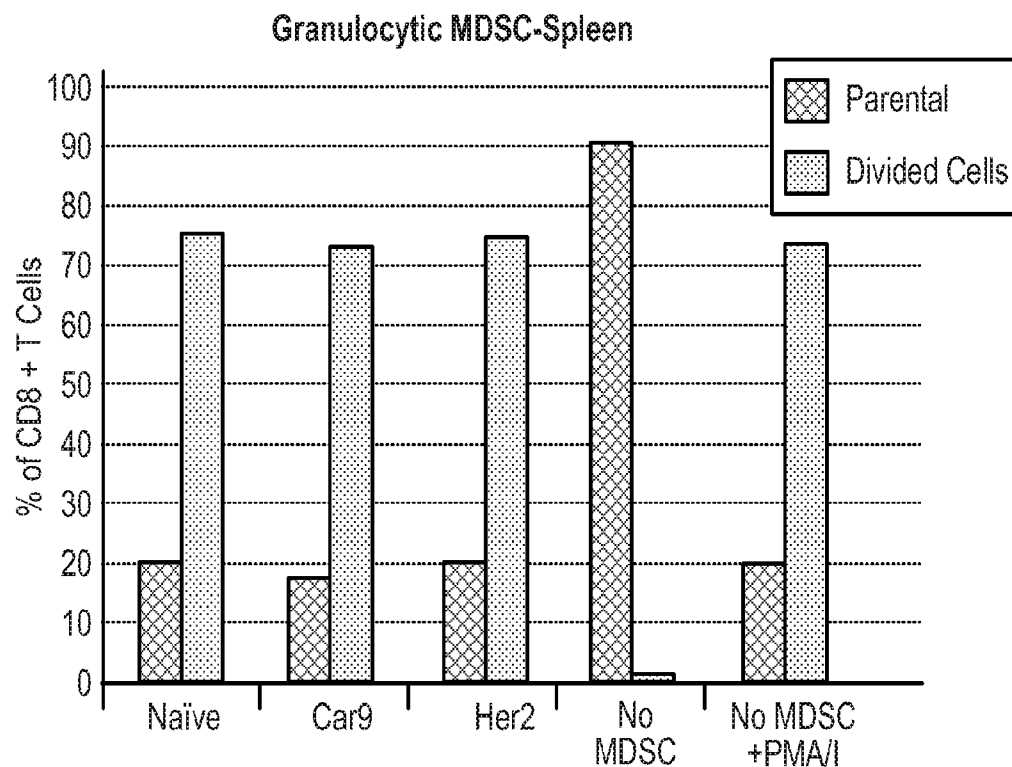


FIG. 15B

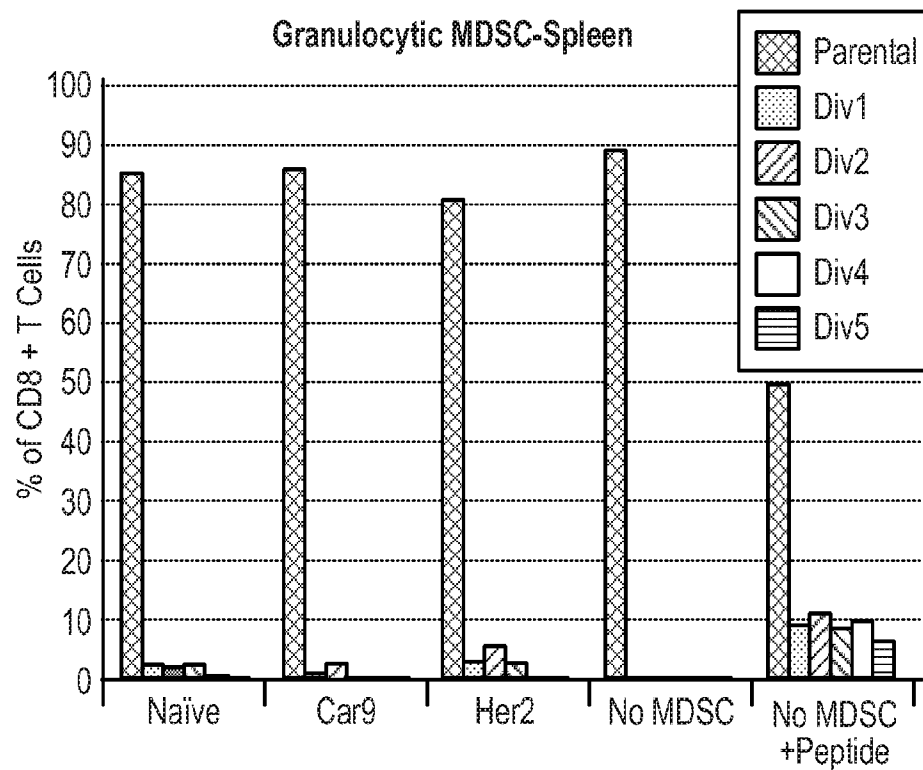


FIG. 15C

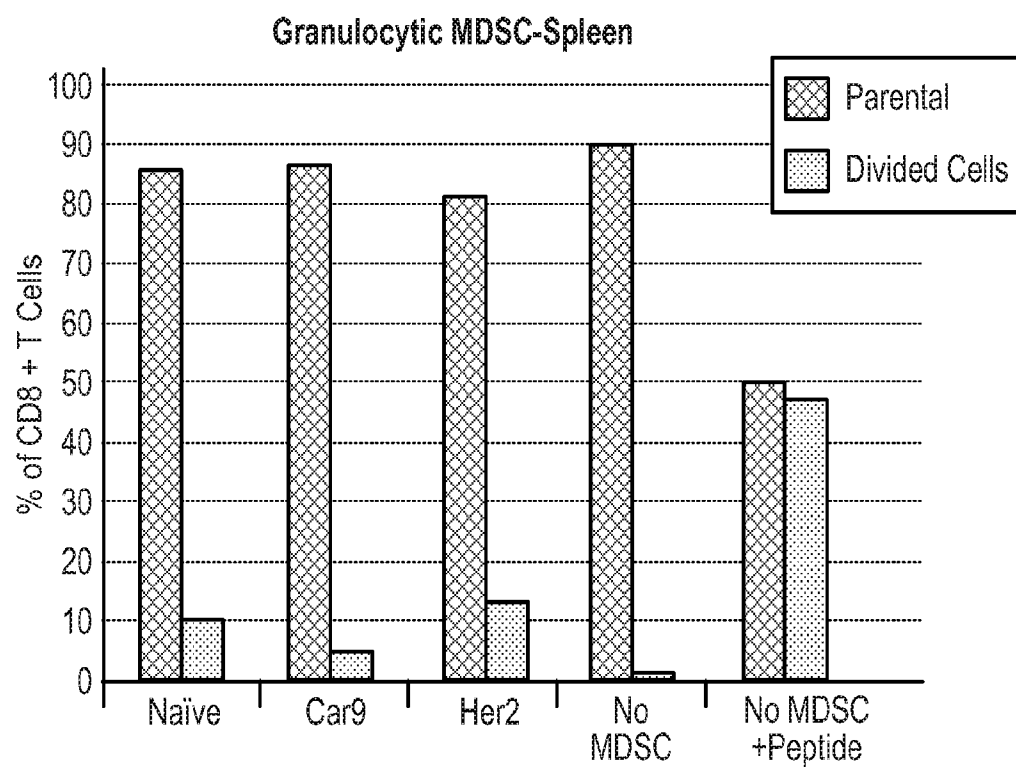


FIG. 15D

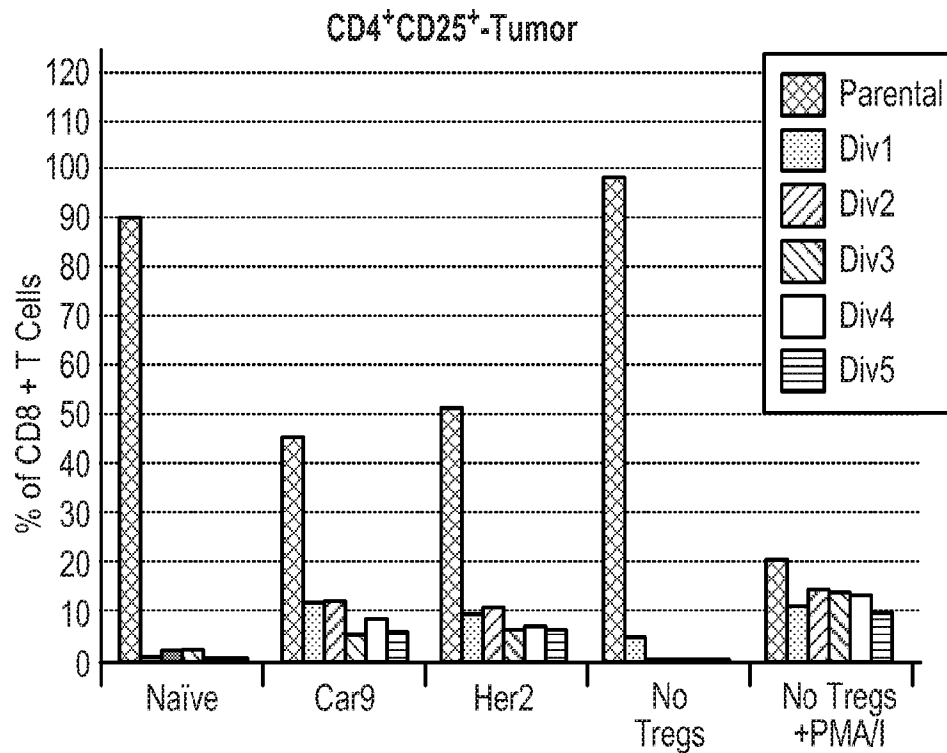


FIG. 16A

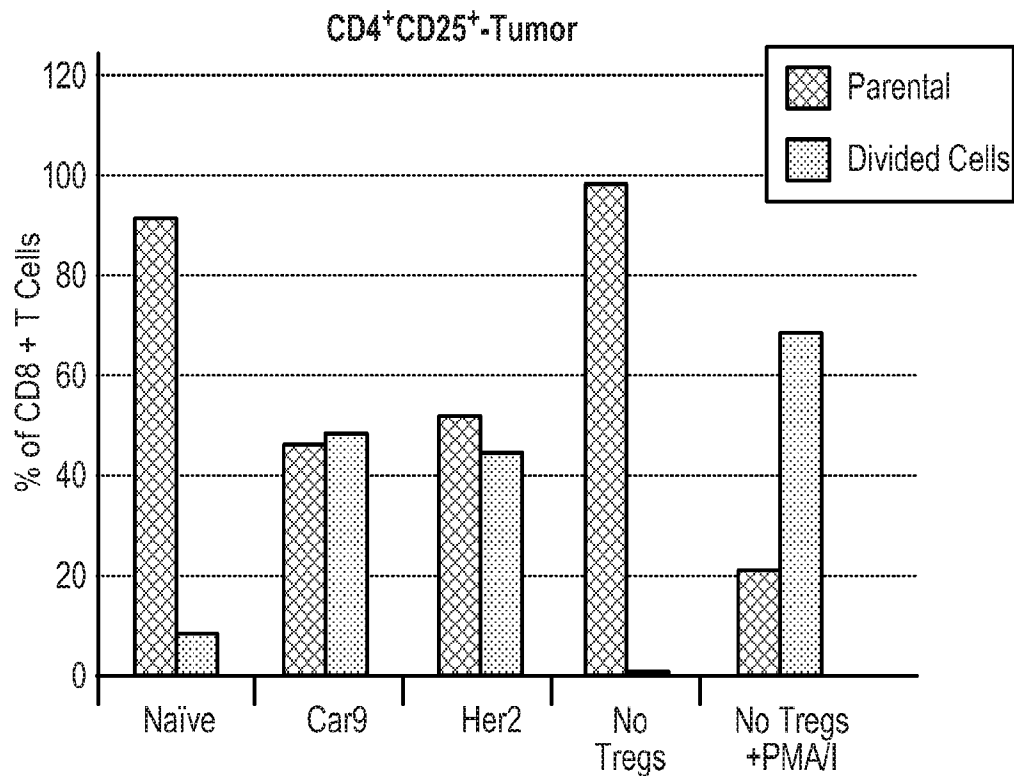


FIG. 16B

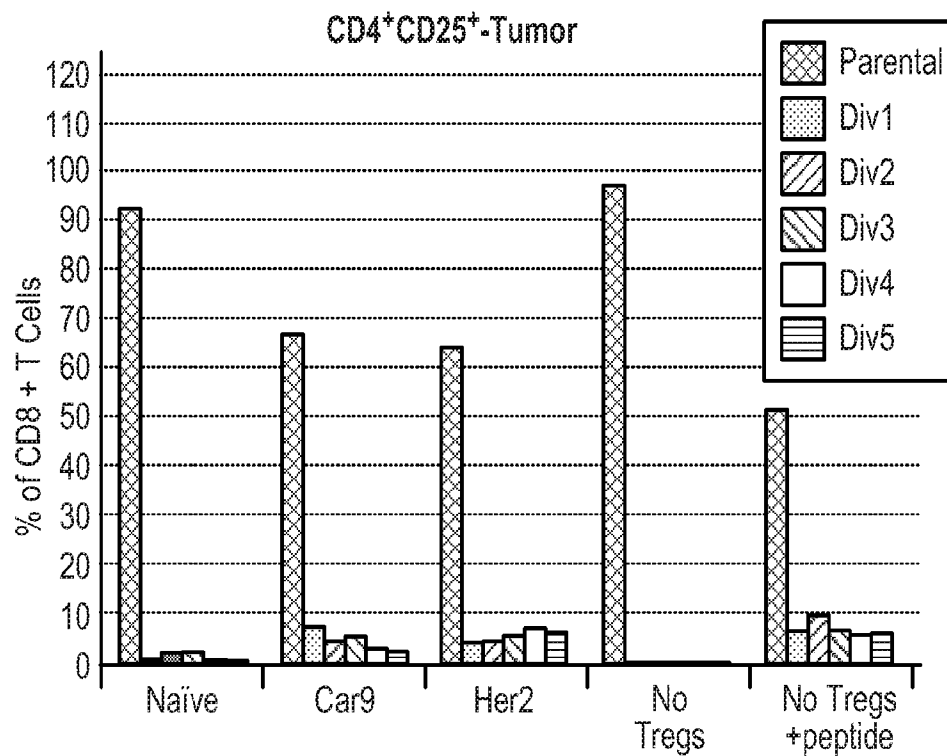


FIG. 16C

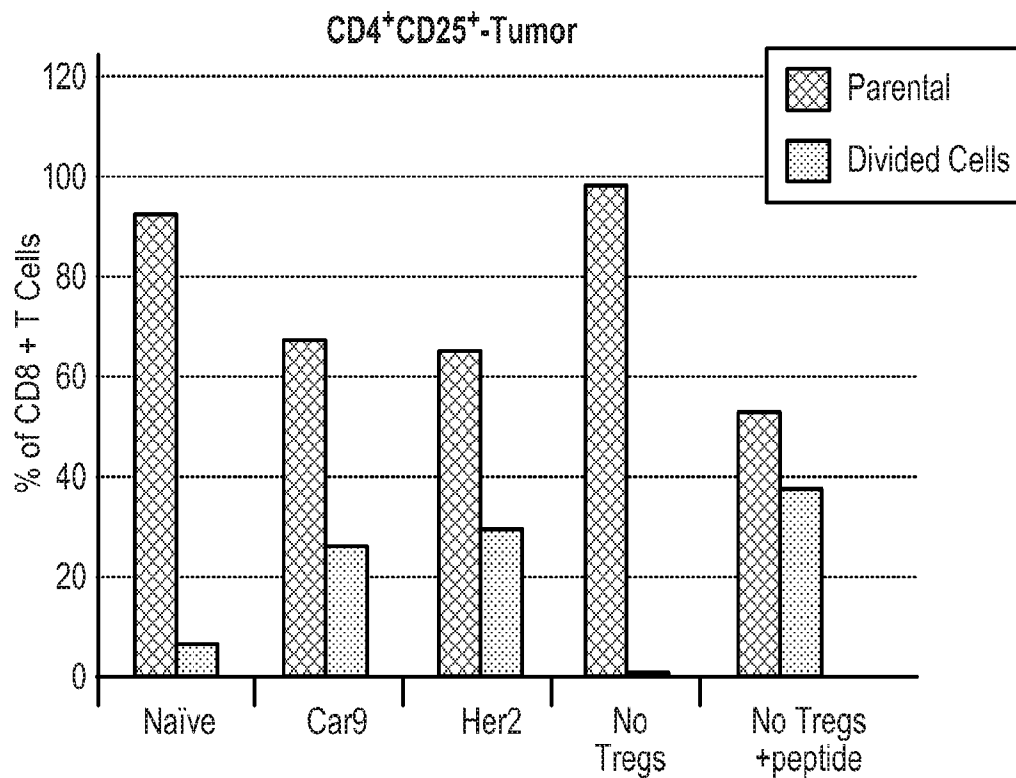


FIG. 16D

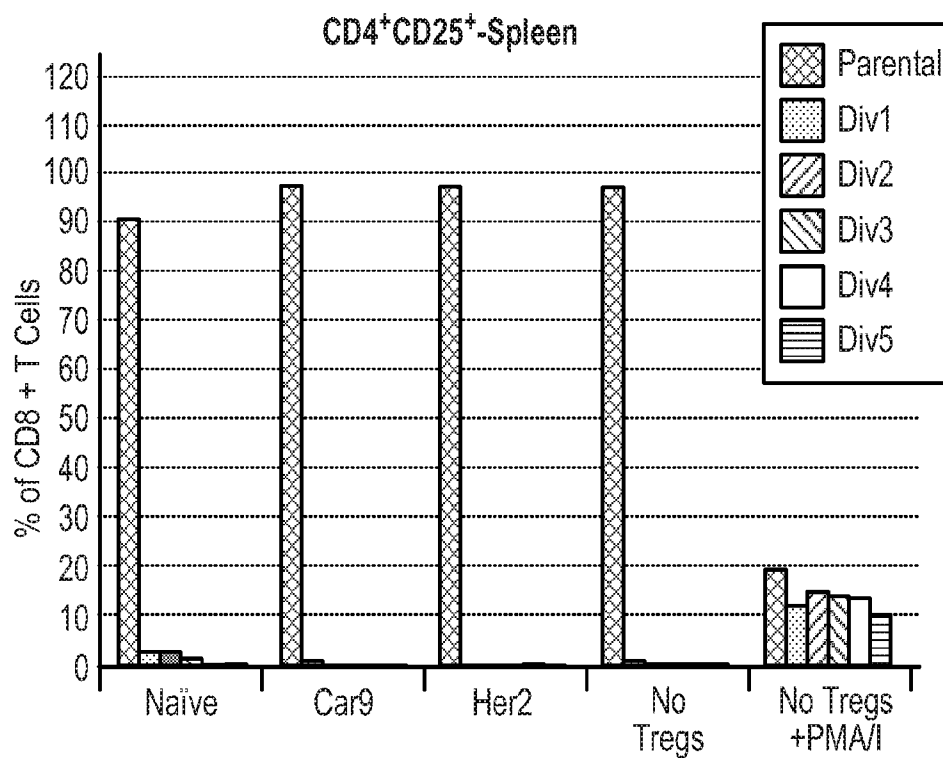


FIG. 17A

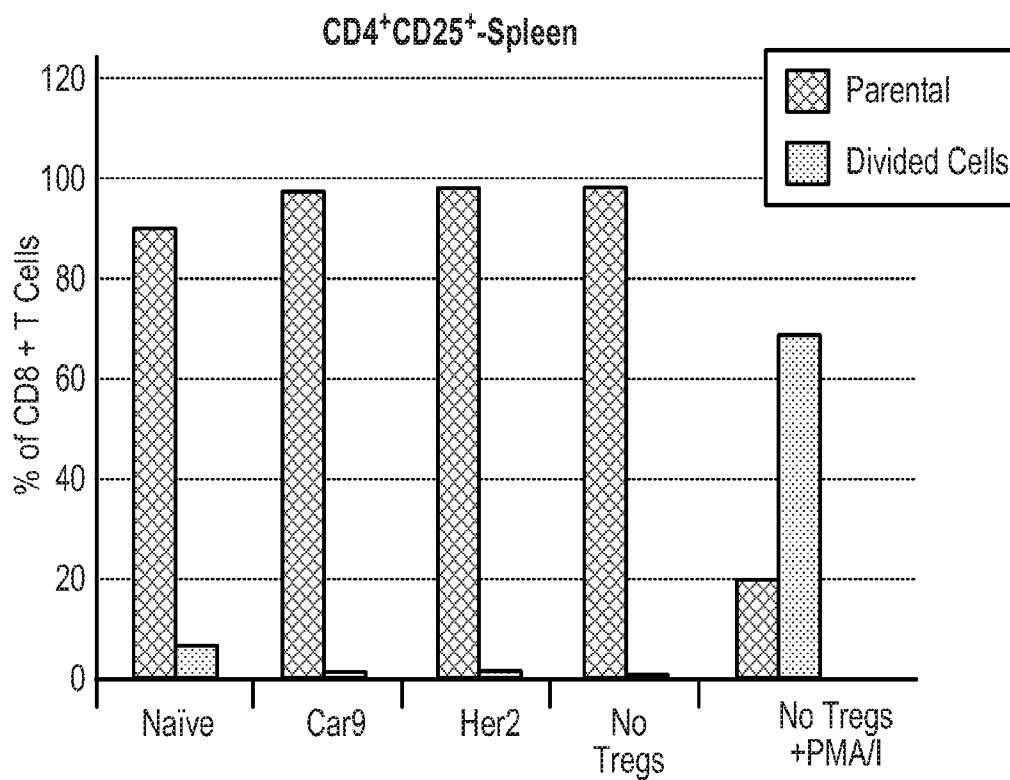


FIG. 17B

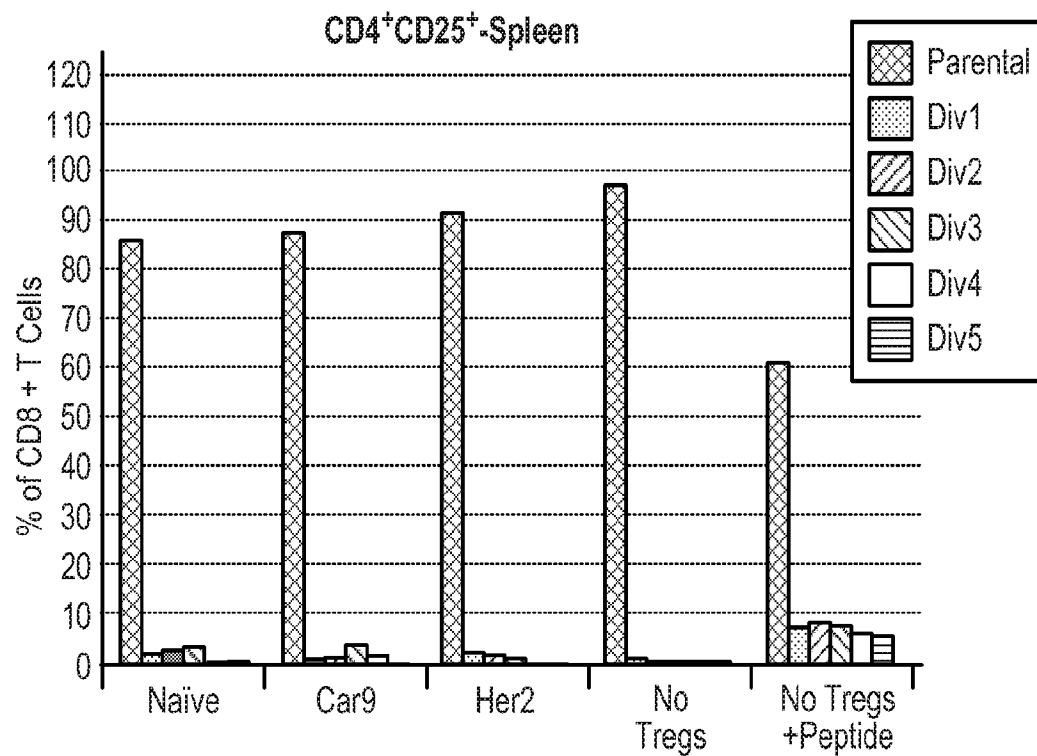


FIG. 17C

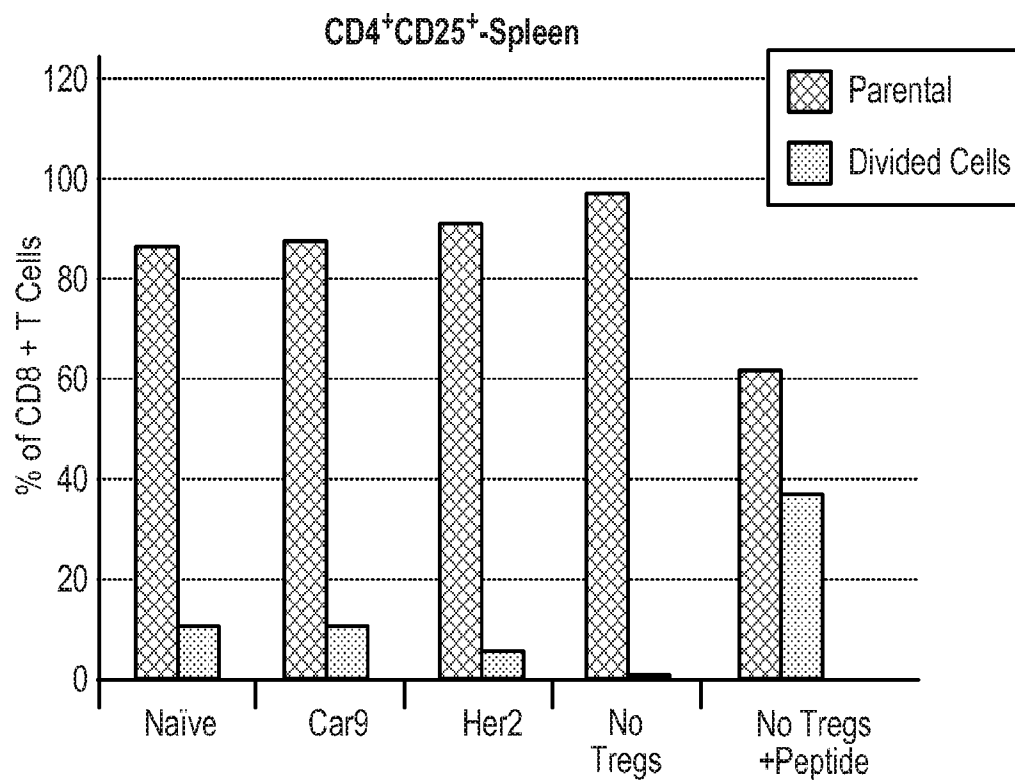
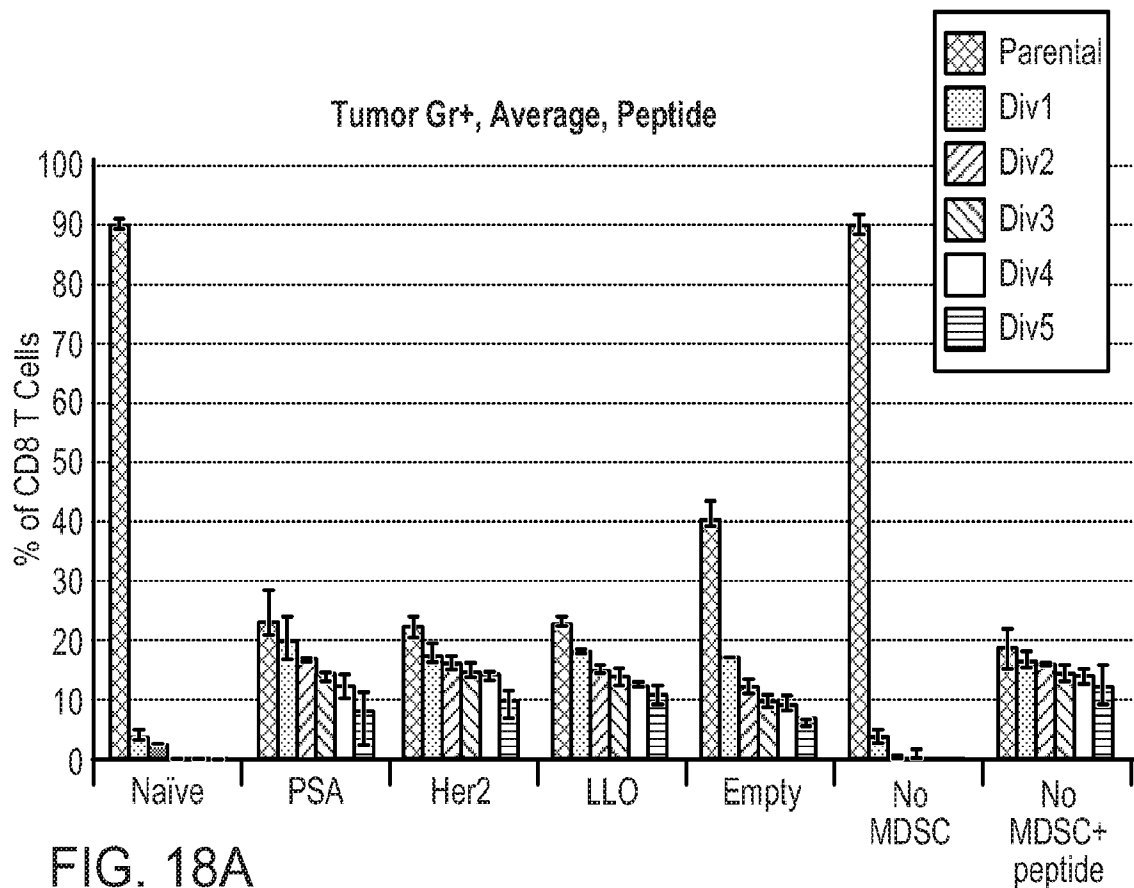
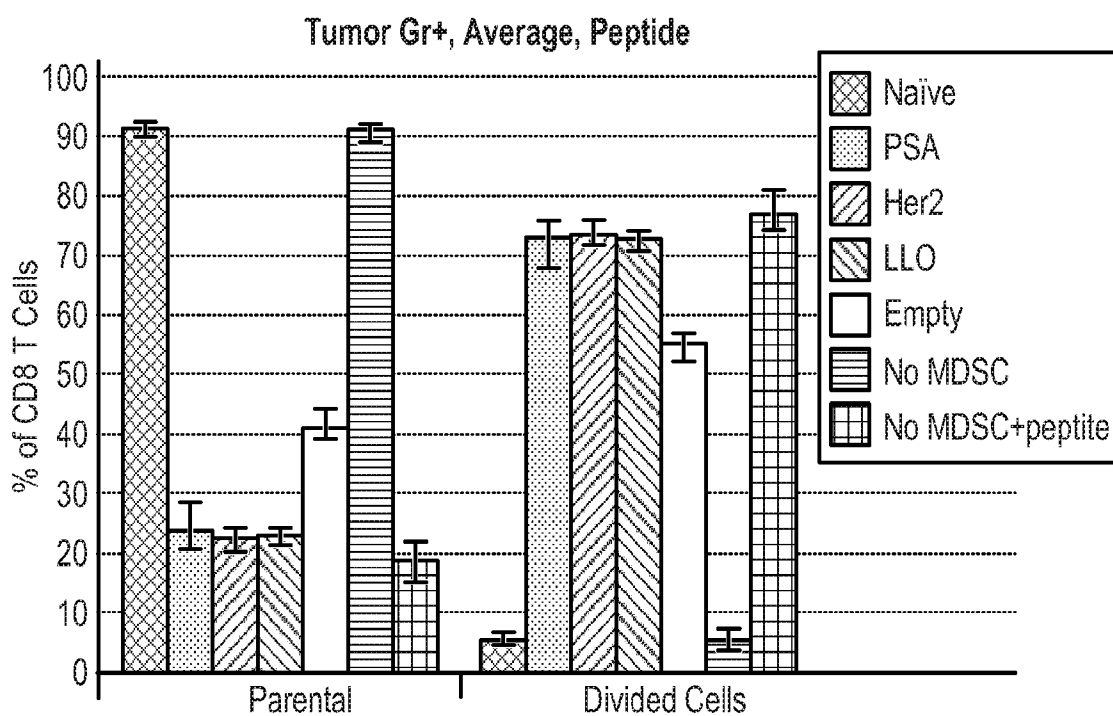


FIG. 17D



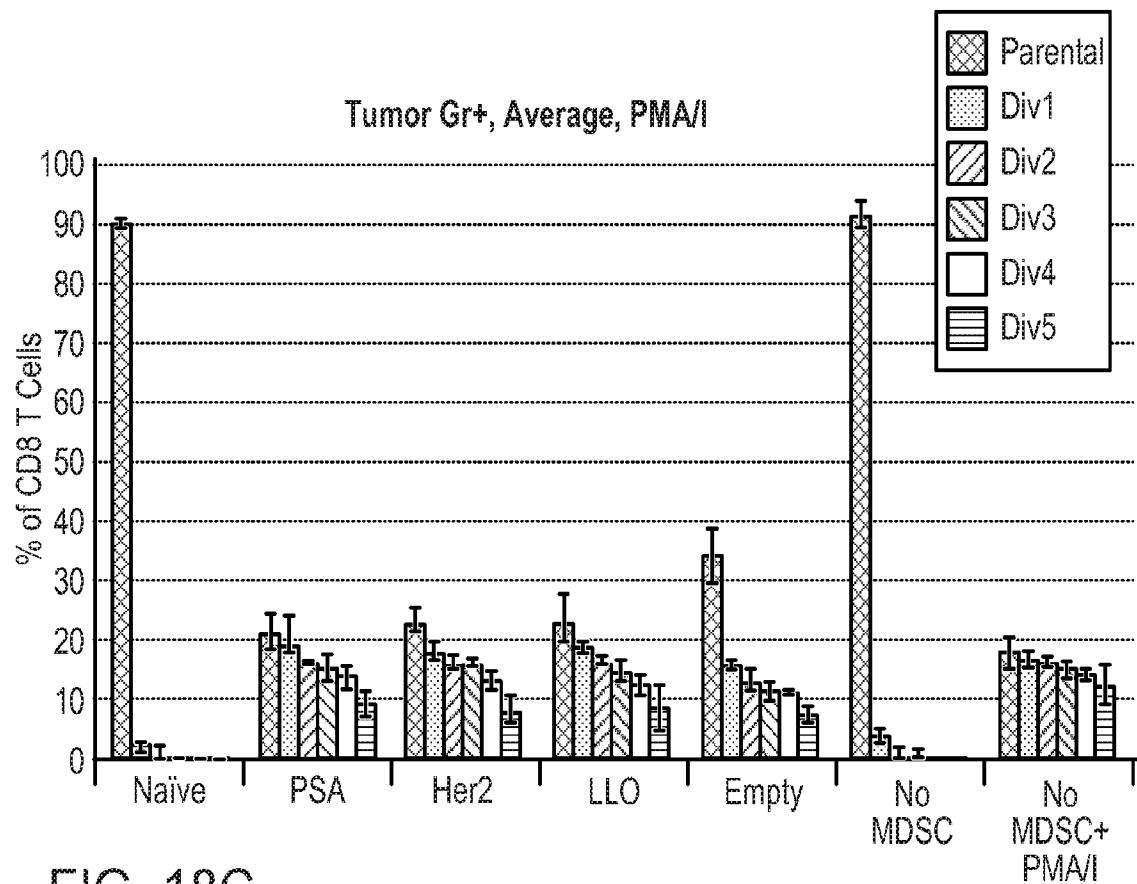


FIG. 18C

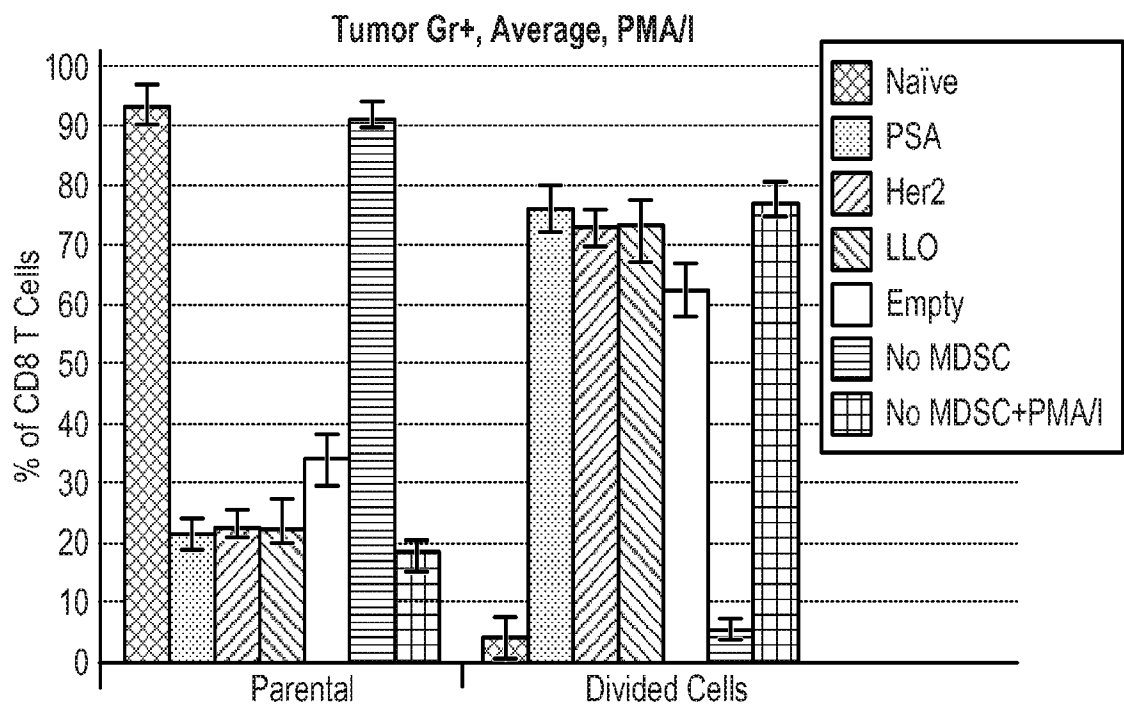


FIG. 18D

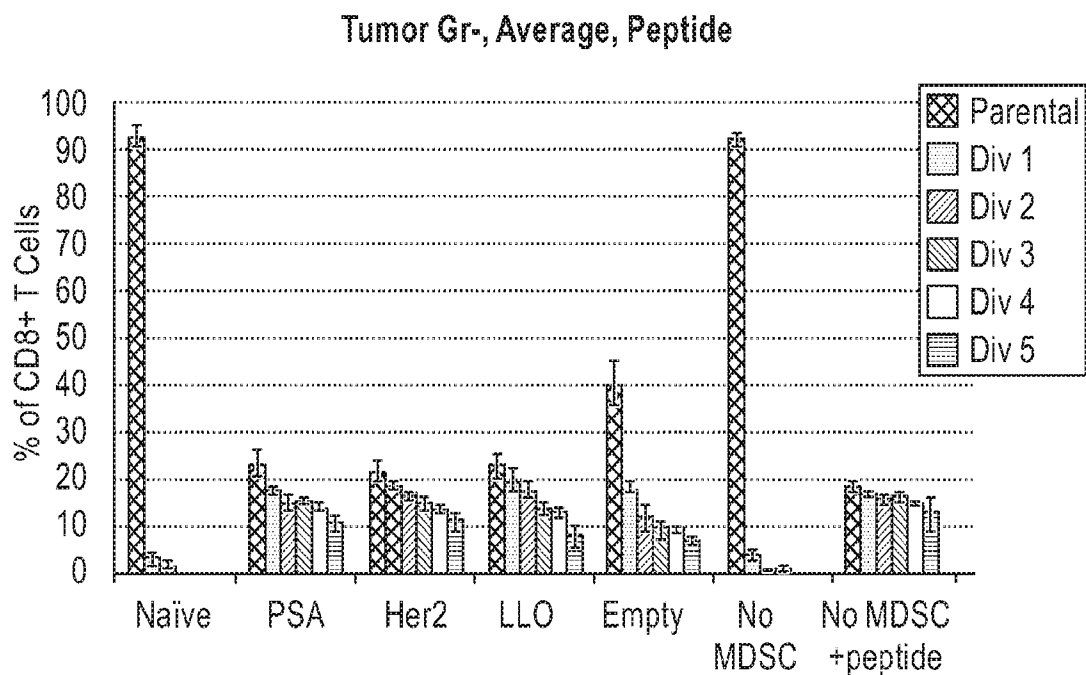


FIG. 19A

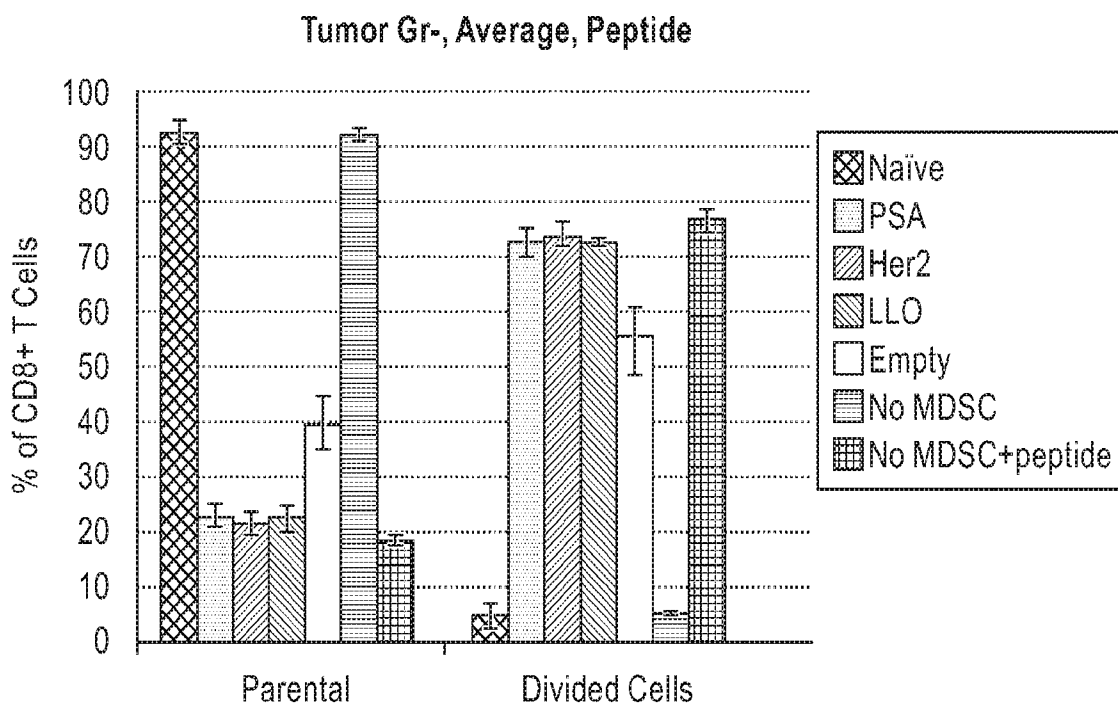


FIG. 19B

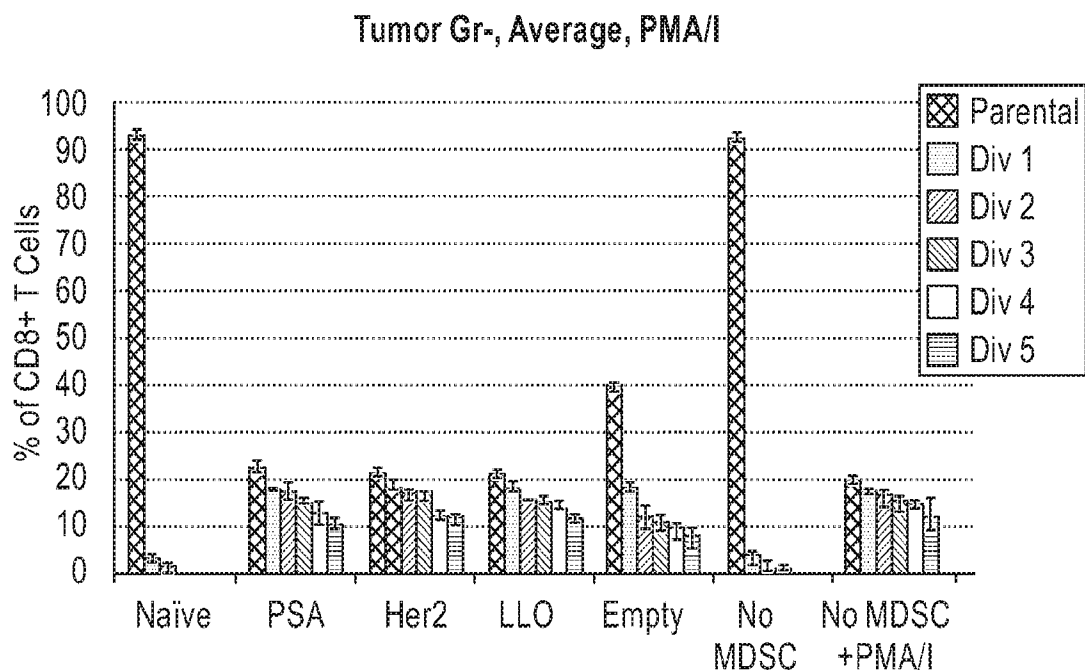


FIG. 19C

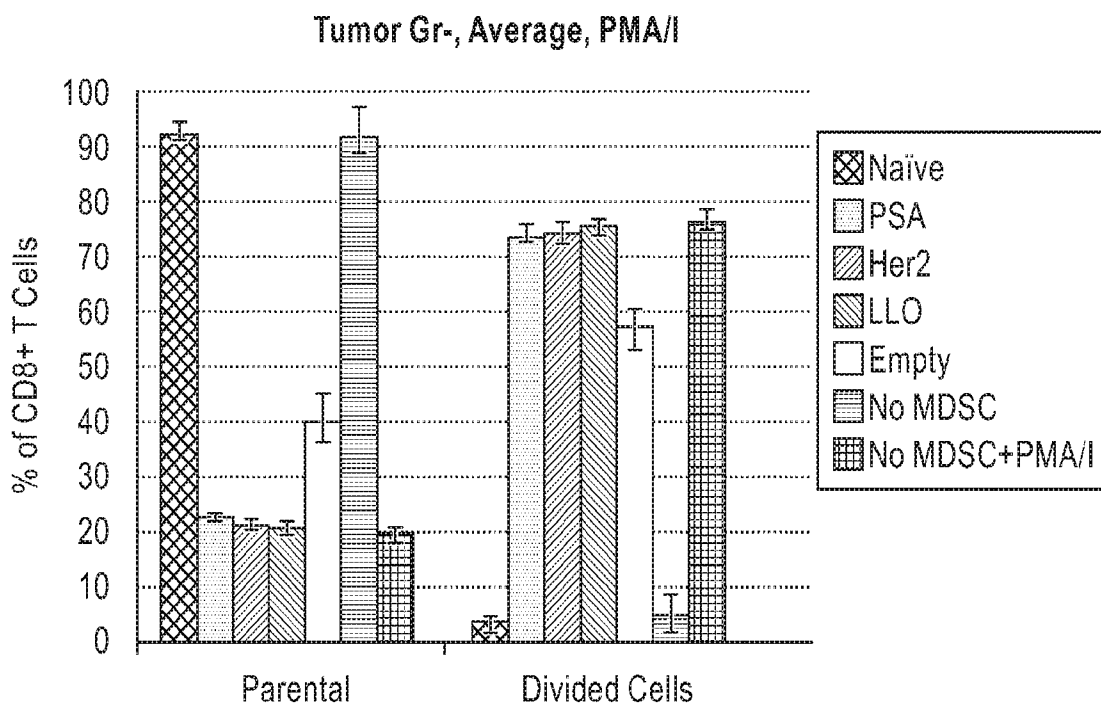


FIG. 19D

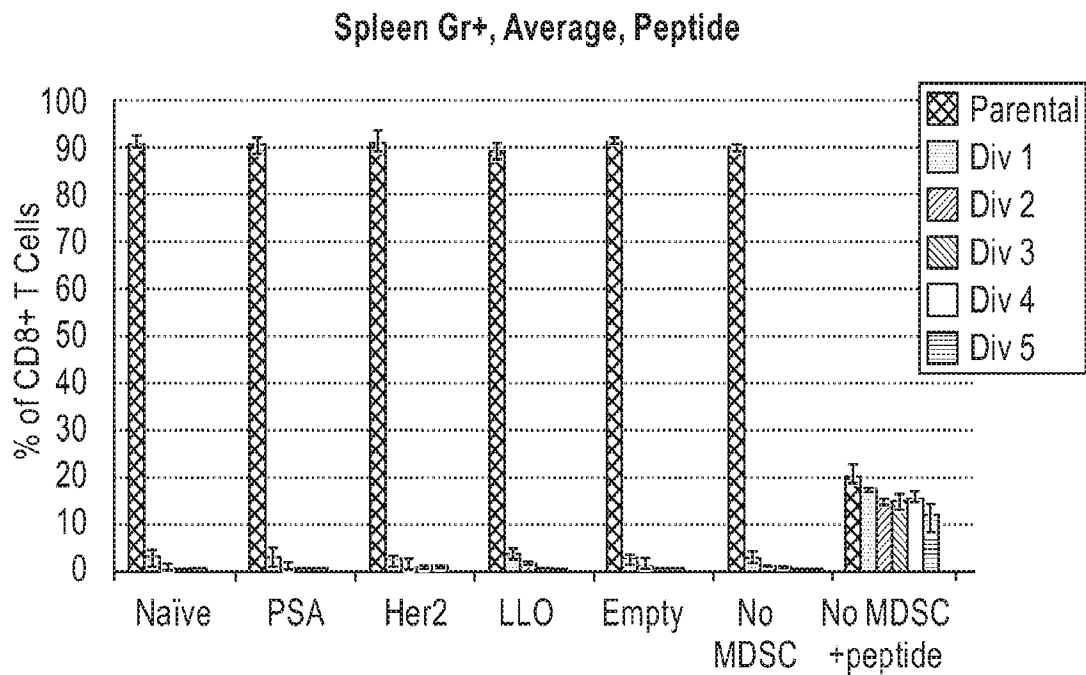


FIG. 20A

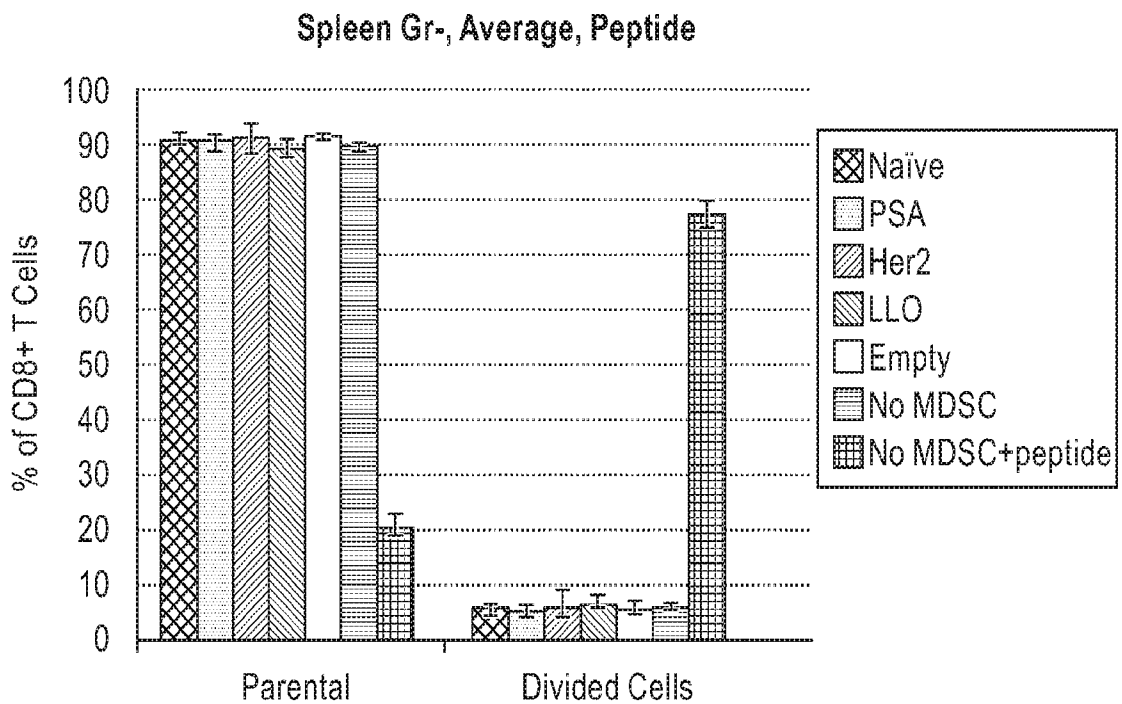


FIG. 20B

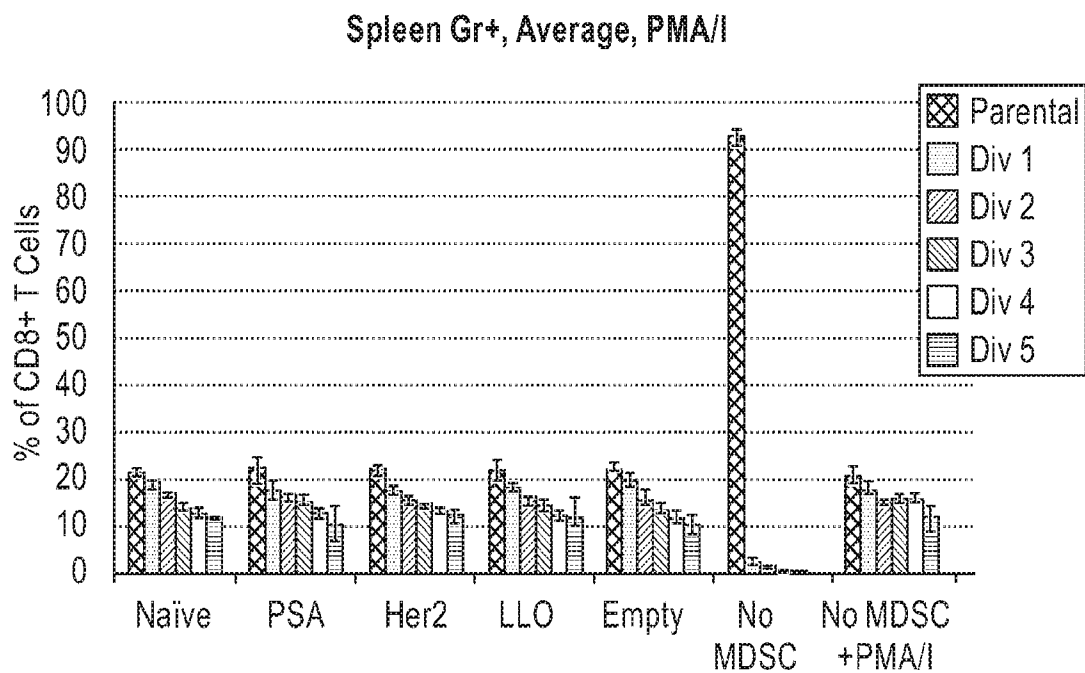


FIG. 20C

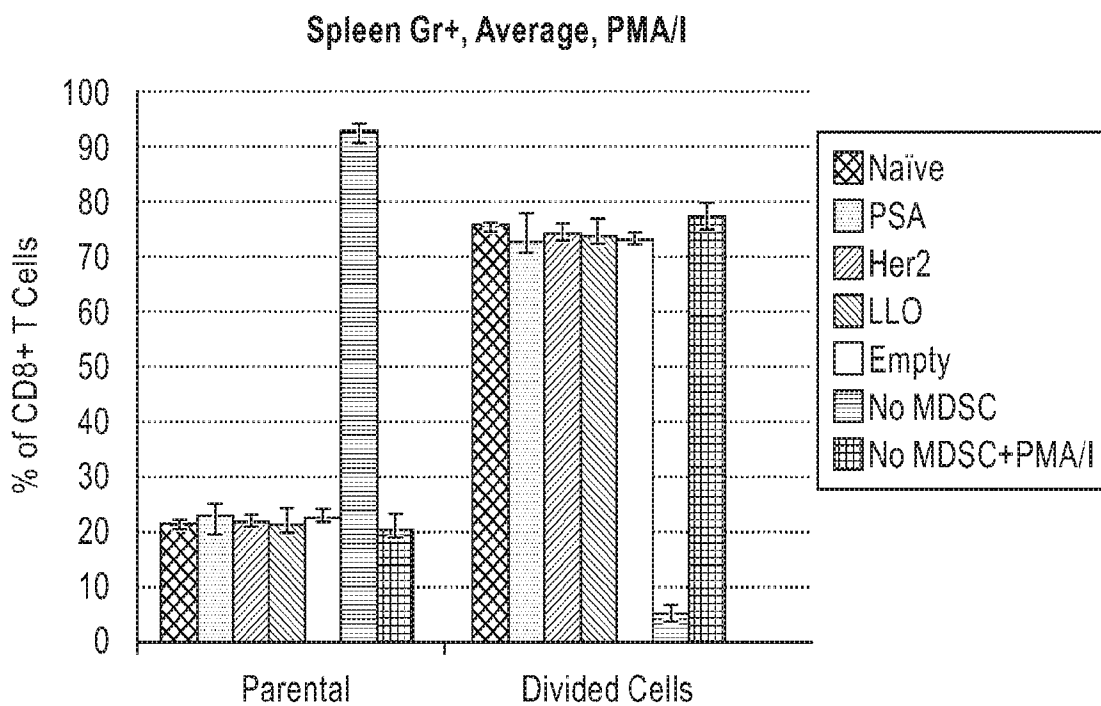


FIG. 20D

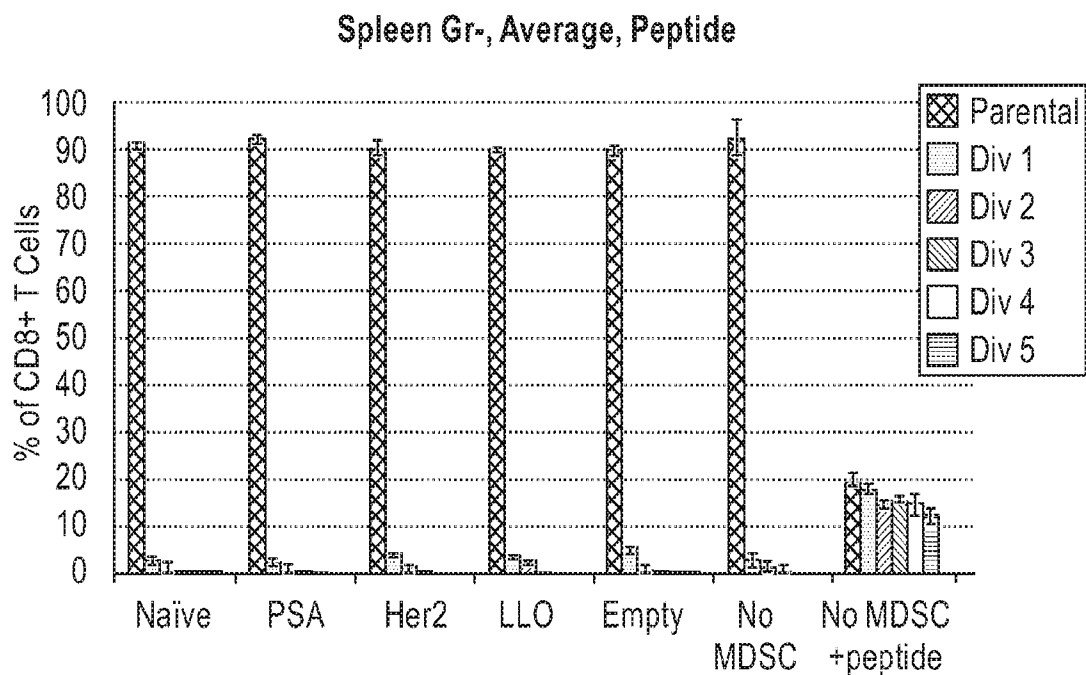


FIG. 21A

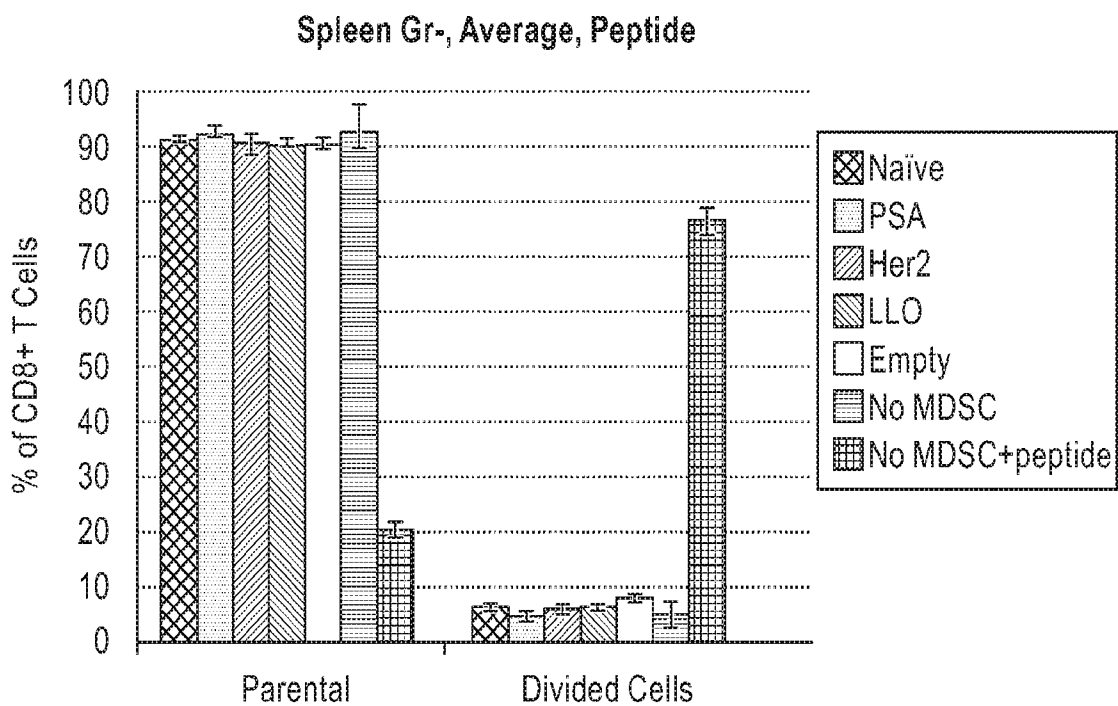


FIG. 21B

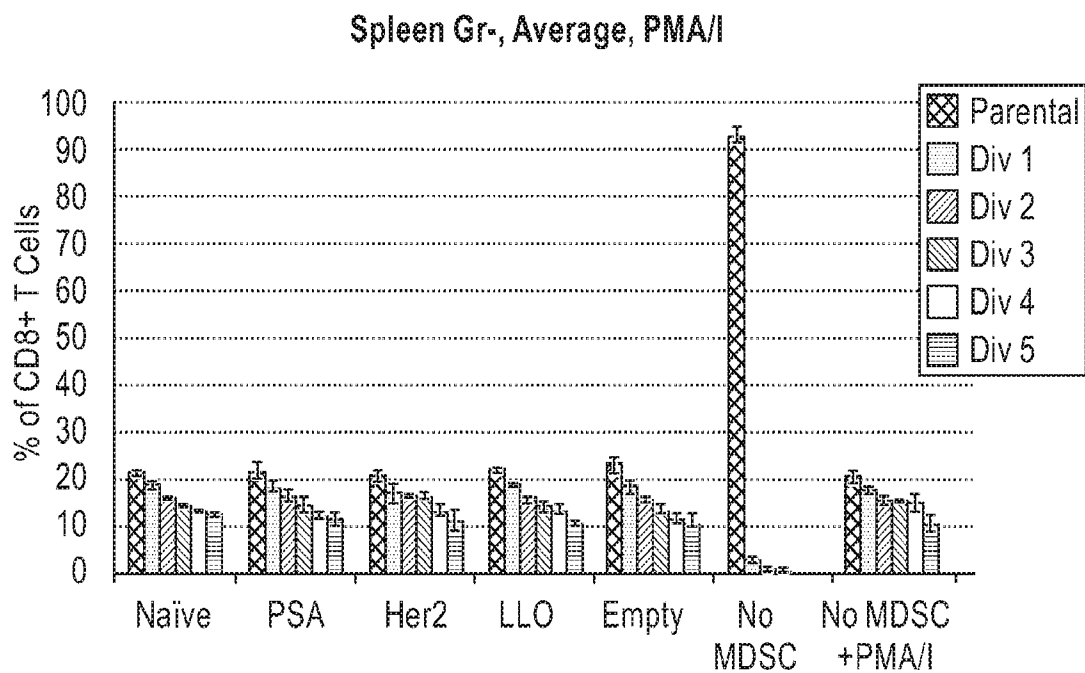


FIG. 21C

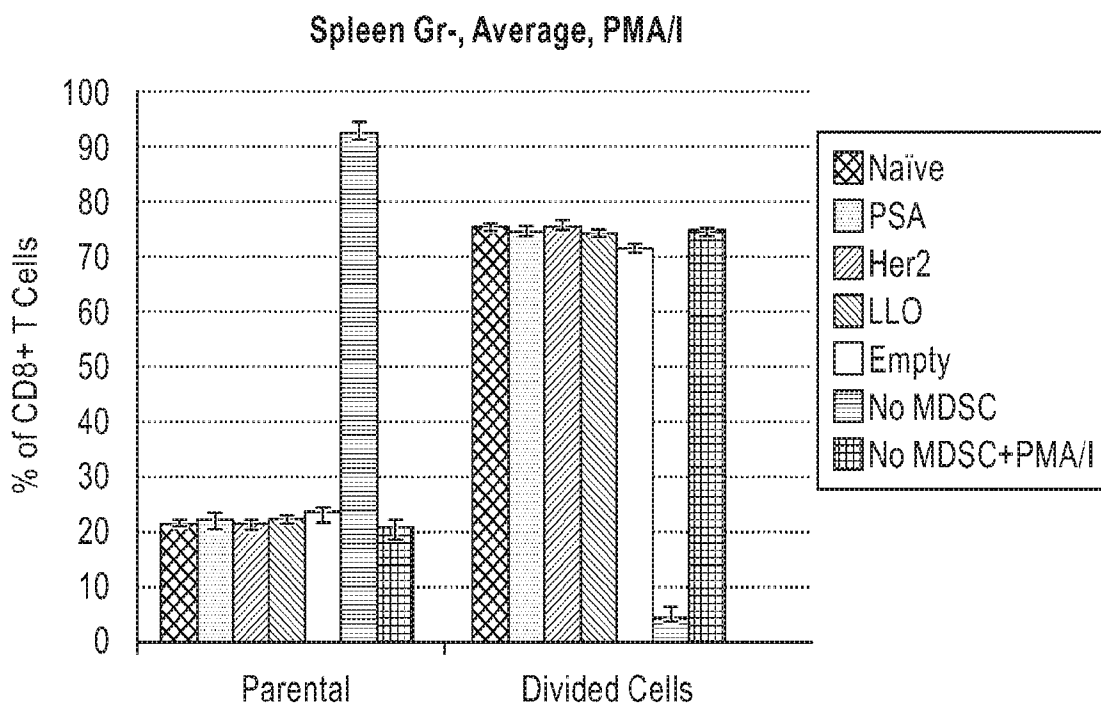


FIG. 21D

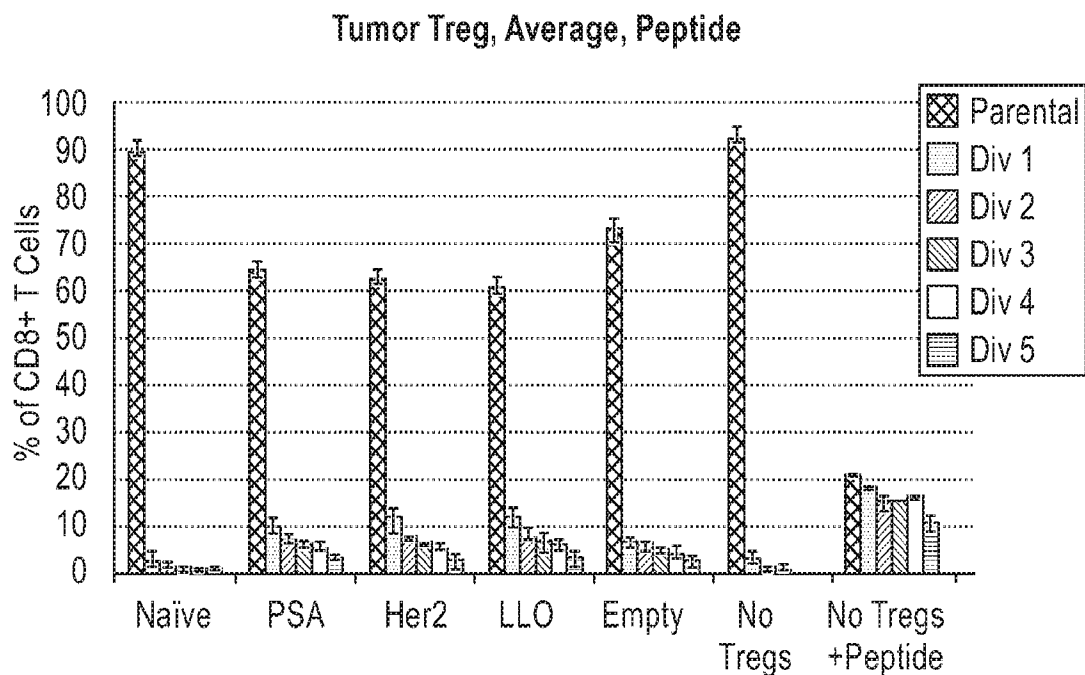


FIG. 22A

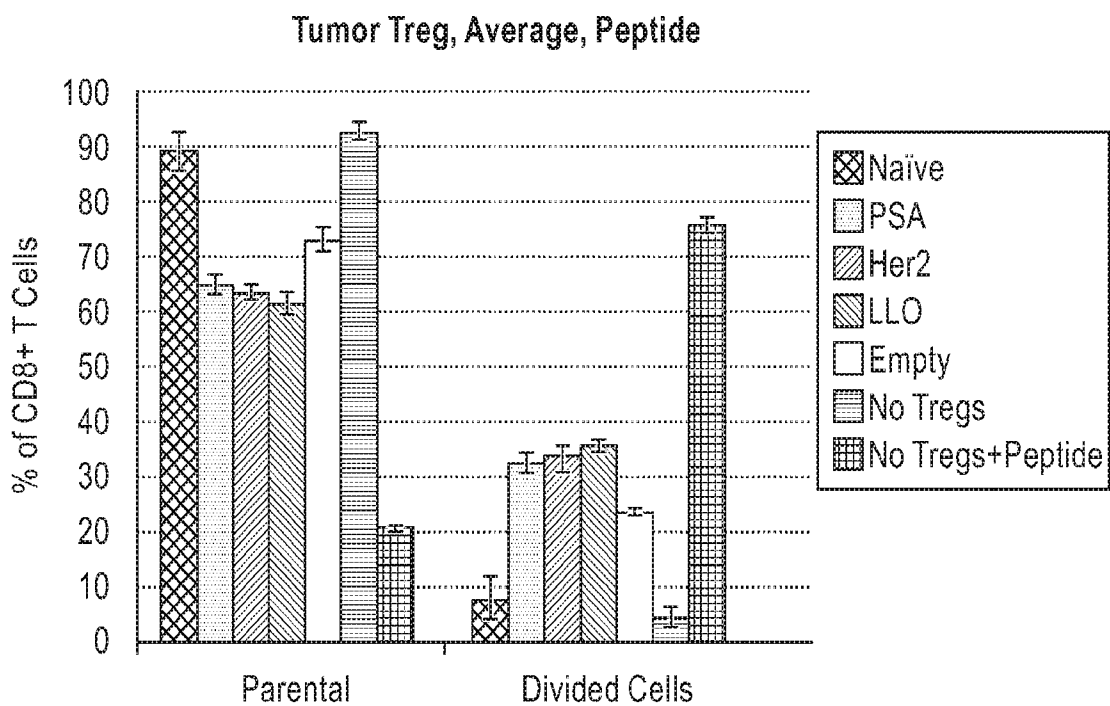


FIG. 22B

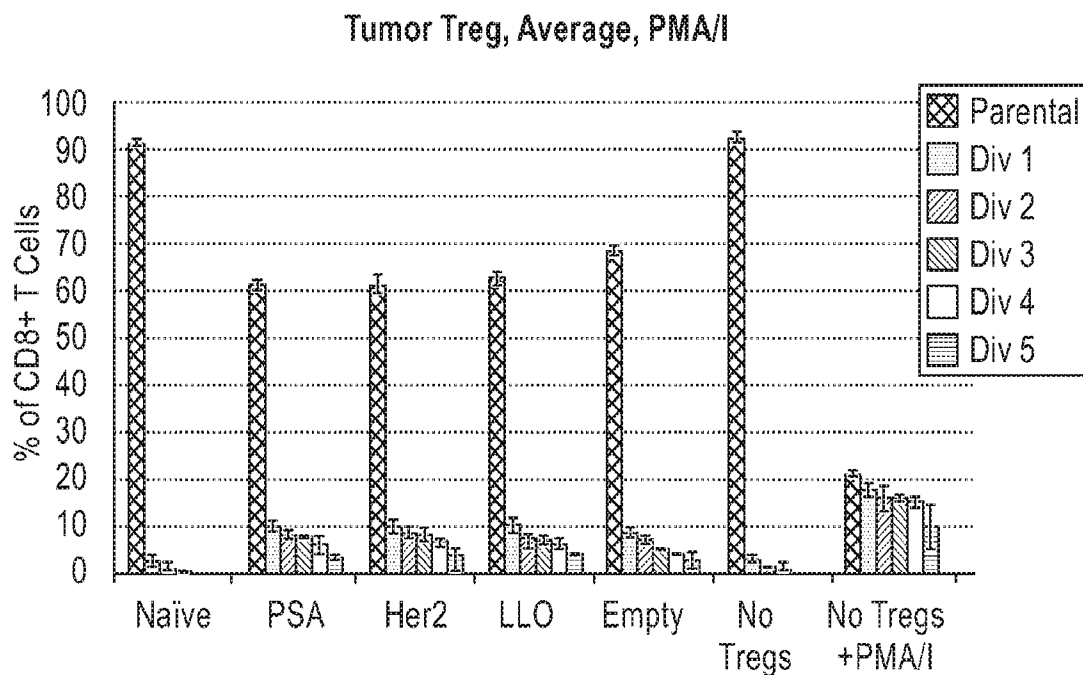


FIG. 22C

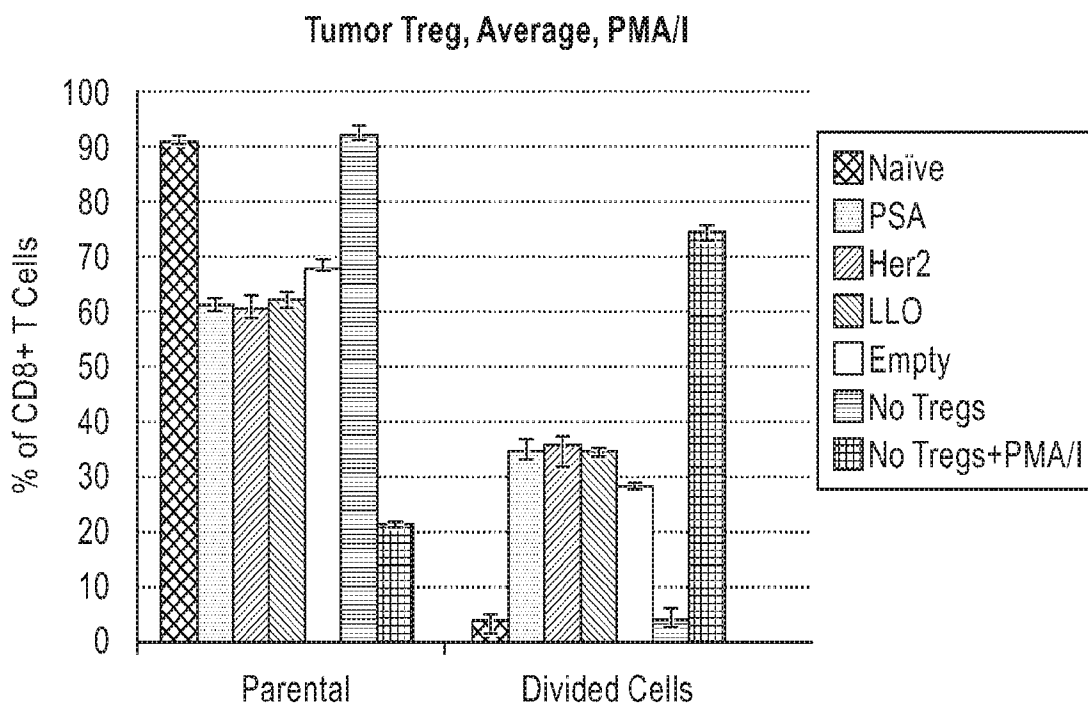


FIG. 22D

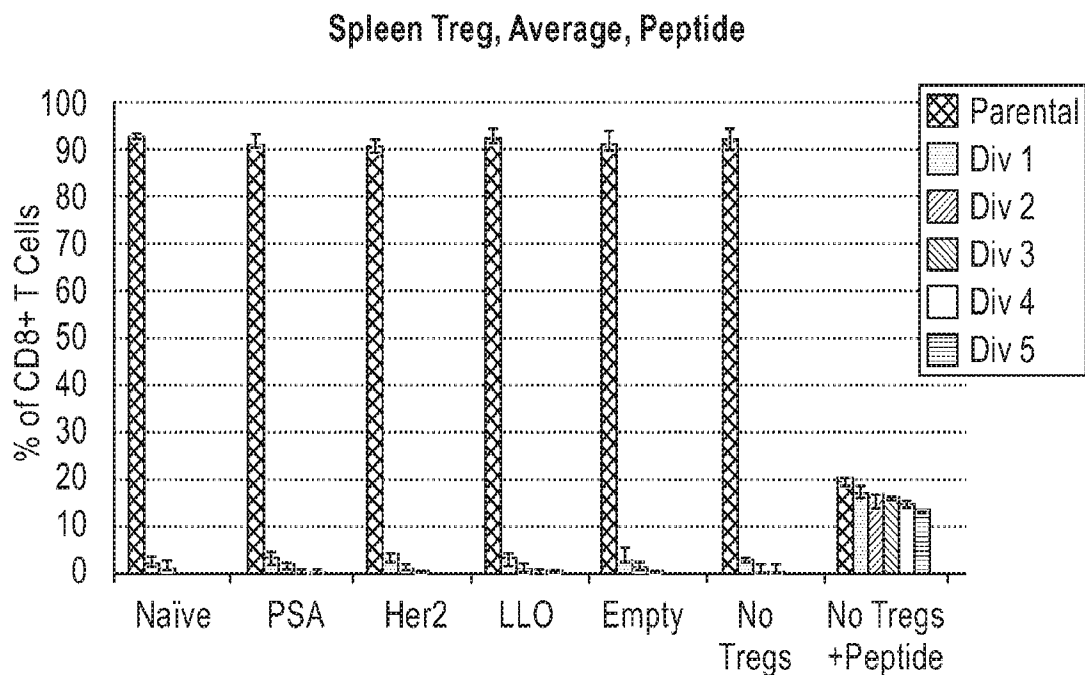


FIG. 23A

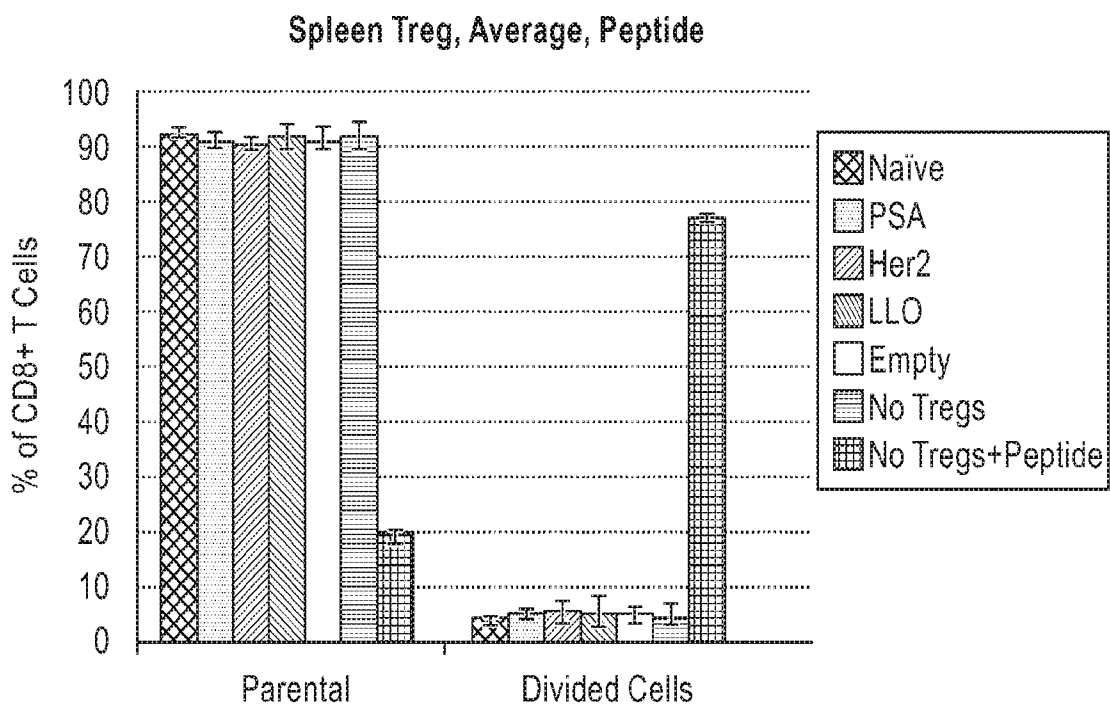


FIG. 23B

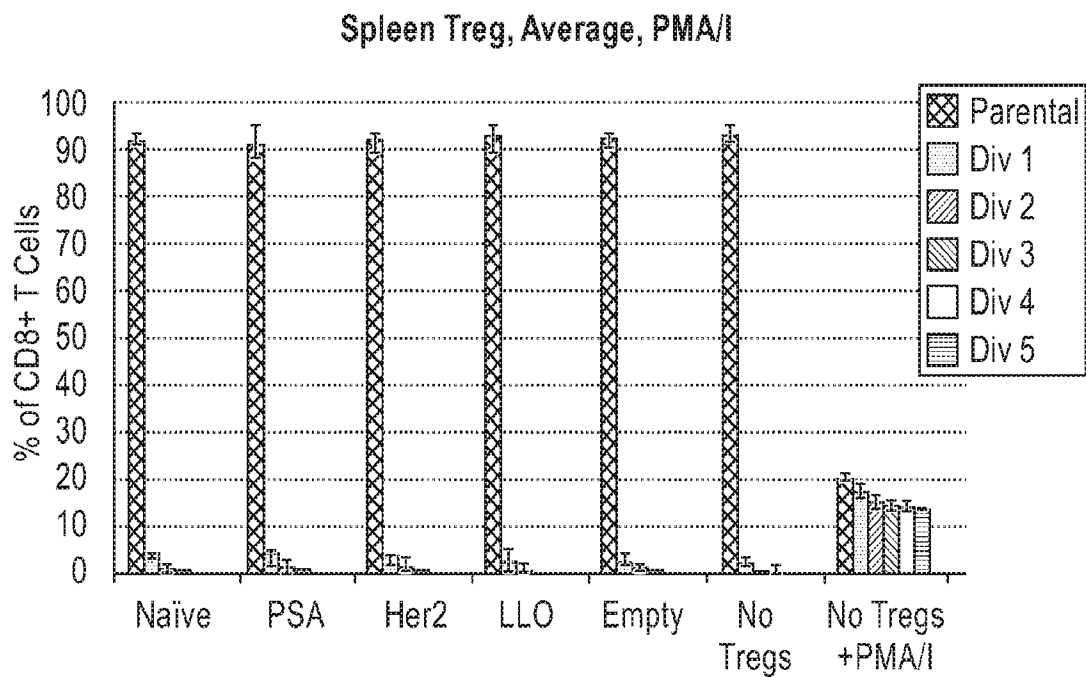


FIG. 23C

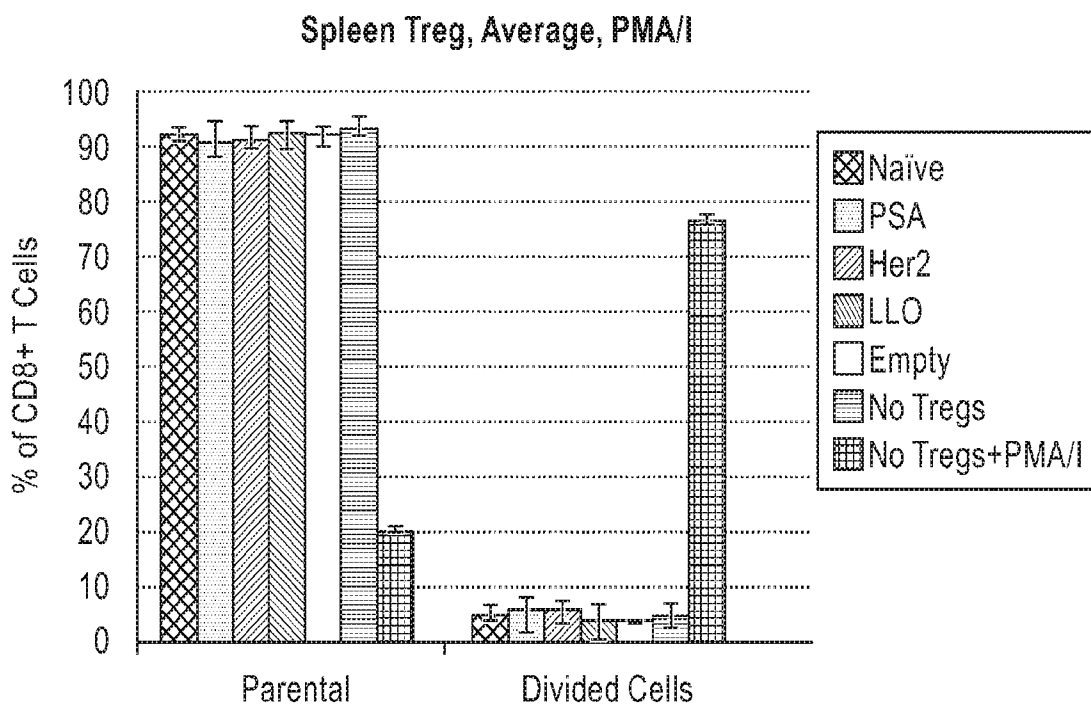


FIG. 23D

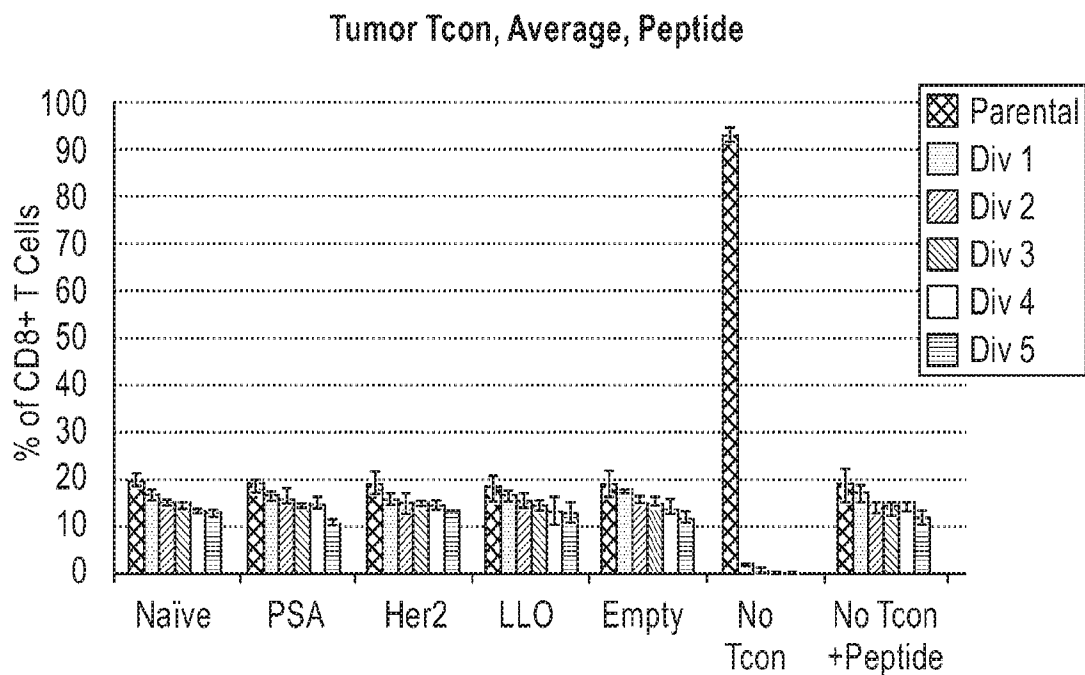


FIG. 24A

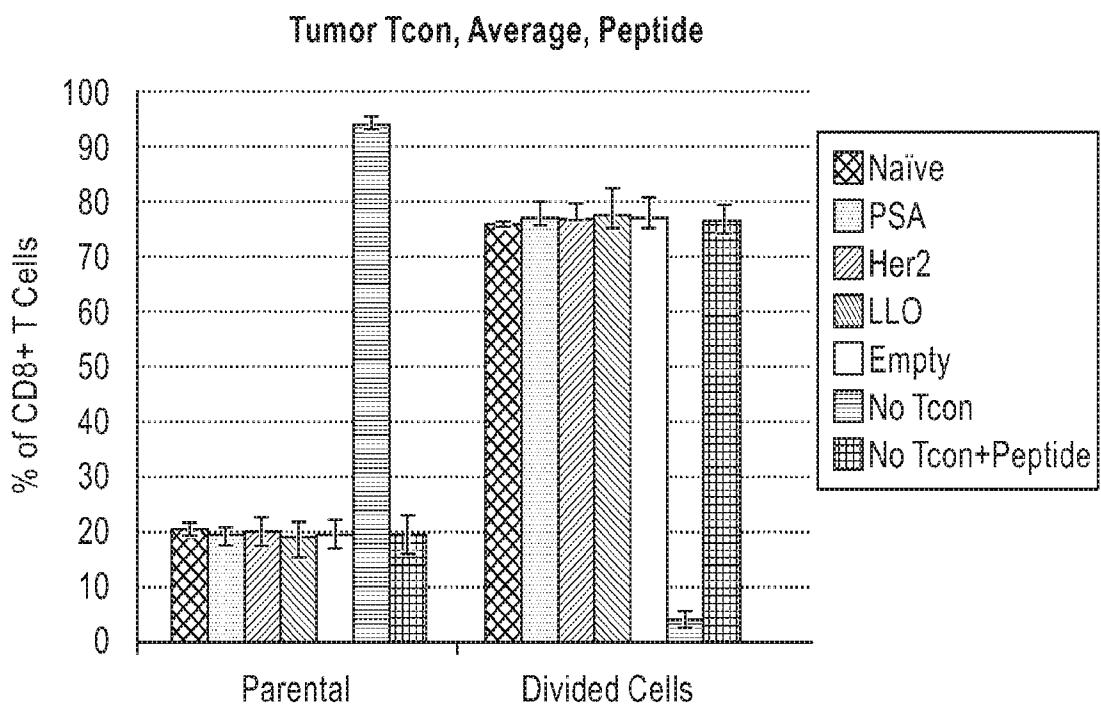


FIG. 24B

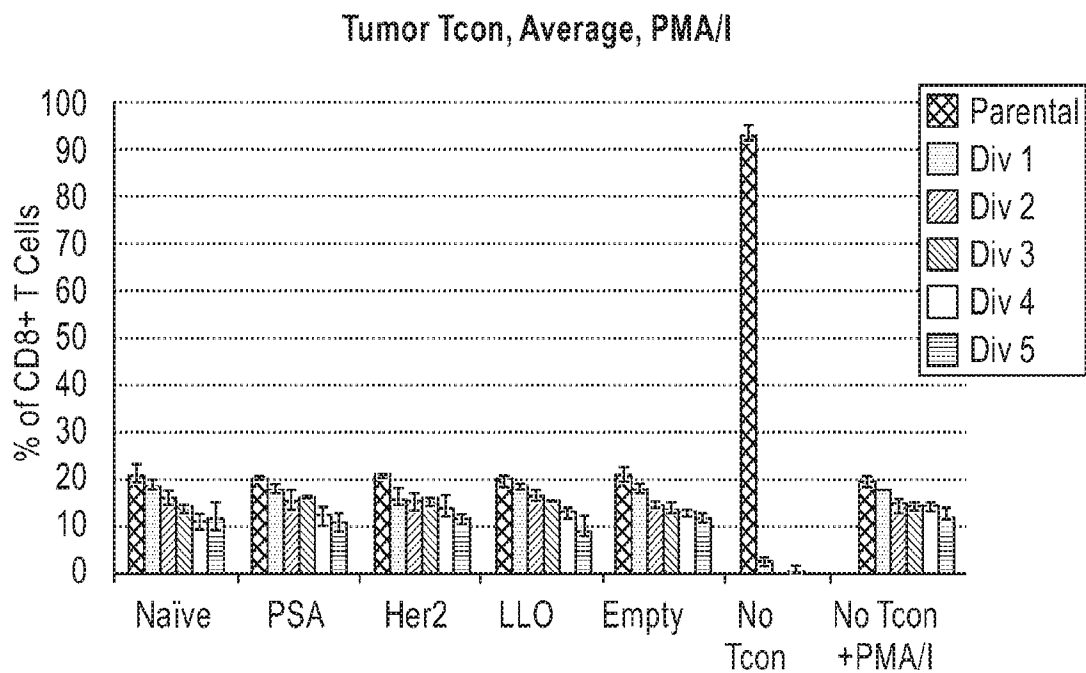


FIG. 24C

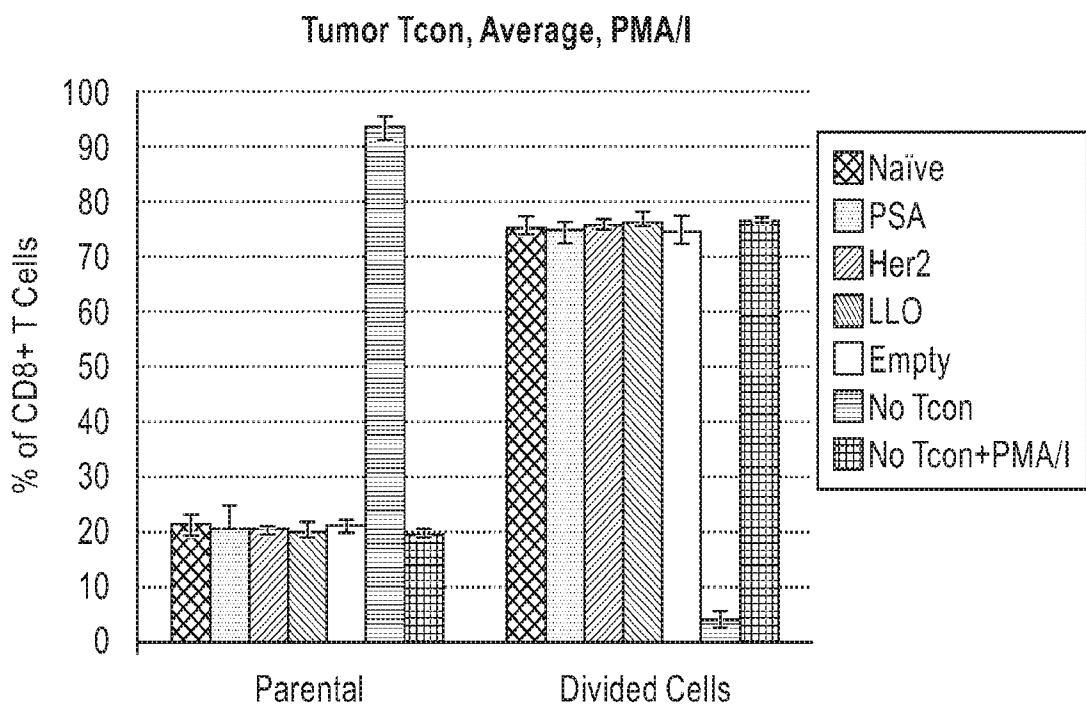


FIG. 24D

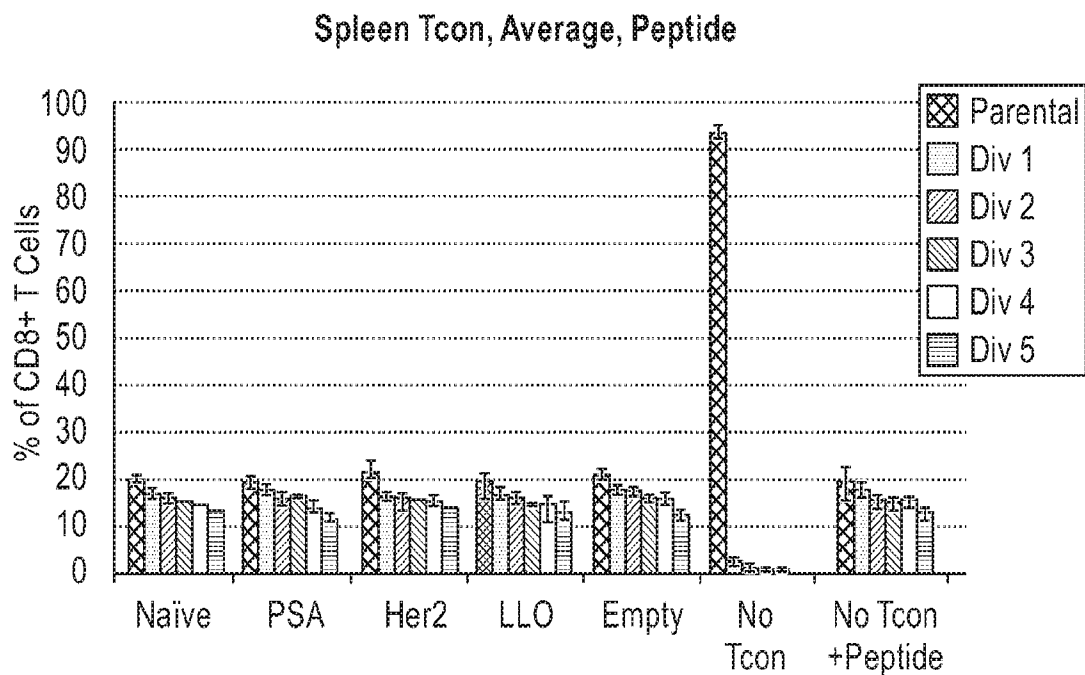


FIG. 25A

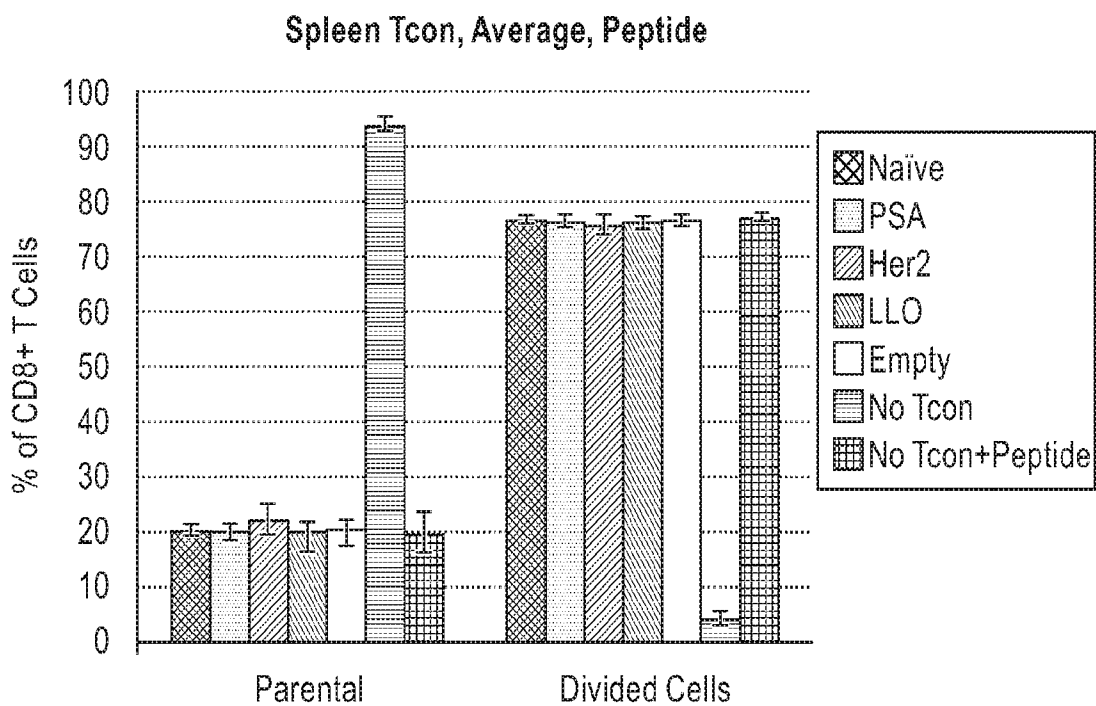


FIG. 25B

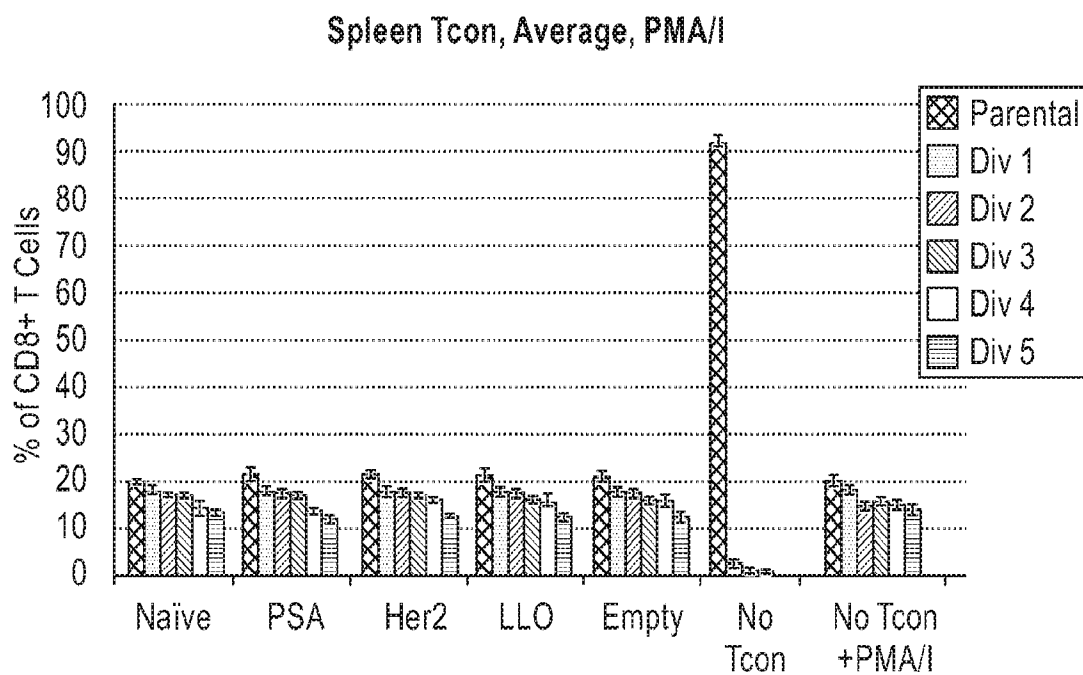


FIG. 25C

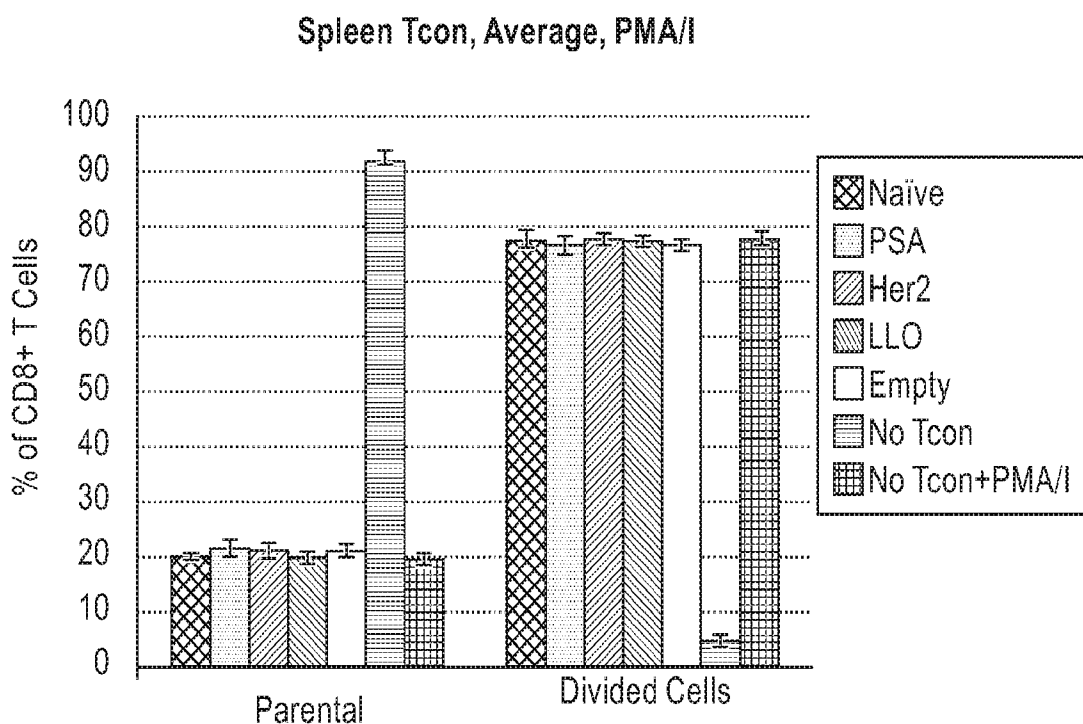


FIG. 25D

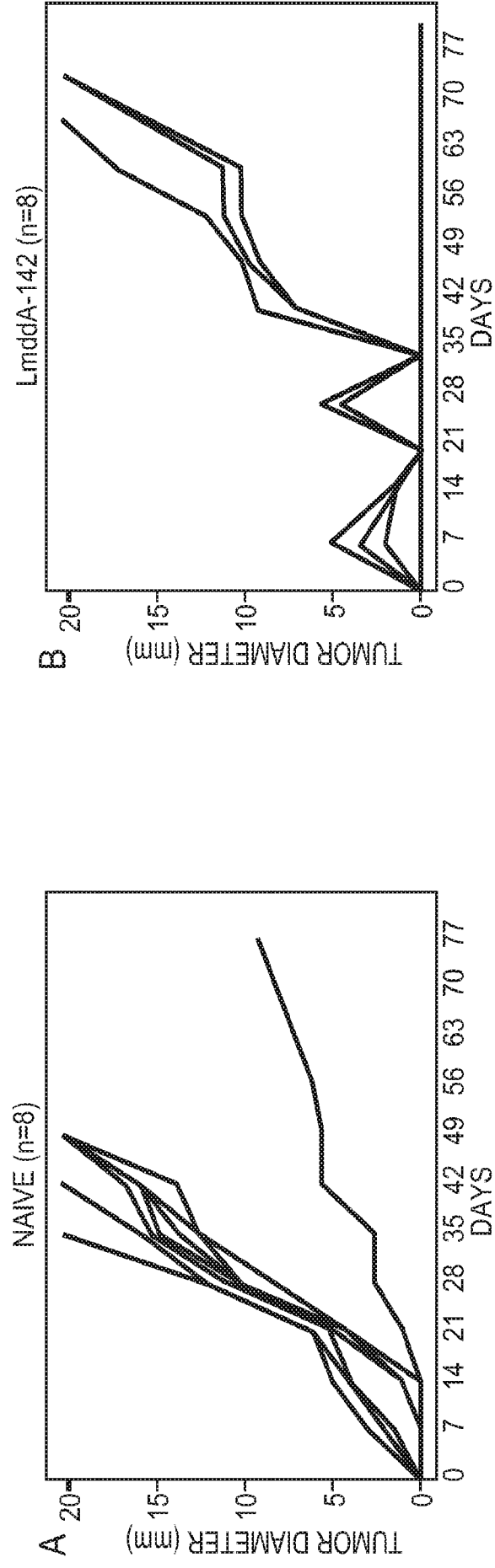


FIG. 26B

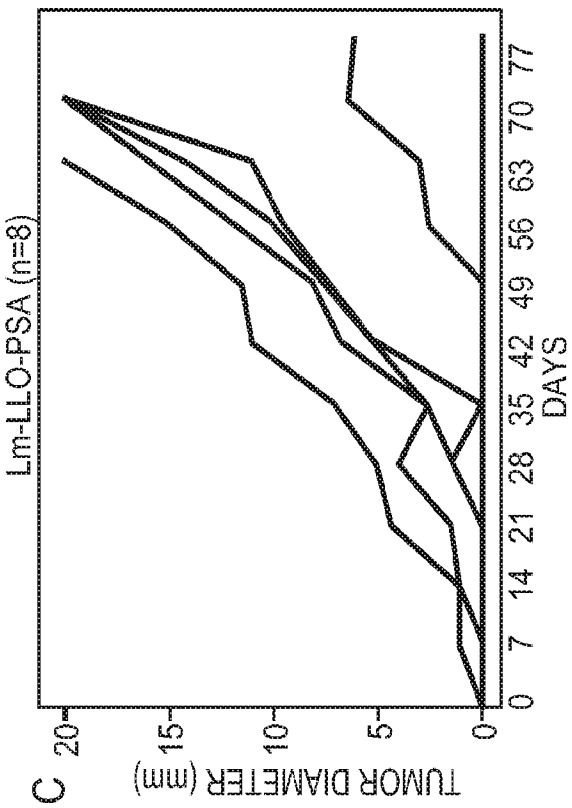


FIG. 26C

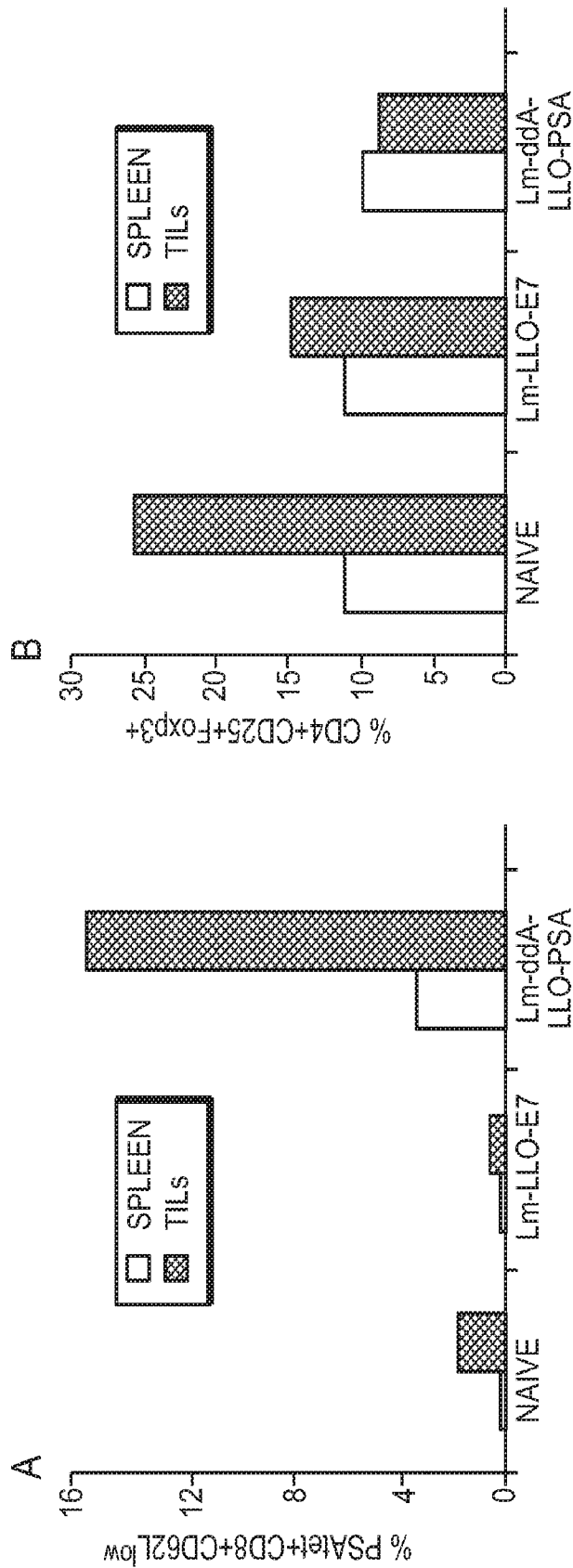


FIG. 27B

FIG. 27A

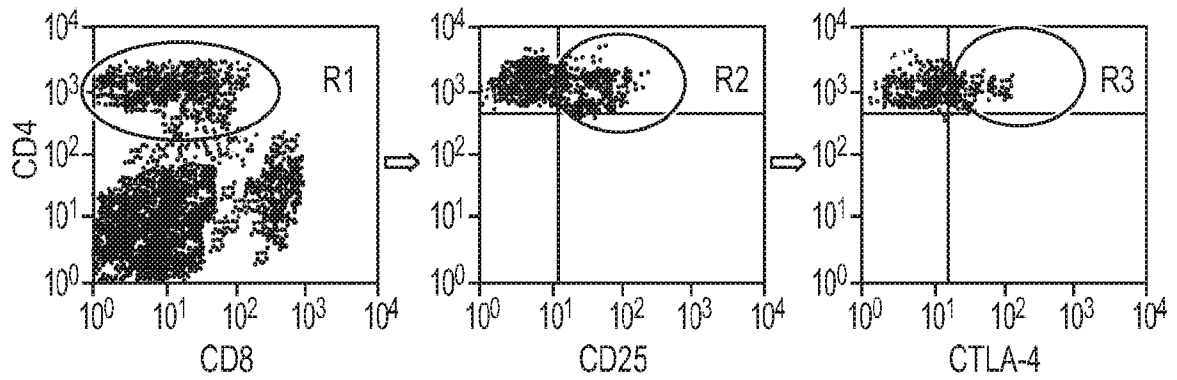


FIG. 28A

% GATED CELLS	CONTROL	TC1	LmE7	LmLLOE7
R1 = CD4	10.3	10.36	15.3	26
R2 = CD4+CD25+	13.9	14	14.1	9.14
R3 = CD4+CD25+CTLA+	13.7	28.7	27.98	27.6

FIG. 28B

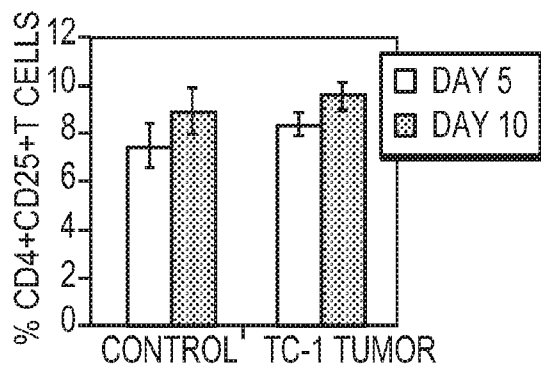


FIG. 29A

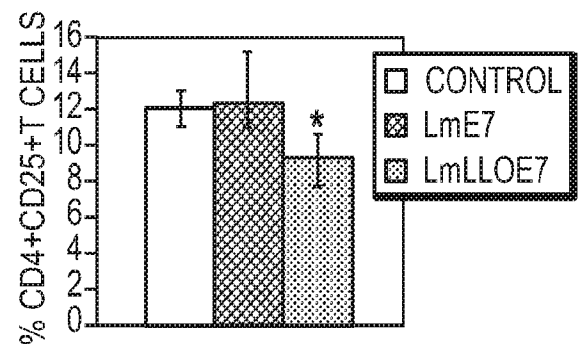


FIG. 29B

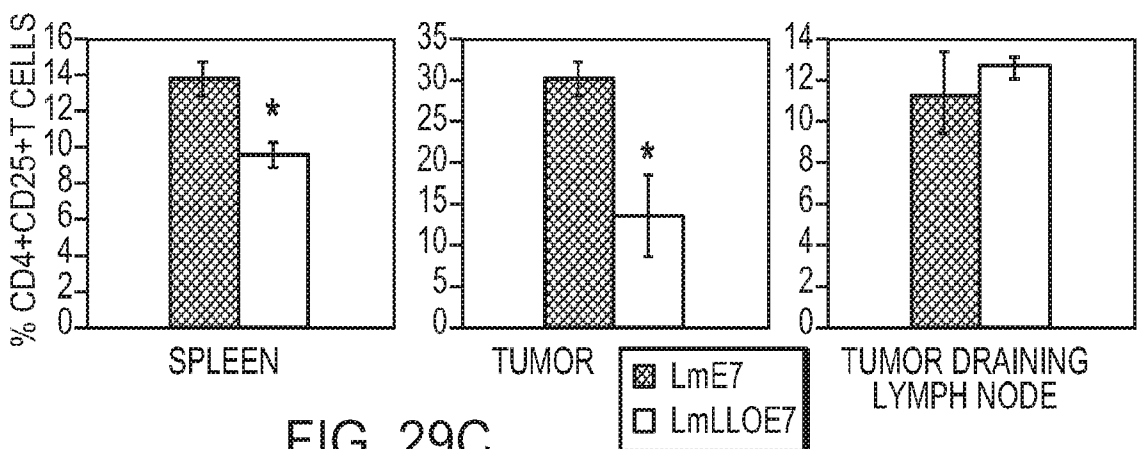


FIG. 29C

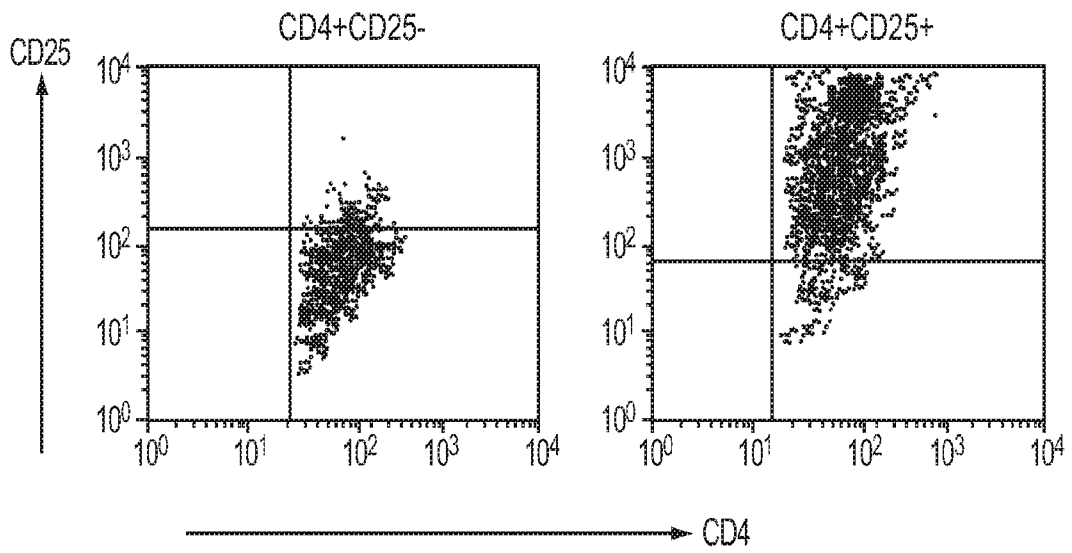
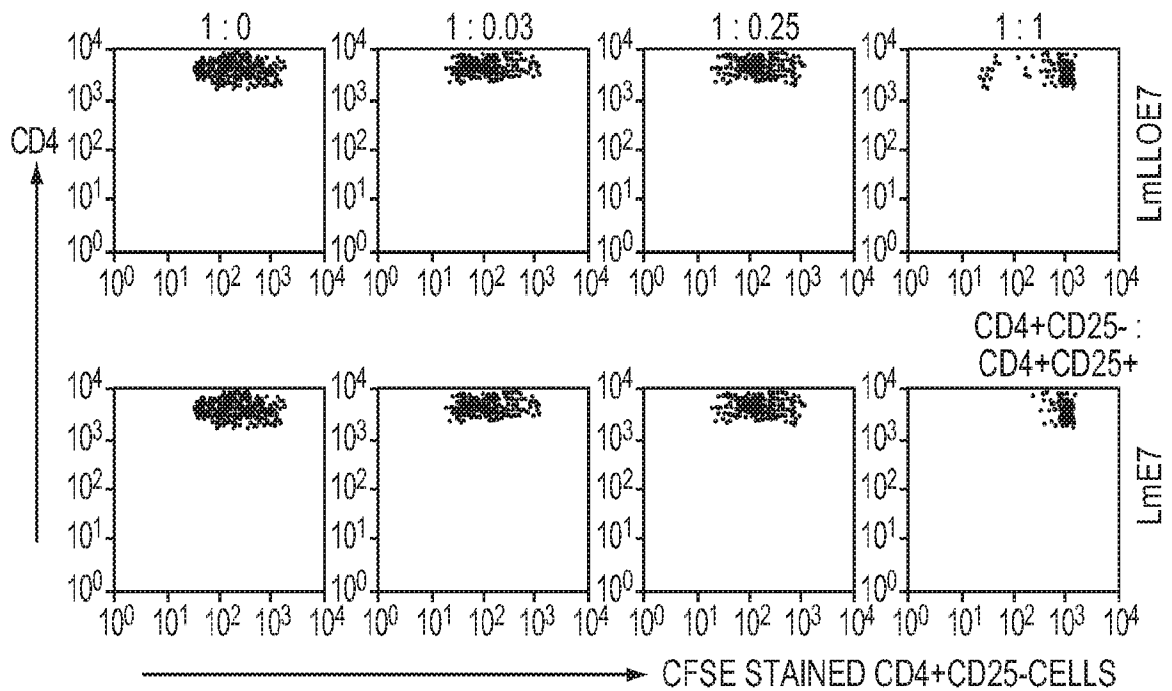


FIG. 30A



CD4+CD25- : CD4+CD25+	# OF DIVISIONS	Lm-E7 VACCINE	Lm-LLO-E7 VACCINE
1 : 1	DIVISION 0	60.2%	61.1%
	DIVISION 1	7.2%	3.7%
	DIVISION 2	0.4%	0.0%
	DIVISION 3	0.0%	0.0%
	DIVISION 4	0.7%	0.0%
1 : 0.25	DIVISION 0	20.4%	24.2%
	DIVISION 1	24.0%	26.4%
	DIVISION 2	14.0%	14.8%
	DIVISION 3	5.25%	5.89%
	DIVISION 4	1.22%	1.13%
1 : 0.03	DIVISION 0	14.17%	13.2%
	DIVISION 1	18.4%	19.5%
	DIVISION 2	24.6%	24.0%
	DIVISION 3	22.2%	24.0%
	DIVISION 4	6.6%	4.9%
1 : 0	DIVISION 0	8.2%	13.9%
	DIVISION 1	11.7%	14.0%
	DIVISION 2	20.4%	23.7%
	DIVISION 3	27.6%	27.6%
	DIVISION 4	16.8%	7.9%

FIG. 30B

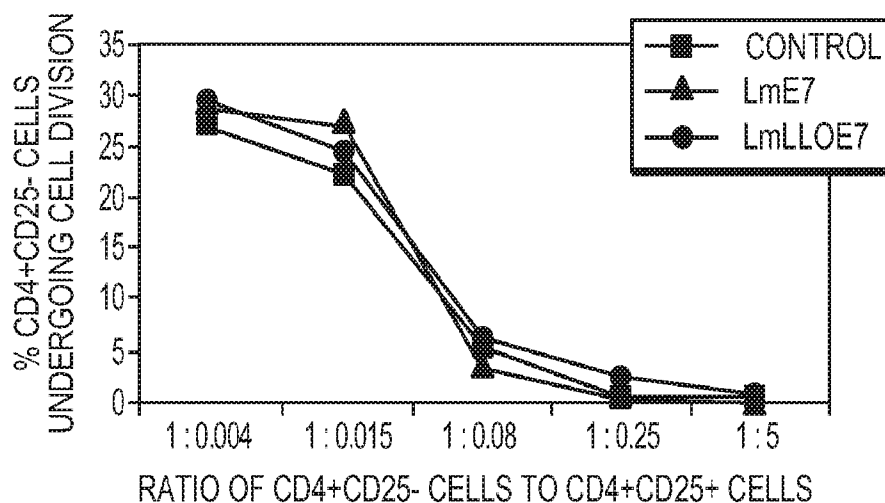


FIG. 30C

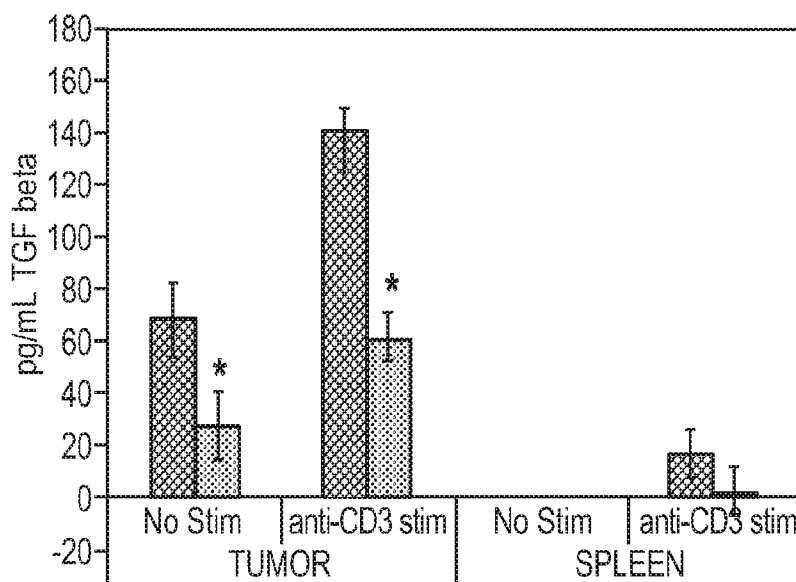


FIG. 31A

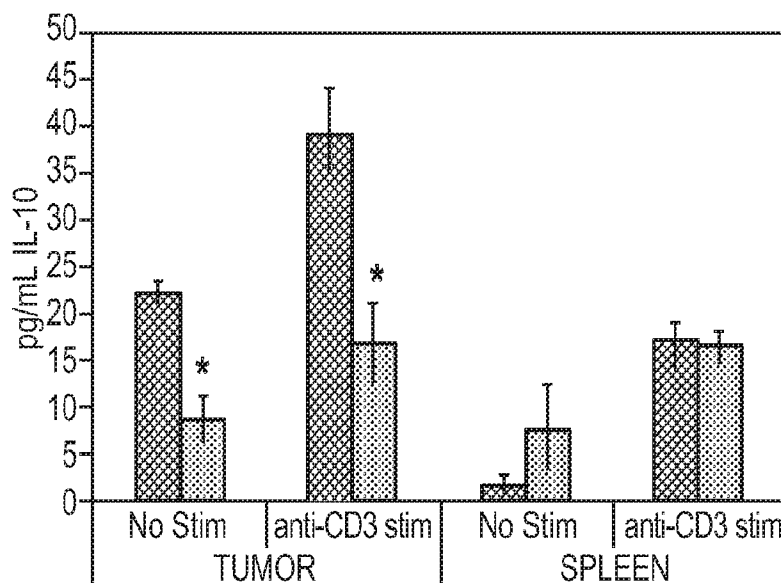


FIG. 31B

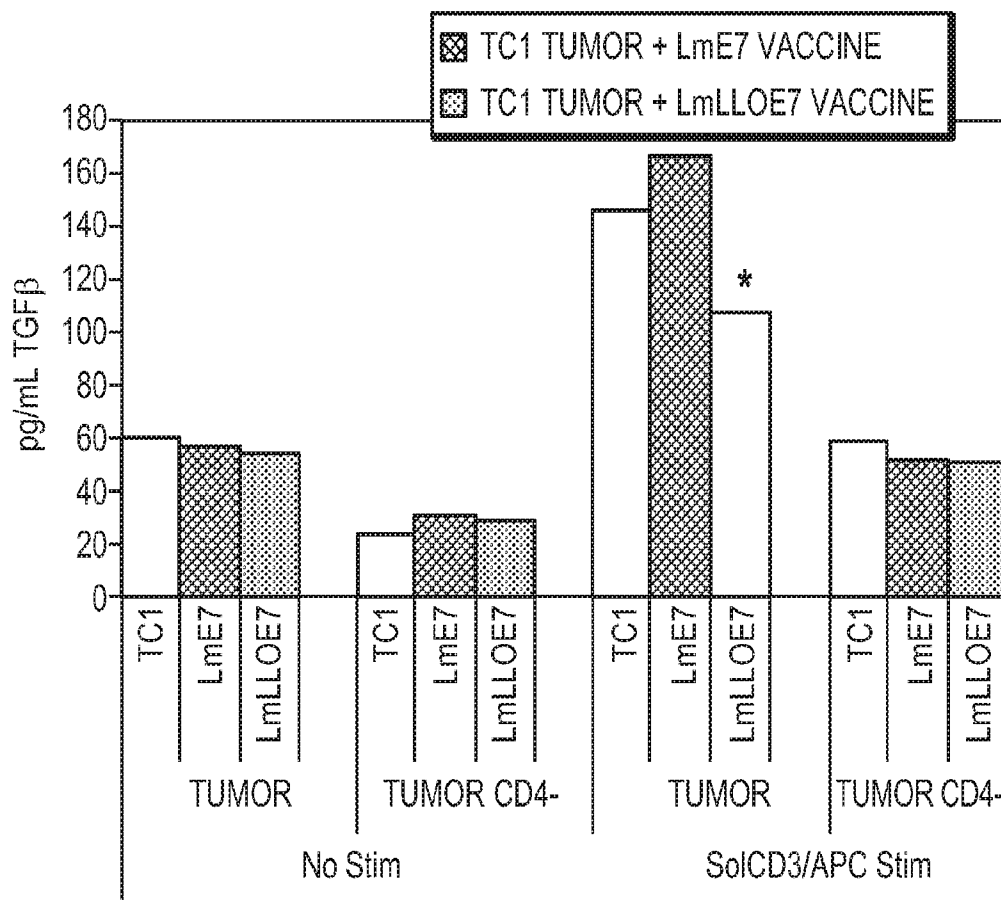


FIG. 31C

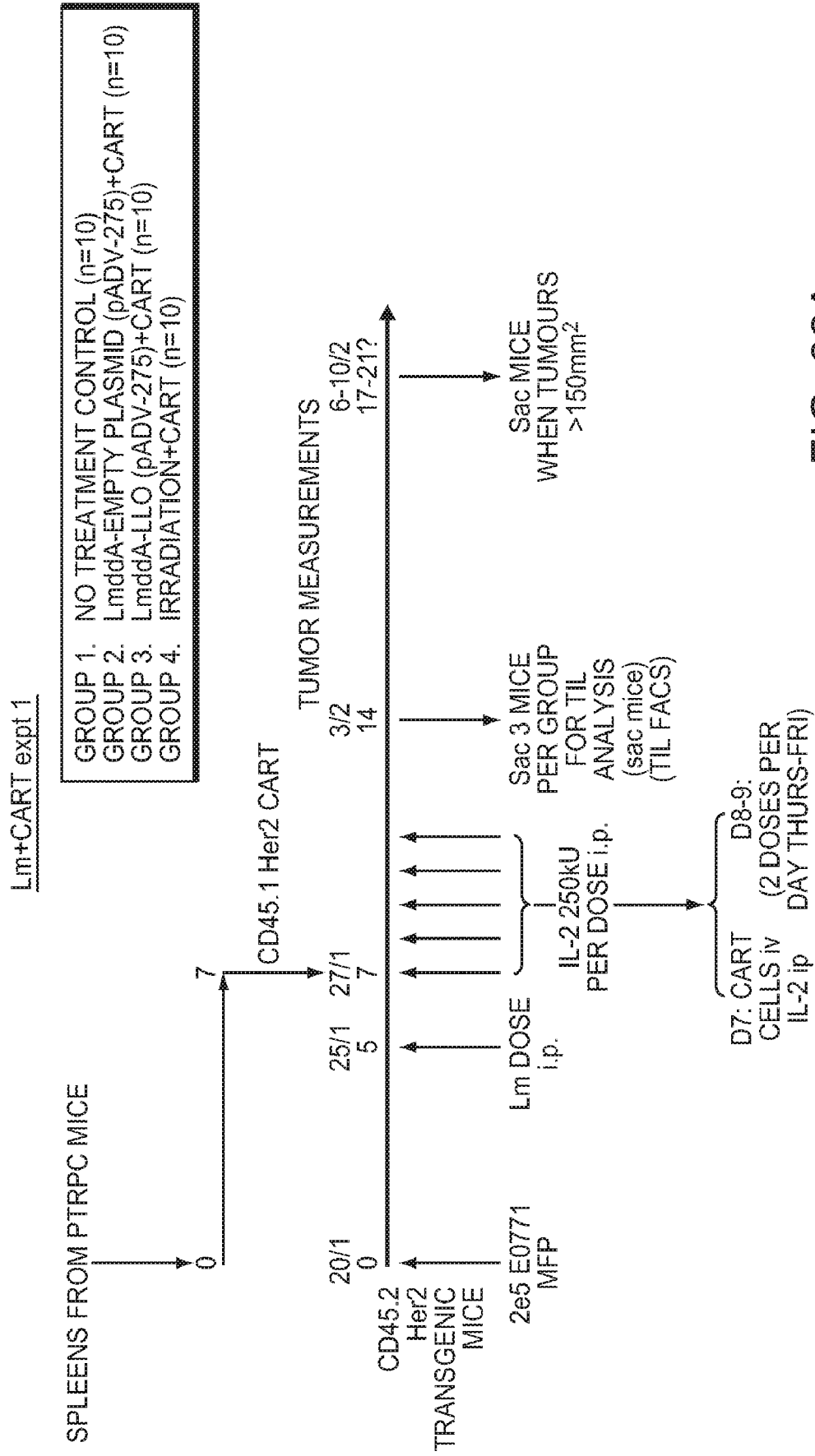


FIG. 32A

TIC ANALYSIS BY FACS

	FITC	PE	APC	APCCy7	PECy7	PB	BV711	BV785	VIABILITY
PANEL 1	Ly5.1	IFNg	Ki-67	CD45.2	CD4	FoxP3	CD8	CD103	FIX YELLOW
PANEL 2	Ly5.1	PD-1	TCRb	CD45.2	CD4	CD62L	CD8	CD44	FIX YELLOW
PANEL 3	Ly6C (AL-21)	Ly6G (1A8)	CD11c	CD11b					FIX YELLOW

FIG. 32B

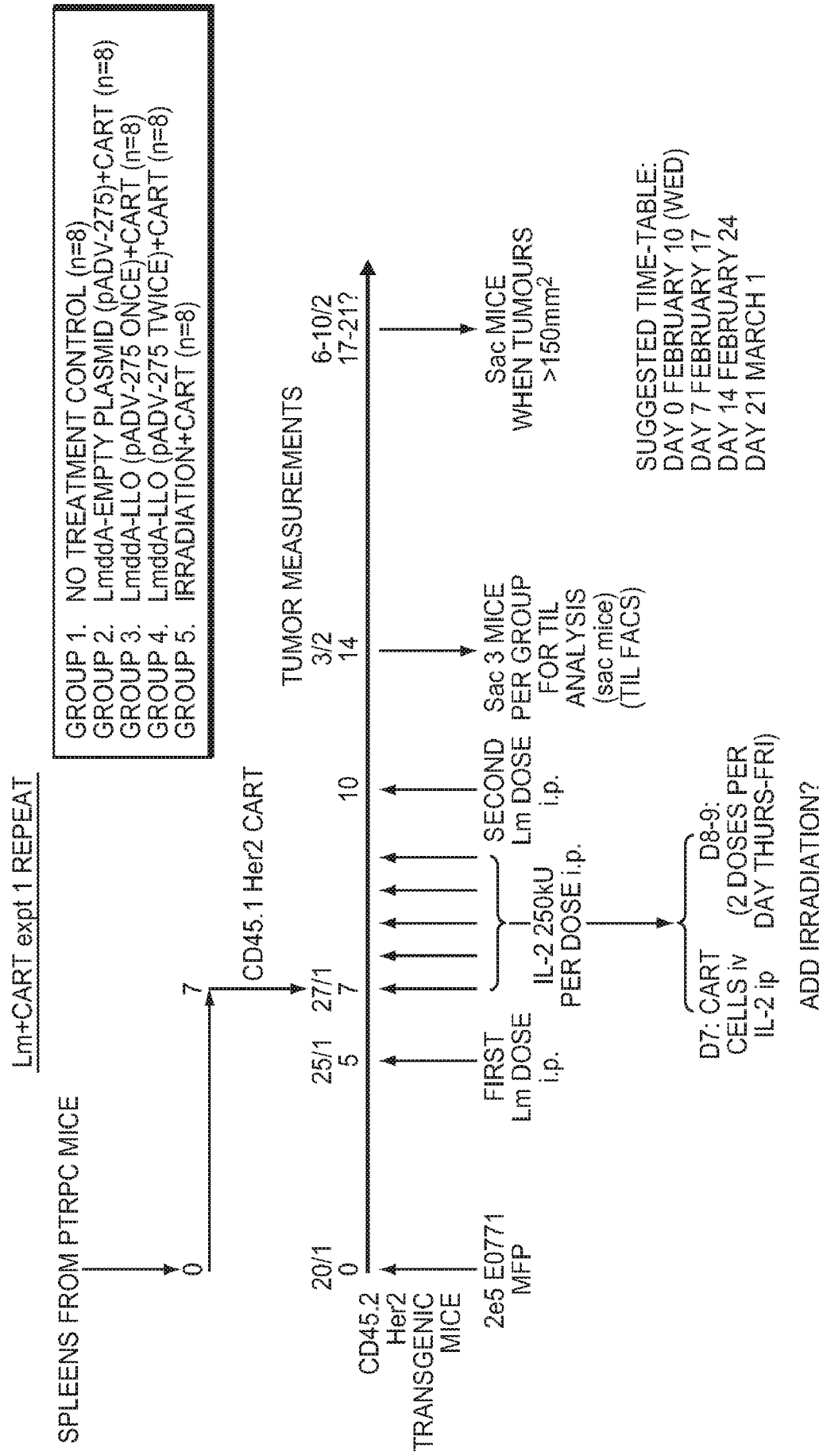


FIG. 33A

TIL ANALYSIS BY FACS

	FITC	PE	APC	APCCy7	PECy7	PB	BV711	BV785	VIABILITY
PANEL 1	Ly5.1	IFNg	Ki-67	CD45.2	CD4	FoxP3	CD8	CD103	FIX YELLOW
PANEL 2	Ly5.1	PD-1	TCRb	CD45.2	CD4	CD62L	CD8	CD44	FIX YELLOW
PANEL 3	Ly6C (AL-21)	Ly6G (1A8)	CD11c	CD11b					FIX YELLOW

FIG. 33B