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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 June 2016 (23.06.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/100924 A1

(51) International Patent Classification:

C12N 15/70 (2006.01)	*C07K 14/705* (2006.01)
C12N 15/62 (2006.01)	*C07K 16/28* (2006.01)
C07K 14/195 (2006.01)	*A61K 39/00* (2006.01)
C07K 14/47 (2006.01)	*A61K 39/02* (2006.01)
C07K 14/025 (2006.01)	*A61K 39/39* (2006.01)

(21) International Application Number:
PCT/US2015/066885

(22) International Filing Date:
18 December 2015 (18.12.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/094,349	19 December 2014 (19.12.2014)	US
62/094,472	19 December 2014 (19.12.2014)	US
62/147,463	14 April 2015 (14.04.2015)	US
62/262,896	3 December 2015 (03.12.2015)	US

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

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

Published:

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

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

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: COMBINATION THERAPIES WITH RECOMBINANT LISTERIA STRAINS

(57) Abstract: The disclosure is directed to compositions comprising an oncolytic virus, chimeric antigen receptor T cells (CAR T cells), a therapeutic or immunomodulating monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or an adoptively transferred cells incorporating engineered T cell receptors, and a live attenuated recombinant Listeria strain comprising a fusion protein of a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a tumor-associated antigen. The disclosure is further directed to methods of treating, protecting against, and inducing an immune response against a tumor, comprising the step of administering the same, with or without an additional radiation therapy treatment.

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COMBINATION THERAPIES WITH RECOMBINANT LISTERIA STRAINS

FIELD OF INTEREST

[001] The disclosure is directed to combination therapies comprising use of compositions comprising a live attenuated recombinant *Listeria* strain comprising a fusion protein of a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a tumor-associated antigen, wherein the compositions further comprise or are co-administered with an additional active agent. The disclosure is further directed to combination therapies comprising use of these compositions comprising live attenuated recombiant *Listeria* strains, in conjuction with a targeted radiation therapy for treating, protecting against, and/or inducing an immune response against a tumor.

BACKGROUND

[002] *Listeria monocytogenes* (Lm) is a Gram-positive facultative intracellular pathogen that causes listeriolysis. Once invading a host cell, Lm can escape from the phagolysosome through production of a pore-forming protein listeriolysin O (LLO) to lyse the vascular membrane, allowing it to enter the cytoplasm, where it replicates and spreads to adjacent cells based on the mobility of actin-polymerizing protein (ActA). In the cytoplasm, Lm-secreting proteins are degraded by the proteasome and processed into peptides that associate with MHC class I molecules in the endoplasmic reticulum. This unique characteristic makes it a very attractive cancer immunotherapeutic vector in that tumor antigen can be presented with MHC class I molecules to activate tumor-specific cytotoxic T lymphocytes (CTLs).

[003] In addition, once phagocytized, Lm may then be processed in the phagolysosomal compartment and peptides presented on MHC Class II for activation of Lm-specific CD4-T cell responses. Alternatively, Lm can escape the phagosome and enter the cytosol where recognition of peptidoglycan by nuclear oligomerization domain-like receptors and Lm DNA by DNA sensor, AIM2, activate inflammatory cascades. This combination of inflammatory responses and efficient delivery of antigens to the MHC I and MHC II pathways makes Lm a powerful immunotherapeutic vector in treating, protecting against, and inducing an immune response against a tumor.

[004] However, tumor cells often induce an immunosuppressive microenvironment, which favors the development of immunosuppressive populations of immune cells, such as myeloid-derived suppressor cells and regulatory T cells. Understanding the complexity of immunomodulation by tumors is important for the development of immunotherapy. Various strategies are being developed to enhance anti-tumor immune responses and to overcome 'immune checkpoints'. In addition, administration of combination immunotherapies may provide

a more efficacious and enduring response.

[005] For example, one of several mechanisms of tumor-mediated immune suppression is the expression of T-cell co-inhibitory molecules by tumor. Upon engagement to their ligands these molecules can suppress effector lymphocytes in the periphery and in the tumor microenvironment.

[006] Presently, there remains a need to provide effective combination therapies for tumor targeting methods that can eliminate tumor growth and cancer. The disclosure addresses this need by providing a combination of a *Listeria*-based immunotherapy with various therapies including addition of active agents such as oncolytic viruses, chimeric antigen receptor engineered cells (CAR T cells), therapeutic or immunomodulating monoclonal antibodies, a targeting thymidine kinase inhibitor, and/or adoptively transferred cells that may incorporate engineered T cell receptors, which may further be used in combination with additional therapies such as targeted radiation therapy.

[007] Oncolytic viruses (OVs) are self-amplifying biotherapeutics that have been selected or engineered to preferentially infect and kill cancer cells *in vivo*. Generated from a multitude of viral species including adenoviruses, reoviruses, alphaviruses, Herpes Simplex virus, Newcastle disease virus, Coxsackie B virus, Coxsackie A21 virus, Sindbis virus, measles virus, poliovirus, vesicular stomatitis virus, myxoma virus, vaccinia virus and other poxviruses, Sendai virus, and influenza virus. OVs exploit cancer-associated cellular defects arising from genetic perturbations including mutations and epigenetic reprograming. Among others, these cellular defects lead to dysfunctional anti-viral responses and immune evasion, increased cell proliferation and metabolism, and leaky tumor vasculature. These characteristics in turn provide a fertile ground for viral replication and subsequent lysis of tumor cells and permit the growth of genetically attenuated OVs that are otherwise harmless to normal cells.

[008] In addition to the direct killing of cancer cells, OVs can also trigger a potent anti-tumor immune response. Infected tumor cells induce the release of pro-inflammatory cytokines and expose both viral and tumor-associated antigens to patrolling immune cells, promoting the differentiation of antigen-presenting cells and T-cell activation. How much tumor infection and lysis are necessary to trigger these responses remains a topic of debate; however, it is clear that the combination of direct oncolysis and activation of anti-tumor immunity can lead to durable cures in pre-clinical mouse models of cancer.

[009] Another immune therapy targeted approach involves engineering patients' own immune cells to recognize and attack their tumors. This approach is often known as adoptive cell transfer. For example, using recombinant technology known in the art the receptors present on T cells may have both polypeptide chains engineered to have a selected specificity (Receptor

engineered T cells). In certain instances, T cells are engineered to have a chimeric antigen receptor, wherein one of the polypeptide chains is from the T cell receptor and that other polypeptide chain is from an antibody. These cells are known as chimeric antigen receptor T cells (CAR T cells).Adoptive cell transfer involves administration of T cells comprising engineer receptors, wherein the cells may be Receptor engineered T cell or CAR T cells, each engineered to produce special receptors on their surface, either engineer T cell receptors or chimeric T cell receptors called chimeric antigen receptors (CARs). CARs are proteins that allow the T cells to recognize a specific protein antigen on a tumor cell. CAR T cells are then administered to patient, wherein these engineered T cells can recognize and kill cancer cells that harbor the specific antigen on their surfaces. A combination therapy administering CAR T cells and a *Listeria*-based immunotherapy may provide another therapy to eliminate tumor growth and cancer. There remains a need to optimize the dosage and schedule for administrating these two treatments. The disclosure further addresses this need by providing a combination of a *Listeria*-based immunotherapy with targeted CAR T cell administration.

[0010] Another immune therapy targeted approach involves the use of monoclonal antibodies developed to specifically target antigens expressed on the surface of cancerous cells. Due to immunotolerance, a person's immune system does not always recognize cancer cells as foreign targets. A monoclonal antibody can be directed to attach to antigens on the surface of a cancer cell. In this way, the antibody marks the cancer cell and makes it easier for the immune system to find. Alternatively, antibodies targeting growth signals may help prevent a tumor from developing a blood supply so that the tumor fails to growth or remains small. In the case of a tumor with an already-established network of blood vessels, blocking the growth signals could cause the blood vessels to die and the tumor to shrink. A combination therapy administering a therapeutic and/or immunomodulatory antibody and a *Listeria*-based immunotherapy may provide another therapy to eliminate tumor growth and cancer. There remains a need to optimize the dosage and schedule for administrating these two treatments. The disclosure further addresses this need by providing a combination of a *Listeria*-based immunotherapy with therapeutic and/or immunomodulatory antibody administration.

[0011] Another immune therapy targeted approach is the administration of Tyrosine Kinase Inhibitor (TKI) anticancer treatments. TKI are chemical compounds that inhibit the activity of tyrosine kinase enzyme inside the body. Often, tyrosine kinases provide an activity that aids in the growth and metastasis of tumors. Therefore, incorporation of a TKI may prevent growth and spreading of a cancer. A combination therapy administering a TKI and a *Listeria* may provide another therapy to eliminate tumor growth and cancer. There remains a need to optimize the dosage and schedule for administrating these two treatments. The disclosure further addresses

this need by providing a combination of a *Listeria*-based immunotherapy with TKI administration.

[0012] Evidence has recently emerged revealing the capacity of targeted radiation therapy (RT) to induce antitumor responses, suggesting a possible combination therapy combining RT with *Listeria* based immunotherapy to promote tumor-specific immunity. Since RT and Lm vaccine therapy each induce a different aspect of antitumor immunity, a combination of these therapies may result in an overall increase in intratumoral numbers of activated T cells, antigen specific CD8+ T cells, natural killer cells and levels of effector molecules, such as interferon -γ (IFN-γ) and granzyme B. There remains a need to optimize the dosage and schedule for administrating these two treatments. The disclosure further addresses this need by providing a combination of a *Listeria*-based immunotherapy with targeted radiation therapy regimes.

[0013] Given the complex nature of certain diseases, including cancer, a need exists for a combined approach in treating the same. As seen in the Detailed Description below, these combination therapies may improve the overall anti-tumor efficacy of immunotherapy.

SUMMARY OF THE DISCLOSURE

[0014] In one aspect, the disclosure relates to an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, said composition further comprising an additional active agent.

[0015] In a related aspect, the disclosure relates to a method of inhibiting tumor-mediated immunosuppression in a subject, said method comprising the step of administering to said subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein:

(a) said composition further comprises an additional active agent;

(b) said method further comprises a step of administering an effective amount of a composition comprising an additional active agent to said subject; or

(c) said method further comprises a step of administering a targeted radiation therapy to said subject; or

any combination thereof of (a)-(c).

[0016] In another related aspect, the disclosure relates to a method of eliciting an enhanced anti-tumor T cell response in a subject, said method comprising the step of administering to said subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein:

(a) said composition further comprises an additional active agent;

(b) said method further comprises a step of administering an effective amount of a composition comprising an additional active agent to said subject; or

(c) said method further comprises a step of administering a targeted radiation therapy to said subject; or any combination thereof of (a)-(c).

[0017] In another related aspect, the disclosure relates to a method of treating a tumor or cancer in a subject, said method comprising the step of administering to said subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein:

(a) said composition further comprises an additional active agent;

(b) said method further comprises a step of administering an effective amount of a composition comprising an additional active agent to said subject; or

(c) said method further comprises a step of administering a targeted radiation therapy to said subject; or

any combination thereof of (a)-(c).

[0018] In another related aspect, an additional active agent disclosed herein comprises an oncolytic virus, a T cell receptor engineered T cell (Receptor engineered T cells), a chimeric antigen receptor engineered T cell (CAR T cells), a therapeutic or immunomodulating monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or an adoptively transferred cell incorporating engineered T cell receptors, or any combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] 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:

[0020] **Figure 1.** (**Figure 1A**) Schematic representation of the chromosomal region of the *Lmdd*-143 and *LmddA*-143 after *klk3* integration and *actA* deletion; (**Figure B**) The *klk3* gene is integrated into the *Lmdd* and *LmddA* chromosome. PCR from chromosomal DNA preparation from each construct using *klk3* specific primers amplifies a band of 714 bp corresponding to the *klk3* gene, lacking the secretion signal sequence of the wild type protein.

[0021] **Figure 2.** (**Figure 2A**) Map of the pADV134 plasmid. (**Figure 2B**) Proteins from *LmddA*-134 culture supernatant were precipitated, separated in a SDS-PAGE, and the LLO-E7 protein detected by Western-blot using an anti-E7 monoclonal antibody. The antigen expression cassette consists of *hly* promoter, ORF for truncated LLO and human PSA gene (*klk3*). (**Figure 2C**) Map of the pADV142 plasmid. (**Figure 2D**) Western blot showed the expression of LLO-PSA fusion protein using anti-PSA and anti-LLO antibody.

[0022] **Figure 3.** (**Figure 3A**) Plasmid stability *in vitro* of LmddA-LLO-PSA if cultured with and without selection pressure (D-alanine). Strain and culture conditions are listed first and plates used for CFU determination are listed after. (**Figure 3B**) Clearance of LmddA-LLO-PSA *in vivo* and assessment of potential plasmid loss during this time. Bacteria were injected i.v. and isolated from spleen at the time point indicated. CFUs were determined on BHI and BHI + D-alanine plates.

[0023] **Figure 4.** (**Figure 4A**) *In vivo* clearance of the strain LmddA-LLO-PSA after administration of 10^8 CFU in C57BL/6 mice. The number of CFU were determined by plating on BHI/str plates. The limit of detection of this method was 100 CFU. (**Figure 4B**) Cell infection assay of J774 cells with 10403S, LmddA-LLO-PSA and XFL7 strains.

[0024] **Figure 5.** (**Figure 5A**) PSA tetramer-specific cells in the splenocytes of naïve and LmddA-LLO-PSA immunized mice on day 6 after the booster dose. (**Figure 5B**) Intracellular cytokine staining for IFN-γ in the splenocytes of naïve and LmddA-LLO-PSA immunized mice were stimulated with PSA peptide for 5 h. Specific lysis of EL4 cells pulsed with PSA peptide with in vitro stimulated effector T cells from LmddA-LLO-PSA immunized mice and naïve mice at different effector/target ratio using a caspase based assay (**Figure 5C**) and a europium based assay (**Figure 5D**). Number of IFNγ spots in naïve and immunized splenocytes obtained after stimulation for 24 h in the presence of PSA peptide or no peptide (**Figure 5E**).

[0025] **Figure 6.** Immunization with *LmddA*-142 induces regression of Tramp-C1-PSA (TPSA) tumors. Mice were left untreated (n=8) (**Figure 6A**) or immunized i.p. with *LmddA*-142 ($1x10^8$ CFU/mouse) (n=8) (**Figure 6B**) or Lm-LLO-PSA (n=8) (**Figure 6C**) 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.

[0026] **Figure 7.** (**Figure 7A**) 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 7B**) 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.

[0027] **Figure 8.** (**Figure 8A**) Schematic representation of the chromosomal region of the Lmdd-143 and LmddA-143 after *klk3* integration and actA deletion; (**Figure 8B**) The *klk3* gene is integrated into the Lmdd and LmddA chromosome. PCR from chromosomal DNA preparation from each construct using *klk3* specific primers amplifies a band of 760 bp corresponding to the *klk3* gene.

[0028] **Figure 9.** (**Figure 9A**) *Lmdd*-143 and *LmddA*-143 secretes the LLO-PSA protein. Proteins from bacterial culture supernatants were precipitated, separated in a SDS-PAGE and LLO and LLO-PSA proteins detected by Western-blot using an anti-LLO and anti-PSA antibodies; (**Figure 9B**) LLO produced by *Lmdd*-143 and *LmddA*-143 retains hemolytic activity. Sheep red blood cells were incubated with serial dilutions of bacterial culture supernatants and hemolytic activity measured by absorbance at 590nm; (**Figure 9C**) *Lmdd*-143 and *LmddA*-143 grow inside the macrophage-like J774 cells. J774 cells were incubated with bacteria for 1 hour followed by gentamicin treatment to kill extracellular bacteria. Intracellular growth was measured by plating serial dilutions of J774 lysates obtained at the indicated timepoints. *Lm* 10403S was used as a control in these experiments.

[0029] **Figure 10.** Immunization of mice with *Lmdd*-143 and *LmddA*-143 induces a PSA-specific immune response. C57BL/6 mice were immunized twice at 1-week interval with $1x10^8$ CFU of *Lmdd*-143, *LmddA*-143 or *LmddA*-142 and 7 days later spleens were harvested. Splenocytes were stimulated for 5 hours in the presence of monensin with 1 μM of the PSA_{65-74} peptide. Cells were stained for CD8, CD3, CD62L and intracellular IFN-γ and analyzed in a FACS Calibur cytometer.

[0030] **Figures 11A-B** show a decrease in MDSCs and Tregs in tumors. The number of MDSCs (right-hand panel) and Tregs (left-hand panel) following Lm vaccination (LmddAPSA and LmddAE7).

[0031] **Figure 12.** Figures 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 12A and 12B** Phorbol-Myristate-Acetate and Ionomycin

(PMA/I) represents non-specific stimulation. In **Figures 12C and 12D** 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 12A and 12C** show individual cell division cycles for each group. **Figures 12B and 12D** show pooled division cycles.

[0032] **Figure 13** 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 13A and 13B** PMA/I represents non-specific stimulation. In **Figures 13C and 13D** 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 13A and 13C** show individual cell division cycles for each group. **Figures 13B and 13D** show pooled division cycles.

[0033] **Figure 14** 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 14A and 14B** PMA/I represents non-specific stimulation. In **Figures 14C and 14D** 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 14A and 14C** show individual cell division cycles for each group. **Figures 14B and 14D** show pooled percentage division.

[0034] **Figure 15** 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 15A and 15B** PMA/I represents non-specific stimulation. In **Figures 15C and 15D** 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 15A and 15C** show individual cell division cycles for each group. **Figures 15B and 15D** show pooled percentage division.

[0035] **Figure 16** 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 16A and 16B** PMA/I represents non-specific

stimulation. In **Figures 16C and 16D** 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 16A and 16C** show individual cell division cycles for each group. **Figures 16B and 16D** show pooled percentage division.

[0036] **Figure 17** shows suppressor assay data demonstrating that splenic Tregs are still suppressive. In **Figures 17A and 17B** PMA/I represents non-specific stimulation. In **Figures 17C and 17D** 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 17A and 17C** show individual cell division cycles for each group. **Figures 17B and 17D** show pooled percentage division.

[0037] **Figure 18** 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 18A and 18B** PMA/I represents non-specific stimulation. In **Figures 18C and 18D** 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 18C-18D** show data from pooled percentage division.

[0038] **Figure 19** 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 19A and 19B** PMA/I represents non-specific stimulation. In **Figures 19C and 19D** 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 19A and 19C** show individual cell division cycles for each group. **Figures 19B and 19D** show pooled percentage division.

[0039] **Figure 20** show suppressor assay data demonstrating that there is no *Listeria*-specific

effect on splenic monocytic MDSCs. In **Figures 20A and 20B** PMA/I represents non-specific stimulation. In **Figures 20C and 20D** 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 20A and 20C** show individual cell division cycles for each group. **Figures 20B and 20D** show pooled percentage division.

[0040] **Figure 21** 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 21A and 21B** PMA/I represents non-specific stimulation. In **Figures 21C and 21D** 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 21A and 21C** show individual cell division cycles for each group. **Figures 21B and 21D** shows pooled percentage division.

[0041] **Figure 22** present suppressor assay data demonstrating that there is no *Listeria*-specific effect on splenic granulocytic MDSCs. In **Figures 22A and 22B** PMA/I represents non-specific stimulation. In **Figures 22C and 22D** 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 22A and 22C** show individual cell division cycles for each group. **Figures 22B and 22D** show pooled percentage division.

[0042] **Figure 23** present suppressor assay data demonstrating that decrease in the suppressive ability of Tregs from 4T1 tumors (Her2 expressing tumors) after *Listeria* vaccination. In **Figures 23A and 23B** PMA/I represents non-specific stimulation. In **Figures 23C and 23D** 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 23A and 23C** show individual cell division cycles for each group. **Figures**

23B and 23D show pooled percentage division.

[0043] **Figure 24** 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 whether or not they are antigen specific. In **Figures 24A and 24B** PMA/I represents non-specific stimulation. In **Figures 24C and 24D** the term "peptide" represents specific antigen stimulation. Percent (%) CD8+ represents % effector (responder) T cells. **Figures 24A and 24C** show individual cell division cycles for each group. **Figures 24B and 24D** show pooled percentage division.

[0044] **Figure 25** 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 25A and 25C**) show individual cell division cycles for each group. Right-hand panels (**Figures 25B and 25D**) show pooled percentage division.

[0045] **Figure 26** 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 26A and 26C**) show individual cell division cycles for each group. Right-hand panels (**Figures 26B and 26D**) show pooled percentage division.

[0046] **Figure 27** 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 27A and 27B**). However, after non-specific stimulation, activated T cells (with PMA/ionomycin) are still capable of dividing (**Figures 27C and 27D**). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled percentage division.

[0047] **Figure 28** show suppressor assay data demonstrating that monocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination (**Figures 28A and 28B**). However, after non-specific activation (stimulated by PMA/ionomycin), T cells are still capable of dividing (**Figures 28C and 28D**). Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled percentage division.

[0048] **Figure 29** show suppressor assay data demonstrating that Tregs purified from the

tumors of any of the *Lm*-treated groups have a slightly diminished ability to suppress the division of the responder T cells, regardless of whether the responder cells are antigen specific (**Figures 29A and 29B**) or non-specifically (**Figures 29C and 29D**) activated. Left-hand panels show individual cell division cycles for each group. Right-hand panels show pooled percentage division.

[0049] **Figure 30** show suppressor assay data demonstrating that Tregs purified from the spleen are still capable of suppressing the division of both antigen specific (**Figures 30A-30B**) and non-specifically (**Figures 30C and 30D**) activated responder T cells.

[0038] **Figure 31** 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 31A and 31B**) or non-specifically activated (**Figures 31C and 31D**).

[0050] **Figure 32** 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 32A and 32B**) or non-specifically activated (**Figures 32C and 32D**).

[0051] **Figure 33. Construction of ADXS31-164. (Figure 33A)** Plasmid map of pAdv164, which harbors *bacillus subtilis dal* gene under the control of constitutive *Listeria* p60 promoter for complementation of the chromosomal *dal-dat* deletion in *LmddA* strain. It also contains the fusion of truncated $LLO_{(1-441)}$ to the chimeric human Her2/neu gene, which was constructed by the direct fusion of 3 fragments the Her2/neu: EC1 (aa 40-170), EC2 (aa 359-518) and ICI (aa 679-808). **(Figure 33B)** Expression and secretion of tLLO-ChHer2 was detected in *Lm*-LLO-ChHer2 (Lm-LLO-138) and *LmddA*-LLO-ChHer2 (ADXS31-164) by western blot analysis of the TCA precipitated cell culture supernatants blotted with anti-LLO antibody. A differential band of ~104 KD corresponds to tLLO-ChHer2. The endogenous LLO is detected as a 58 KD band. *Listeria* control lacked ChHer2 expression.

[0052] **Figure 34. Immunogenic properties of ADXS31-164 (Figure 34A)** Cytotoxic T cell responses elicited by Her2/neu *Listeria*-based vaccines in splenocytes from immunized mice were tested using NT-2 cells as stimulators and 3T3/neu cells as targets. Lm-control was based on the *LmddA* background that was identical in all ways but expressed an irrelevant antigen (HPV16-E7). **(Figure 34B)** IFN-γ secreted by the splenocytes from immunized FVB/N mice into the cell culture medium, measured by ELISA, after 24 hours of *in vitro* stimulation with mitomycin C treated NT-2 cells. **(Figure 34C)** IFN-γ secretion by

splenocytes from HLA-A2 transgenic mice immunized with the chimeric vaccine, in response to *in vitro* incubation with peptides from different regions of the protein. A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide groups constituted the negative controls as listed in the figure legend. IFN-γ secretion was detected by an ELISA assay using cell culture supernatants harvested after 72 hours of co-incubation. Each data point was an average of triplicate data +/- standard error. * P value < 0.001.

[0053] **Figure 35. Tumor Prevention Studies for *Listeria*-ChHer2/neu Vaccines** Her2/neu transgenic mice were injected six times with each recombinant *Listeria*-ChHer2 or a control *Listeria* strain. Immunizations started at 6 weeks of age and continued every three weeks until week 21. Appearance of tumors was monitored on a weekly basis and expressed as percentage of tumor free mice. *p<0.05, N = 9 per group.

[0054] **Figure 36. Effect of immunization with ADXS31-164 on the % of Tregs in Spleens.** FVB/N mice were inoculated s.c. with 1 x 10^6 NT-2 cells and immunized three times with each vaccine at one week intervals. Spleens were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. dot-plots of the Tregs from a representative experiment showing the frequency of $CD25^+/FoxP3^+$ T cells, expressed as percentages of the total $CD3^+$ or $CD3^+CD4^+$ T cells across the different treatment groups.

[0055] **Figure 37. Effect of immunization with ADXS31-164 on the % of tumor infiltrating Tregs in NT-2 tumors.** FVB/N mice were inoculated s.c. with 1 x 10^6 NT-2 cells and immunized three times with each vaccine at one week intervals. Tumors were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. (**Figure 37A**). dot-plots of the Tregs from a representative experiment. (**Figure 37B**). Frequency of $CD25^+/FoxP3^+$ T cells, expressed as percentages of the total $CD3^+$ or $CD3^+CD4^+$ T cells (left panel) and intratumoral CD8/Tregs ratio (right panel) across the different treatment groups. Data is shown as mean±SEM obtained from 2 independent experiments.

[0056] **Figure 38. Vaccination with ADXS31-164 can delay the growth of a breast cancer cell line in the brain.** Balb/c mice were immunized thrice with ADXS31-164 or a control *Listeria* strain. EMT6-Luc cells (5,000) were injected intracranially in anesthetized mice. (**Figure 38A**) *Ex vivo* imaging of the mice was performed on the indicated days using a Xenogen X-100 CCD camera. (**Figure 38B**) Pixel intensity was graphed as number of

photons per second per cm2 of surface area; this is shown as average radiance. (**Figure 38C**) Expression of Her2/neu by EMT6-Luc cells, 4T1-Luc and NT-2 cell lines was detected by Western blots, using an anti-Her2/neu antibody. J774.A2 cells, a murine macrophage like cell line was used as a negative control.

[0057] 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

[0039] 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 disclosure 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 disclosure the disclosure.

[0040] This disclosure provides in one embodiment, an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof and the composition further comprises an additional active agent. In another embodiment, an additional active agent comprise in an immunogenic composition disclosed herein comprises an attenuated oncolytic virus, a T cell receptor engineered T cell (Receptor engineered T cells), a chimeric antigen receptor engineered T cell (CAR T cells), a therapeutic or immunomodulating monoclonal antibody, or a targeting thymidine kinase inhibitor (TKI), or any combination thereof.

[0041] In another embodiment, disclosed herein is an immunogenic composition comprising an oncolytic virus, and a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[0042] In one embodiment, the oncolytic virus is attenuated to eliminate viral functions that are expendable in tumor cells, but not in normal cells, thus making the virus safer and more tumor-specific. Hence, in another embodiment, disclosed herein is an immunogenic composition comprising an attenuated oncolytic virus, and a recombinant *Listeria* strain

comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[0043] In another embodiment, disclosed herein is an immunogenic composition comprising chimeric antigen receptor-engineered T cells (CAR T cells), and a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[0044] In another embodiment, disclosed herein is an immunogenic composition comprising a therapeutic or immunmodulating antibody, and a recombinant *isteria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof. In another embodiment, the immunomodulating antibody is a monoclonal antibody. In another embodiment, the monoclonal antibody recognizes an epitope of said heterologous antigen on a cancer cell.

[0045] In another embodiment, disclosed herein is an immunogenic composition comprising targeting thymidine kinase (TKI), and a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[0046] In another embodiment, disclosed herein is an immunogenic composition comprising a T cell receptor engineered T cell (Receptor engineered T cells), and a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[0047] In one embodiment, this disclosure provides a method of eliciting an enhanced anti-tumor T cell response in a subject, the method comprising the step of administering to the subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen

or fragment thereof, wherein: (a) the composition further comprises an additional active agent; (b) the method further comprises a step of administering an effective amount of a composition comprising an additional active agent to the subject; or (c) the method further comprises a step of administering a targeted radiation therapy to the subject; or any combination thereof of (a)-(c).

[0048] In another embodiment, a method disclosed herein is for inhibiting tumor-mediated immunosuppression in a subject, the method comprising the step of administering to the subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein: (a) the composition further comprises an additional active agent; (b) the method further comprises a step of administering an effective amount of a composition comprising an additional active agent to the subject; or (c) the method further comprises a step of administering a targeted radiation therapy to the subject; or any combination thereof of (a)-(c).

[0049] In another embodiment, a method disclosed herein is for a method for increasing the ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor of a subject, the method comprising the step of administering to the subject an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein: (a) the composition further comprises an additional active agent; (b) the method further comprises a step of administering an effective amount of a composition comprising an additional active agent to the subject; or (c) the method further comprises a step of administering a targeted radiation therapy; or any combination thereof of (a)-(c).

Recombinant *Listeria* strains

[0050] In one embodiment, a recombinant *Listeria* strain disclosed herein comprises a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof. In one embodiment, the recombinant *Listeria* strain is attenuated.

[0051] In one embodiment a Truncated LLO, a truncated ActA or a PEST-sequence peptide Truncated LLO, a truncated ActA or a PEST-sequence peptide comprises a truncated

listeriolysin O (LLO) protein or a truncated actA protein. In one embodiment, a Truncated LLO, a truncated ActA or a PEST-sequence peptide is a truncated LLO protein. In another embodiment, a Truncated LLO, a truncated ActA or a PEST-sequence peptide is a truncated actA protein. In one embodiment, a Truncated LLO, a truncated ActA or a PEST-sequence peptide is a full-length LLO protein. In another embodiment, a Truncated LLO, a truncated ActA or a PEST-sequence peptide is a full-length ActA protein.

[0052] In one embodiment, a PEST amino acid (AA) sequence comprises a truncated LLO sequence. In another embodiment, the PEST amino acid sequence is KENSISSMAPPASPPASPKTPIEKKHADEIDK (SEQ ID NO: 1). In another embodiment, fusion of an antigen to other LM PEST AA sequences from *Listeria* will also enhance immunogenicity of the antigen.

[0053] The N-terminal LLO protein fragment of methods and compositions of the disclosure comprises, in another embodiment, SEQ ID No: 3. In another embodiment, the fragment comprises an LLO signal peptide. In another embodiment, the fragment comprises SEQ ID No: 4. In another embodiment, the fragment consists approximately of SEQ ID No: 4. In another embodiment, the fragment consists essentially of SEQ ID No: 4. In another embodiment, the fragment corresponds to SEQ ID No: 4. In another embodiment, the fragment is homologous to SEQ ID No: 4. In another embodiment, the fragment is homologous to a fragment of SEQ ID No: 4. The ΔLLO used in some of the Examples was 416 AA long (exclusive of the signal sequence), as 88 residues from the amino terminus which is inclusive of the activation domain containing cysteine 484 were truncated. It will be clear to those skilled in the art that any ΔLLO without the activation domain, and in particular without cysteine 484, are suitable for methods and compositions of the disclosure. In another embodiment, fusion of a heterologous antigen to any ΔLLO, including the PEST AA sequence, SEQ ID NO: 1, enhances cell mediated and anti-tumor immunity of the antigen.

[0054] It will be appreciated by the skilled artisan that the term "PEST sequence- peptide" or "PEST sequence-containing protein" may encompass a truncated LLO protein, which in one embodiment is a N-terminal LLO, and a truncated ActA protein which in one embodiment is an N-terminal LLO, or fragments thereof. It will also be appreciated by the skilled artisan that the term "PEST-sequence peptide" may encompass a PEST sequence peptide or peptide fragments of an LLO protein or an ActA protein thereof. PEST sequence peptides are known in the art and are described in US Patent Serial No. 7,635,479, and in US Patent Publication Serial No. 2014/0186387, both of which are hereby incorporated in their entirety herein.

[0055] In another embodiment, the a PEST sequence of prokaryotic organisms can be identified routinely in accordance with methods such as described by Rechsteiner and Roberts

(TBS 21:267-271,1996) for *L. monocytogenes*. Alternatively, PEST amino acid sequences from other prokaryotic organisms can also be identified based by this method. Other prokaryotic organisms wherein PEST amino acid sequences would be expected to include, but are not limited to, other *Listeria* species. For example, the *L. monocytogenes* protein ActA contains four such sequences. These are KTEEQPSEVNTGPR (SEQ ID NO: 5), KASVTDTSEGDLDSSMQSADESTPQPLK (SEQ ID NO: 6), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 7), and RGGIPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 8). Also Streptolysin O from *Streptococcus* sp. contain a PEST sequence. For example, *Streptococcus pyogenes* Streptolysin O comprises the PEST sequence KQNTASTETTTTNEQPK (SEQ ID NO: 9) at amino acids 35-51 and *Streptococcus equisimilis* Streptolysin O comprises the PEST-like sequence KQNTANTETTTTNEQPK (SEQ ID NO: 10) at amino acids 38-54. Further, it is believed that the PEST sequence can be embedded within the antigenic protein. Thus, for purposes of the disclosure herein, by "fusion" when in relation to PEST sequence fusions, it is meant that the antigenic protein comprises both the antigen and the PEST amino acid sequence either linked at one end of the antigen or embedded within the antigen.

[0056] In another embodiment, the construct or nucleic acid molecule is expressed from an episomal or plasmid vector, with a nucleic acid sequence encoding a truncated LLO, a truncated ActA or a PEST-sequence peptide. In another embodiment, the plasmid is stably maintained in the recombinant *Listeria* strain in the absence of antibiotic selection. In another embodiment, the plasmid does not confer antibiotic resistance upon the recombinant *Listeria*. In another embodiment, the fragment is a functional fragment. In another embodiment, the fragment is an immunogenic fragment.

[0057] The LLO protein utilized to construct vaccines of the disclosure has, in another embodiment, the sequence:

[0058] MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSMAPPASPPASPKTPIEK
KHADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQN
NADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIV
VKNATKSNVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAF
KAVNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALG
VNAENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNS
SFKAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVI
KNNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYDPEGNEIVQHKNWSENN
KSKLAHFTSSIYLPGNARNINVYAKECTGLAWEWWRTVIDDRNLPLVKNRNISIWGTT
LYPKYSNKVDNPIE (GenBank Accession No. P13128; SEQ ID NO: 2; nucleic acid

sequence is set forth in GenBank Accession No. X15127). The first 25 AA of the proprotein corresponding to this sequence are the signal sequence and are cleaved from LLO when it is secreted by the bacterium. Thus, in this embodiment, the full length active LLO protein is 504 residues long. In another embodiment, the above LLO fragment is used as the source of the LLO fragment incorporated in a immunotherapy of the disclosure. In another embodiment, the N-terminal fragment of an LLO protein utilized in compositions and methods of the disclosure has the sequence:

[0059] MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSVAPPASPPASPKTPIEKK HADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNN ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVV KNATKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIAKFGTAFK AVNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGV NAENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSS FKAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK NNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYD (SEQ ID NO: 3).

[0060] In one embodiment, the term "vaccine" and "immunotherapy" or their plural form, have the same meanings and qualifications for the purposes of the disclosure and are used interchangeably herein.

[0061] In another embodiment, the LLO fragment corresponds to about AA 20-442 of an LLO protein utilized herein.

[0062] In another embodiment, the LLO fragment has the sequence:

[0063] MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSVAPPASPPASPKTPIEKK HADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNN ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVV KNATKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIAKFGTAFK AVNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGV NAENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSS FKAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK NNSEYIETTSKAYTD (SEQ ID NO: 4).

[0064] In another embodiment, "truncated LLO" or "ΔLLO" refers to a fragment of LLO that comprises the PEST-like domain. In another embodiment, the terms refer to an LLO fragment that comprises a PEST sequence.

[0065] 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,

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. 8,771,702, which is incorporated by reference herein.

[0066] 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.

[0067] In another embodiment, the LLO fragment consists of about residues 1-25. In another embodiment, the LLO fragment consists of about residues 1-50. In another embodiment, the LLO fragment consists of about residues 1-75. In another embodiment, the LLO fragment consists of about residues 1-100. In another embodiment, the LLO fragment consists of about residues 1-125. In another embodiment, the LLO fragment consists of about residues 1-150. In another embodiment, the LLO fragment consists of about residues 1175. In another embodiment, the LLO fragment consists of about residues 1-200. In another embodiment, the LLO fragment consists of about residues 1-225. In another embodiment, the LLO fragment consists of about residues 1-250. In another embodiment, the LLO fragment consists of about residues 1-275. In another embodiment, the LLO fragment consists of about residues 1-300. In another embodiment, the LLO fragment consists of about residues 1-325. In another embodiment, the LLO fragment consists of about residues 1-350. In another embodiment, the LLO fragment consists of about residues 1-375. In another embodiment, the LLO fragment consists of about residues 1-400. In another embodiment, the LLO fragment consists of about residues 1-425. Each possibility represents a separate embodiment of the disclosure.

[0068] 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.

[0069] In another embodiment, a homologous LLO refers to identity to an LLO sequence disclosed herein of greater than 70%. In another embodiment, a homologous LLO refers to identity to an LLO sequence disclosed herein of greater than 72%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 75%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of

greater than 78%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 80%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 82%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 83%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 85%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 87%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 88%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 90%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 92%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 93%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 95%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 96%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 97%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 98%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of greater than 99%. In another embodiment, a homologous refers to identity to an LLO sequence disclosed herein of 100%. Each possibility represents a separate embodiment of the disclosure.

[0070] In one embodiment, an ActA protein comprises the sequence set forth in SEQ ID NO: 11:

[0071] MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSE VNTGPRYETAREVSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKGPNINNNNSEQTE NAAINEEASGADRPAIQVERRHPGLPSDSAAEIKKRRKAIASSDSELESLTYPDKPTKV NKKKVAKESVADASESDLDSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRD KIDENPEVKKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGF NAPATSEPSSFEFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTEDELEIIRET ASSLDSSFTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGGRPTSEEFSSLNSGDFTD DENSETTEEEIDRLADLRDRGTGKHSRNAGFLPLNPFASSPVPSLSPKVSKISDRALISDI TKKTPFKNPSQPLNVFNKKTTTKTVTKKPTPVKTAPKLAELPATKPQETVLRENKTPFI EKQAETNKQSINMPSLPVIQKEATESDKEEMKPQTEEKMVEESESANNANGKNRSAGI EEGKLIAKSAEDEKAKEEPGNHTTLILAMLAIGVFSLGAFIKIIQLRKNN. 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: 11 above. In another embodiment, an ActA polypeptide or peptide does not include the signal sequence, AA 1-29 of SEQ ID NO: 11 above.

[0072] In one embodiment, a truncated ActA protein comprises an N-terminal fragment of an ActA protein. In another embodiment, a truncated ActA protein is an N-terminal fragment of an Acta protein. In one embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 12:

[0073] MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSEVNTGPR YETAREVSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKGPNINNNNSEQTENAAINE EASGADRPAIQVERRHPGLPSDSAAEIKKRRKAIASSDSELESLTYPDKPTKVNKKKVA KESVADASESDLDSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKIDENPE VKKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSE PSSFEFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTEDELEIIRETASSLDSS FTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGGRP. In another embodiment, the ActA fragment comprises the sequence set forth in SEQ ID NO: 12.

[0074] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 13: MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEE EKTEEQPSEVNTGPRYETAREVSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKG.

[0075] In one embodiment, a truncated ActA protein comprises an N-terminal fragment of an ActA protein additionally lacking all or a portion of the ActA signal sequence, AA 1-29 of SEQ ID NO: 11 above. In another embodiment, a truncated ActA protein is an N-terminal fragment of an ActA protein additionally lacking all or a portion of the ActA signal sequence, AA 1-29 of SEQ ID NO: 11 above. In another embodiment a truncated ActA protein lacks AA 1-29, which is the ActA signal sequence of SEQ ID NO: 11 above. In another embodiment, a truncated ActA protein comprises at least one PEST sequence.

[0076] In one embodiment, the full length ActA protein comprises a PEST region, the sequence of which is set forth in SEQ ID NO: 14. In one embodiment, the fusion protein disclosed herein comprises SEQ ID NO: 14. In one embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 14:

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: 14). In one embodiment, a truncated ActA protein is the sequence set forth in SEQ ID NO: 14.

[0077] In one embodiment, a truncated ActA protein comprises one to four PEST sequences. In one embodiment, the full length ActA protein comprises a PEST region comprising one to

four PEST sequences, the sequence of which is set forth in SEQ ID NO: 15. In one embodiment, the fusion protein disclosed herein comprises SEQ ID NO: 15. In one embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 15:

[0078] 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: 15). In one embodiment, a truncated ActA protein is the sequence set forth in SEQ ID NO: 15.

[0079] In one embodiment, a truncated ActA protein comprises one to four PEST sequences. In one embodiment, the full length ActA protein comprises a PEST region comprising one to four PEST sequences, the sequence of which is set forth in SEQ ID NO: 16. In one embodiment, the fusion protein disclosed herein comprises SEQ ID NO: 16. In one embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 16:

[0080] 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 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 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: 16). In one embodiment, a truncated ActA protein is the sequence set forth in SEQ ID NO: 16.

[0081] In one embodiment, a truncated ActA protein comprises one to four PEST sequences. In one embodiment, the full length ActA protein comprises a PEST region comprising one to four PEST sequences, the sequence of which is set forth in SEQ ID NO: 17. In one embodiment, the fusion protein disclosed herein comprises SEQ ID NO: 17. In one embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 17:

[0082] 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 NO: 17). In one embodiment, a truncated ActA protein is the sequence set forth in SEQ ID NO: 17.

[0083] In another embodiment, the ActA fragment is any other ActA fragment known in the art. Each possibility represents a separate embodiment of the disclosure.

[0084] In another embodiment, the recombinant nucleotide encoding anActA protein comprises the sequence set forth in SEQ ID NO: 18:

[0085] Atgcgtgcgatgatggtggttttcattactgccaattgcattacgattaaccccgacataatatttgcagcgacagatagcgaa
gattctagtctaaacacagatgaatgggaagaagaaaaaacagaagagcaaccaagcgaggtaaatacgggaccaagatacgaaact
gcacgtgaagtaagttcacgtgatattaaagaactagaaaaatcgaataaagtgagaaatacgaacaaagcagacctaatagcaatgttga
aagaaaaagcagaaaaaggtccaaatatcaataataacaacagtgaacaaactgagaatgcggctataaatgaagaggcttcaggagcc
gaccgaccagctatacaagtggagcgtcgtcatccaggattgccatcggatagcgcagcggaaattaaaaaaagaaggaaagccatag
catcatcggatagtgagcttgaaagccttacttatccggataaaccaacaaaagtaaataagaaaaaagtggcgaaagagtcagttgcgg
atgcttctgaaagtgacttagattctagcatgcagtcagcagatgagtcttcaccacaacctttaaaagcaaaccaacaaccatttttccctaa
agtatttaaaaaaataaaagatgcggggaaatgggtacgtgataaaatcgacgaaaatcctgaagtaaagaaagcgattgttgataaaagt
gcagggttaattgaccaattattaaccaaaaagaaaagtgaagaggtaaatgcttcggacttcccgccaccacctacggatgaagagttaa
gacttgctttgccagagacaccaatgcttcttggttttaatgctcctgctacatcagaaccgagctcattcgaatttccaccaccacctacgga
tgaagagttaagacttgctttgccagagacgccaatgcttcttggttttaatgctcctgctacatcggaaccgagctcgttcgaatttccaccg
cctccaacagaagatgaactagaaatcatccgggaaacagcatcctcgctagattctagttttacaagaggggatttagctagtttgagaaa
tgctattaatcgccatagtcaaaatttctctgatttcccaccaatcccaacagaagaagagttgaacgggagaggcggtagacca. In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 18. In another embodiment, the recombinant nucleotide comprises any other sequence that encodes a fragment of an ActA protein.

[0086] In another embodiment, “truncated ActA” or “ΔActA” refers to a fragment of ActA that comprises the PEST-like domain. In another embodiment, the terms refer to an ActA fragment that comprises a PEST sequence.

[0087] In one embodiment LM PEST sequences and PEST sequences derived from other prokaryotic organisms will enhance immunogenicity of the antigen. In one embodiment, the

PEST sequence is a PEST sequence from the LM ActA protein. In another embodiment, the terms "PEST- sequence peptide," and "PEST sequence" are used interchangeably herein. In another embodiment, the PEST sequence is KTEEQPSEVNTGPR (SEQ ID NO: 19), KASVTDTSEGDLDSSMQSADESTPQPLK (SEQ ID NO: 20), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 21), or RGGIPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 22). In another embodiment, the PEST-like sequence is from Streptolysin O protein of *Streptococcus sp.* In another embodiment, the PEST-like sequence is from *Streptococcus pyogenes* Streptolysin O, e.g. KQNTASTETTTTNEQPK (SEQ ID NO: 23) at AA 35-51. In another embodiment, the PEST-like sequence is from *Streptococcus equisimilis* Streptolysin O, e.g. KQNTANTETTTTNEQPK (SEQ ID NO: 24) at AA 38-54. In another embodiment, the PEST-like sequence is another PEST AA sequence derived from a prokaryotic organism. In another embodiment, the PEST sequence is any other PEST sequence known in the art.

[0088] In another embodiment, the ActA fragment consists of about the first 100 AA of the ActA protein.

[0089] 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.

[0090] 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.

[0091] In another embodiment, a homologous ActA refers to identity to an ActA sequence disclosed herein of greater than 70%. In another embodiment, a homologous ActA refers to identity to an ActA sequence of greater than 72%. In another embodiment, a homologous refers to identity to an ActA sequence of greater than 75%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 78%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 80%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 82%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 83%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 85%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 87%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 88%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein greater than 90%. In another embodiment, a homologous

refers to identity to one of SEQ ID No: 11of greater than 92%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 93%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 95%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 96%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 97%. In another embodiment, a homologous refers to identity to an ActA sequence disclosed herein of greater than 98%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11of greater than 99%. In another embodiment, a homologous refers to identity to identity to an ActA sequence of 100%.

[0092] As used herein, the term "homology," when in reference to any nucleic acid sequence disclosed herein refers in one embodiment to a percentage of nucleotides in a candidate sequence that is identical with the nucleotides of a corresponding native nucleic acid sequence .

[0093] 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), LALIGN, GENPEPT and TREMBL packages.

[0094] In another embodiment, "homology" refers to identity to a sequence selected from the sequences disclosed herein of greater than 68%. In another embodiment, "homology" refers to identity to a sequence selected from the sequences disclosed herein of greater than 70%. In another embodiment, "homology" refers to identity to a sequence selected from the sequences disclosed herein of greater than 72%. In another embodiment, the identity is greater than 75%. In another embodiment, the identity is greater than 78%. In another embodiment, the identity is greater than 80%. In another embodiment, the identity is greater than 82%. In another embodiment, the identity is greater than 83%. In another embodiment, the identity is greater than 85%. In another embodiment, the identity is greater than 87%. In another embodiment, the identity is greater than 88%. In another embodiment, the identity is greater than 90%. In another embodiment, the identity is greater than 92%. In another embodiment, the identity is greater than 93%. In another embodiment, the identity is greater than 95%. In another embodiment, the identity is greater than 96%. In another embodiment, the identity is greater than 97%. In another embodiment, the identity is greater than 98%. In another embodiment, the identity is greater than 99%. In another embodiment, the identity is 100%.

[0095] 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 ºC 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 .

[0096] In one embodiment, the recombinant *Listeria* strain disclosed herein lacks antibiotic resistance genes. In another embodiment, the recombinant *Listeria* strain disclosed herein comprises a plasmid comprising a nucleic acid encoding an antibiotic resistance gene.

[0097] In one embodiment, the recombinant *Listeria* disclosed herein is capable of escaping the phagolysosome.

[0098] In one embodiment, the *Listeria* genome comprises a deletion of the endogenous ActA gene, which in one embodiment is a virulence factor. In one embodiment, the heterologous antigen or antigenic polypeptide is integrated in frame with LLO in the *Listeria* chromosome. In another embodiment, the integrated nucleic acid molecule is integrated in frame with ActA into the ActA locus. In another embodiment, the chromosomal nucleic acid encoding ActA is replaced by a nucleic acid molecule encoding an antigen.

[0099] In one embodiment, a heterologous antigen is a tumor-associated antigen. In another embodiment, the 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.

[00100] In one embodiment, the nucleic acid molecule disclosed herein comprises a first open reading frame encoding recombinant polypeptide comprising a heterologous antigen or fragment thereof. In another embodiment, the recombinant polypeptide further comprises a truncated LLO protein, a truncated ActA protein or PEST sequence peptide fused to the heterologous antigen. In another embodiment, the truncated LLO protein is a N-terminal LLO or fragment thereof. In another embodiment, the truncated ActA protein is a N-terminal ActA protein or fragment thereof.

[00101] In one embodiment, "antigenic polypeptide" is used herein to refer 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.

[00102] In one embodiment, the nucleic acid molecule disclosed herein further comprises a second open reading frame encoding a metabolic enzyme. In another embodiment, the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain. In another embodiment, the metabolic enzyme encoded by the second open reading frame is an alanine racemase enzyme (dal). In another embodiment, the metabolic enzyme encoded by the second open reading frame is a D-amino acid transferase enzyme (dat). In another embodiment, the *Listeria* strains disclosed herein comprise a mutation in the endogenous dal/dat genes. In another embodiment, the *Listeria* lacks the dal/dat genes.

[00103] In another embodiment, a nucleic acid molecule of the methods and compositions of the disclosure is operably linked to a promoter/regulatory sequence. In another embodiment, the first open reading frame of methods and compositions of the disclosure is operably linked to a promoter/regulatory sequence. In another embodiment, the second open reading frame of methods and compositions of the 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.

[00104] "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.

[00105] In another embodiment, the recombinant *Listeria* is an attenuated auxotrophic strain. In another embodiment, the recombinant *Listeria* is an Lm-LLO-E7 strain described in US Patent No. 8,114,414, which is incorporated by reference herein in its entirety.

[00106] In one embodiment the attenuated strain is Lm dal(-)dat(-) (*Lmdd*). In another embodiment, the attenuated strains is Lm dal(-)dat(-)ΔactA (*LmddA*). *LmddA* is based on a *Listeria* vector which is attenuated due to the deletion of virulence gene *actA* and retains the plasmid for a desired heterologous antigen or truncated LLO expression *in vivo* and *in vitro* by complementation of *dal* gene.

[00107] In another embodiment the attenuated strain is *LmddA*. In another embodiment, the attenuated strain is LmΔactA. In another embodiment, the attenuated strain is LmΔPrfA. In

another embodiment, the attenuated strain is LmΔPlcB. In another embodiment, the attenuated strain is LmΔPlcA. In another embodiment, the strain is the double mutant or triple mutant of any of the above-mentioned strains. In another embodiment, this strain exerts a strong adjuvant effect which is an inherent property of *Listeria*-based vaccines. In another embodiment, this strain is constructed from the EGD *Listeria* backbone. In another embodiment, the strain used in the disclosure is a *Listeria* strain that expresses a non-hemolytic LLO.

[00108] In another embodiment, the *Listeria* strain is an auxotrophic mutant. In another embodiment, the *Listeria* strain is deficient in a gene encoding a vitamin synthesis gene. In another embodiment, the *Listeria* strain is deficient in a gene encoding pantothenic acid synthase.

[00109] In one embodiment, the generation of AA strains of *Listeria* deficient in D-alanine, for example, may be accomplished in a number of ways that are well known to those of skill in the art, including deletion mutagenesis, insertion mutagenesis, and mutagenesis which results in the generation of frameshift mutations, mutations which cause premature termination of a protein, or mutation of regulatory sequences which affect gene expression. In another embodiment, mutagenesis can be accomplished using recombinant DNA techniques or using traditional mutagenesis technology using mutagenic chemicals or radiation and subsequent selection of mutants. In another embodiment, deletion mutants are preferred because of the accompanying low probability of reversion of the auxotrophic phenotype. In another embodiment, mutants of D-alanine which are generated according to the protocols presented herein may be tested for the ability to grow in the absence of D-alanine in a simple laboratory culture assay. In another embodiment, those mutants which are unable to grow in the absence of this compound are selected for further study.

[00110] In another embodiment, in addition to the aforementioned D-alanine associated genes, other genes involved in synthesis of a metabolic enzyme, as disclosed herein, may be used as targets for mutagenesis of *Listeria*.

[00111] In another embodiment, the metabolic enzyme complements an endogenous metabolic gene that is lacking in the remainder of the chromosome of the recombinant bacterial strain. In one embodiment, the endogenous metabolic gene is mutated in the chromosome. In another embodiment, the endogenous metabolic gene is deleted from the chromosome. In another embodiment, the metabolic enzyme is an amino acid metabolism 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. In another embodiment, the metabolic enzyme is an alanine racemase enzyme. In another embodiment, the metabolic enzyme is a D-amino acid transferase enzyme.

[00112] In one embodiment, the auxotrophic *Listeria* strain comprises an episomal expression vector comprising a metabolic enzyme that complements the auxotrophy of the auxotrophic *Listeria* strain. In another embodiment, the construct is contained in the *Listeria* strain in an episomal fashion. In another embodiment, the foreign antigen is expressed from a vector harbored by the recombinant *Listeria* strain. In another embodiment, the episomal expression vector lacks an antibiotic resistance marker. In one embodiment, an antigen of the methods and compositions as disclosed herein is fused to a polypeptide comprising a PEST sequence. In another embodiment, the polypeptide comprising a PEST sequence is a truncated LLO. In another embodiment, the polypeptide comprising a PEST sequence is ActA.

[00113] In another embodiment, the *Listeria* strain is deficient in an amino acid (AA) metabolism enzyme. In another embodiment, the *Listeria* strain is deficient in a D-glutamic acid synthase gene. In another embodiment, the *Listeria* strain is deficient in the *dat* gene. In another embodiment, the *Listeria* strain is deficient in the *dal* gene. In another embodiment, the *Listeria* strain is deficient in the *dga* gene. In another embodiment, the *Listeria* strain is deficient in a gene involved in the synthesis of diaminopimelic acid. *CysK*. In another embodiment, the gene is vitamin-B12 independent methionine synthase. In another embodiment, the gene is *trpA*. In another embodiment, the gene is *trpB*. In another embodiment, the gene is trpE. In another embodiment, the gene is *asnB*. In another embodiment, the gene is *gltD*. In another embodiment, the gene is *gltB*. In another embodiment, the gene is *leuA*. In another embodiment, the gene is *argG*. In another embodiment, the gene is *thrC*. In another embodiment, the *Listeria* strain is deficient in one or more of the genes described hereinabove.

[00114] In another embodiment, the *Listeria* strain is deficient in a synthase gene. In another embodiment, the gene is an AA synthesis gene. In another embodiment, the gene is *folP*. In another embodiment, the gene is dihydrouridine synthase family protein. In another embodiment, the gene is *ispD*. In another embodiment, the gene is *ispF*. In another embodiment, the gene is phosphoenolpyruvate synthase. In another embodiment, the gene is *hisF*. In another embodiment, the gene is *hisH*. In another embodiment, the gene is *fliI*. In another embodiment, the gene is ribosomal large subunit pseudouridine synthase. In another embodiment, the gene is *ispD*. In another embodiment, the gene is bifunctional GMP synthase/glutamine amidotransferase protein. In another embodiment, the gene is *cobS*. In another embodiment, the gene is *cobB*. In another embodiment, the gene is *cbiD*. In another embodiment, the gene is uroporphyrin-III C-methyltransferase/ uroporphyrinogen-III synthase. In another embodiment, the gene is *cobQ*. In another embodiment, the gene is *uppS*. In another embodiment, the gene is *truB*. In another embodiment, the gene is *dxs*. In another embodiment, the gene is *mvaS*. In another embodiment, the gene is *dapA*. In another embodiment, the gene is

ispG. In another embodiment, the gene is *folC*. In another embodiment, the gene is citrate synthase. In another embodiment, the gene is *argJ*. In another embodiment, the gene is 3-deoxy-7-phosphoheptulonate synthase. In another embodiment, the gene is indole-3-glycerol-phosphate synthase. In another embodiment, the gene is anthranilate synthase/ glutamine amidotransferase component. In another embodiment, the gene is *menB*. In another embodiment, the gene is menaquinone-specific isochorismate synthase. In another embodiment, the gene is phosphoribosylformylglycinamidine synthase I or II. In another embodiment, the gene is phosphoribosylaminoimidazole-succinocarboxamide synthase. In another embodiment, the gene is *carB*. In another embodiment, the gene is *carA*. In another embodiment, the gene is *thyA*. In another embodiment, the gene is *mgsA*. In another embodiment, the gene is *aroB*. In another embodiment, the gene is *hepB*. In another embodiment, the gene is *rluB*. In another embodiment, the gene is *ilvB*. In another embodiment, the gene is *ilvN*. In another embodiment, the gene is *alsS*. In another embodiment, the gene is *fabF*. In another embodiment, the gene is *fabH*. In another embodiment, the gene is pseudouridine synthase. In another embodiment, the gene is *pyrG*. In another embodiment, the gene is *truA*. In another embodiment, the gene is *pabB*. In another embodiment, the gene is an atp synthase gene (e.g. *atpC, atpD-2, aptG, atpA-2*, etc).

[00115] In another embodiment, the gene is *phoP*. In another embodiment, the gene is *aroA*. In another embodiment, the gene is *aroC*. In another embodiment, the gene is *aroD*. In another embodiment, the gene is *plcB*.

[00116] In another embodiment, the *Listeria* strain is deficient in a peptide transporter. In another embodiment, the gene is ABC transporter/ ATP-binding/permease protein. In another embodiment, the gene is oligopeptide ABC transporter/ oligopeptide-binding protein. In another embodiment, the gene is oligopeptide ABC transporter/ permease protein. In another embodiment, the gene is zinc ABC transporter/ zinc-binding protein. In another embodiment, the gene is sugar ABC transporter. In another embodiment, the gene is phosphate transporter. In another embodiment, the gene is ZIP zinc transporter. In another embodiment, the gene is drug resistance transporter of the EmrB/QacA family. In another embodiment, the gene is sulfate transporter. In another embodiment, the gene is proton-dependent oligopeptide transporter. In another embodiment, the gene is magnesium transporter. In another embodiment, the gene is formate/nitrite transporter. In another embodiment, the gene is spermidine/putrescine ABC transporter. In another embodiment, the gene is Na/Pi-cotransporter. In another embodiment, the gene is sugar phosphate transporter. In another embodiment, the gene is glutamine ABC transporter. In another embodiment, the gene is major facilitator family transporter. In another embodiment, the gene is glycine betaine/L-proline ABC transporter. In another embodiment,

the gene is molybdenum ABC transporter. In another embodiment, the gene is techoic acid ABC transporter. In another embodiment, the gene is cobalt ABC transporter. In another embodiment, the gene is ammonium transporter. In another embodiment, the gene is amino acid ABC transporter. In another embodiment, the gene is cell division ABC transporter. In another embodiment, the gene is manganese ABC transporter. In another embodiment, the gene is iron compound ABC transporter. In another embodiment, the gene is maltose/maltodextrin ABC transporter. In another embodiment, the gene is drug resistance transporter of the Bcr/CflA family. In another embodiment, the gene is a subunit of one of the above proteins.

[00117] In one embodiment, disclosed herein is a nucleic acid molecule that is used to transform the *Listeria* in order to arrive at a recombinant *Listeria*. In another embodiment, the nucleic acid disclosed herein used to transform a *Listeria* strain lacks a virulence gene. In another embodiment, the nucleic acid molecule is integrated into the *Listeria* genome and carries a non-functional virulence gene. In another embodiment, the virulence gene is mutated in the recombinant *Listeria*. In yet another embodiment, the nucleic acid molecule is used to inactivate the endogenous gene present in the *Listeria* genome. In yet another embodiment, the virulence gene is an *actA* gene, an *inlA* gene, and *inlB* gene, an *inlC* gene, inlJ gene, a *plbC* gene, a *bsh* gene, or a *prfA* gene. It is to be understood by a skilled artisan, that the virulence gene can be any gene known in the art to be associated with virulence in the recombinant *Listeria*.

[00118] In yet another embodiment the *Listeria* strain is an inlA mutant, an inlB mutant, an inlC mutant, an inlJ mutant, prfA mutant, ActA mutant, a dal/dat mutant, a prfA mutant, a plcB deletion mutant, or a double mutant lacking both plcA and plcB. In another embodiment, the *Listeria* comprise a deletion or mutation of these genes individually or in combination. In another embodiment, the *Listeria* disclosed herein lack each ne of genes. In another embodiment, the *Listeria* disclosed herein lack at least one and up to ten of any gene disclosed herein, including the *actA*, *prfA*, and *dal/dat* genes. In another embodiment, the prfA mutant is a D133V prfA mutant.

[00119] In one embodiment, the live attenuated *Listeria* is a recombinant *Listeria*. In another embodiment, the recombinant *Listeria* comprises a mutation or a deletion of a genomic *internalin C* (*inlC*) gene. In another embodiment, the recombinant *Listeria* comprises a mutation or a deletion of a genomic *actA* gene and a genomic *internalin C* gene. In one embodiment, translocation of *Listeria* to adjacent cells is inhibited by the deletion of the *actA* gene and/or the *inlC* gene, which are involved in the process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a vaccine backbone.

[00120] In one embodiment, the metabolic gene, the virulence gene, etc. is lacking in a

chromosome of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the chromosome and in any episomal genetic element of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the genome of the virulence strain. In one embodiment, the virulence gene is mutated in the chromosome. In another embodiment, the virulence gene is deleted from the chromosome.

[00121] 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. In another embodiment, the recombinant *Listeria* lacks the *inlB* gene. In another embodiment, the recombinant *Listeria* lacks both, the *actA* and *inlB* genes. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *actA* gene. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *inlB* gene. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *inlC* gene. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *actA* and *inlB* genes. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *actA* and *inlC* genes. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *actA*, *inlB*, and *inlC* genes. In another embodiment, the recombinant *Listeria* strain disclosed herein comprise an inactivating mutation of the endogenous *actA*, *inlB*, and *inlC* genes. In another embodiment, the recombinant *Listeria strain* disclosed herein comprise an inactivating mutation in any single gene or combination of the following genes: *actA, dal, dat, inlB, inlC, prfA, plcA, plcB*.

[00122] It will be appreciated by the skilled artisan that the term "mutation" and grammatical equivalents thereof, include any type of mutation or modification to the sequence (nucleic acid or amino acid sequence), and includes a deletion mutation, a truncation, an inactivation, a disruption, a replacement mutation, or a translocation. These types of mutations are readily known in the art.

[00123] In one embodiment, in order to select for an auxotrophic bacteria comprising a plasmid encoding a metabolic enzyme or a complementing gene disclosed herein, transformed auxotrophic bacteria are grown on a media that will select for expression of the amino acid metabolism gene or the complementing gene. In another embodiment, a bacteria auxotrophic for D-glutamic acid synthesis is transformed with a plasmid comprising a gene for D-glutamic

acid synthesis, and the auxotrophic bacteria will grow in the absence of D-glutamic acid, whereas auxotrophic bacteria that have not been transformed with the plasmid, or are not expressing the plasmid encoding a protein for D-glutamic acid synthesis, will not grow. In another embodiment, a bacterium auxotrophic for D-alanine synthesis will grow in the absence of D-alanine when transformed and expressing the plasmid of the 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).

[00124] In another embodiment, once the auxotrophic bacteria comprising the plasmid of the 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* vector by adjusting the volume of the media in which the auxotrophic bacteria comprising the plasmid are growing.

[00125] The skilled artisan will appreciate that, in another embodiment, other auxotroph strains and complementation systems are adopted for the use with this disclosure.

[00126] In one embodiment, the N-terminal LLO protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the genes encoding the N-terminal LLO protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the N-terminal LLO protein fragment and heterologous antigen are operably attached via a linker peptide. In another embodiment, the N-terminal LLO protein fragment and heterologous antigen are attached via a heterologous peptide. In another embodiment, the N-terminal LLO protein fragment is N-terminal to the heterologous antigen. In another embodiment, the N-terminal LLO protein fragment is expressed and used alone, i.e., in unfused form. In another embodiment, the N-terminal LLO protein fragment is the N-terminal-most portion of the fusion protein. In another embodiment, a truncated LLO is truncated at the C-terminal to arrive at an N-terminal LLO.

[00127] In one embodiment, the N-terminal ActA protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the genes encoding the N-terminal ActA protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the N-terminal ActA protein fragment and heterologous antigen are operably

attached via a linker peptide. In another embodiment, the N-terminal ActA protein fragment and heterologous antigen are attached via a heterologous peptide. In another embodiment, the N-terminal ActA protein fragment is N-terminal to the heterologous antigen. In another embodiment, the N-terminal ActA protein fragment is expressed and used alone, i.e., in unfused form. In another embodiment, the N-terminal ActA protein fragment is the N-terminal-most portion of the fusion protein. In another embodiment, a truncated ActA is truncated at the C-terminal to arrive at an N-terminal ActA.

[00128] As disclosed herein, there was an unexpected change in the suppressive ability of the granulocytic MDSC and this is due to the overexpression of tLLO and is independent of the partnering fusion antigen (see Example 13).

[00129] In one embodiment, the recombinant *Listeria* strain disclosed herein expresses the recombinant polypeptide. In another embodiment, the recombinant *Listeria* strain comprises a plasmid that encodes the recombinant polypeptide. In another embodiment, a recombinant nucleic acid disclosed herein is in a plasmid in the recombinant *Listeria* strain disclosed herein. In another embodiment, the plasmid is an episomal plasmid that does not integrate into the recombinant *Listeria* strain's chromosome. In another embodiment, the plasmid is an integrative plasmid that integrates into the *Listeria* strain's chromosome. In another embodiment, the plasmid is a multicopy plasmid.

[00130] In one embodiment, the heterologous antigen is a tumor-associated antigen. In one embodiment, the recombinant *Listeria* strain of the compositions and methods as disclosed herein express a heterologous antigenic polypeptide that is expressed by a tumor cell. 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. In still another embodiment, a tumor-associated antigen is an angiogenic antigen.

[00131] In one embodiment, the recombinant *Listeria* strain of the compositions and methods as disclosed herein comprise a first or second nucleic acid molecule that encodes a Prostate Specific Antigen (PSA), which in one embodiment, is a marker for prostate cancer that is highly expressed by prostate tumors. In one embodiment, PSA is a kallikrein serine protease (KLK3) secreted by prostatic epithelial cells, which in one embodiment, is widely used as a marker for prostate cancer. As used herein, the terms PSA and KLK3 are interchangeable having all the same meanings and qualities.

[00132] In one embodiment, the recombinant *Listeria* strain as disclosed herein comprises a nucleic acid molecule encoding a tumor associated antigen. In one embodiment, a tumor

associated antigen comprises an KLK3 polypeptide or a fragment thereof. In one embodiment, the recombinant *Listeria* strain as disclosed herein comprises a nucleic acid molecule encoding KLK3 protein.

[00133] In another embodiment, the KLK3 protein has the sequence:

[00134] MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG GVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNR FLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLT PKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVL QGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 25; GenBank Accession No. CAA32915). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 25. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 25. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 25. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 25.

[00135] In another embodiment, the KLK3 protein has the sequence:

[00136] IVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWVLTAAHCIRNKSVILL GRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSHDLMLLRLSEPAELT DAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQ KVTKFMLCAGRWTGGKSTCSGDSGGPLVCYGVLQGITSWGSEPCALPERPSLYTKVV HYRKWIKDTIVANP (SEQ ID No: 26). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 26. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 26. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 26. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 26. Each possibility represents a separate embodiment of the methods and compositions as disclosed herein.

[00137] In another embodiment, the KLK3 protein has the sequence: IVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWVLTAAHCIRNKSVILLGRHSL FHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKV MDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKF MLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRK WIKDTIVANP (SEQ ID No: 27; GenBank Accession No. AAA59995.1). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 27. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 27. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 27. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 27.

[00138] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

ggtgtcttaggcacactggtcttggagtgcaaaggatctaggcacgtgaggctttgtatgaagaatcggggatcgtacccacccccctgtttc
tgtttcatcctgggcatgtctcctctgcctttgtcccctagatgaagtctccatgagctacaagggcctggtgcatccagggtgatctagtaatt
gcagaacagcaagtgctagctctccctcccctccacagctctgggtgtgggaggggttgtccagcctccagcagcatggggagggc
cttggtcagcctctgggtgccagcagggcaggggcggagtcctggggaatgaaggttttatagggctcctgggggaggctccccagcc
ccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcaccctgtccgtgacgtggattggtgagagg
ggccatggttgggggatgcaggagagggagccagccctgactgtcaagctgaggctctttcccccccaacccagcaccccagccca
gacagggagctgggctcttttctgtctctcccagccccacttcaagcccatacccccagtcccctccatattgcaacagtcctcactcccac
accaggtccccgctccctcccacttaccccagaactttcttcccatttgcccagccagctccctgctcccagctgctttactaaaggggaagt
tcctgggcatctccgtgtttctctttgtggggctcaaaacctccaaggacctctctcaatgccattggttccttggaccgtatcactggtccatc
tcctgagcccctcaatcctatcacagtctactgacttttcccattcagctgtgagtgtccaaccctatcccagagaccttgatgcttggcctccc
aatcttgccctaggatacccagatgccaaccagacacctccttctttcctagccaggctatctggcctgagacaacaaatgggtccctcagt
ctggcaatgggactctgagaactcctcattccctgactcttagccccagactcttcattcagtggcccacattttccttaggaaaaacatgagc
atccccagccacaactgccagctctctgagtccccaaatctgcatccttttcaaaacctaaaaacaaaaagaaaaacaaataaaacaaaac
caactcagaccagaactgttttctcaacctgggacttcctaaactttccaaaaccttcctcttccagcaactgaacctcgccataaggcactta
tccctggttcctagcacccctttatcccctcagaatccacaacttgtaccaagtttcccttctcccagtccaagaccccaaatcaccacaaagg
acccaatccccagactcaagatatggtctgggcgctgtcttgtgtctcctaccctgatccctgggttcaactctgctcccagagcatgaagc
ctctccaccagcaccagccaccaacctgcaaacctagggaagattgacagaattcccagcctttcccagctccccctgcccatgtcccag
gactcccagccttggttctctgccccccgtgtcttttcaaacccacatcctaaatccatctcctatccgagtcccccagttcccccctgtcaaccct
gattcccctgatctagcacccccctctgcaggcgctgcgcccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattccc
aaccctggcaggtgcttgtggcctctcgtggcagggcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccact
gcatcaggaagtgagtaggggcctggggtctggggagcaggtgtctgtgtcccagaggaataacagctgggcattttccccaggataac
ctctaaggccagccttgggactgggggagagagggaaagttctggttcaggtcacatggggaggcagggttggggctggaccaccctc
cccatggctgcctgggtctccatctgtgtccctctatgtctctttgtgtcgctttcattatgtctcttggtaactggcttcggttgtgtctctccgtgt
gactattttgttctctctctccctctcttctctgtcttcagtctccatatctcccccctctctctgtccttctctggtccctctctagccagtgtgtctcac
cctgtatctctctgccaggctctgtctctcggtctctgtctcacctgtgccttctccctactgaacacacgcacgggatgggcctgggggacc
ctgagaaaaggaagggctttggctgggcgcggtggctcacacctgtaatcccagcactttgggaggccaaggcaggtagatcacctga
ggtcaggagttcgagaccagcctggccaactggtgaaaccccatctctactaaaaatacaaaaaattagccaggcgtggtggcgcatgc
ctgtagtcccagctactcaggagctgagggaggagaattgcattgaacctggaggttgaggttgcagtgagccgagaccgtgccactgc
actccagcctgggtgacagagtgagactccgcctcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaagaaaagaaaagaaaagaaaag
gaagtgttttatccctgatgtgtgtgggtatgagggtatgagagggcccctctcactccattccttctccaggacatccctccactcttgggag
acacagagaagggctggttccagctggagctgggaggggcaattgagggaggaggaaggagaaggggggaaggaaaacagggtat
gggggaaaggaccctggggagcgaagtggaggatacaaccttgggcctgcaggcaggctacctacccacttggaaacccacgccaa
agccgcatctacagctgagccactctgaggcctcccctccccggcggtccccactcagctccaaagtctctctccctttttctctcccacactt
tatcatcccccggattcctctctacttggttctcattcttcctttgacttcctgcttcccttttctcattcatctgtttctcactttctgcctggttttgttctt
ctctctctctttctctggcccatgtctgtttctctatgtttctgtcttttctttctcatcctgtgtattttcggctcaccttgtttgtcactgttctccccctct

gcccttttcattctctctgccctttttaccctcttccttttcccttggttctctcagttctgtatctgcccttcaccctctcacactgctgtttcccaactcg
ttgtctgtattttggcctgaactgtgtcttcccaaccctgtgttttctcactgtttctttttctcttttggagcctcctccttgctcctctgtcccttctctc
tttccttatcatcctcgctcctcattcctgcgtctgcttcctccccagcaaaagcgtgatcttgctgggtcggcacagcctgtttcatcctgaag
acacaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcctgaagaatcgattcctcaggccaggtg
atgactccagccacgacctcatgctgctccgcctgtcagagcctgccgagctcacggatgctgtgaaggtcatggacctgcccacccag
gagccagcactggggaccacctgctacgcctcaggctggggcagcattgaaccagaggagtgtacgcctgggccagatggtgcagcc
gggagcccagatgcctgggtctgagggaggaggggacaggactcctgggtctgagggaggagggccaaggaaccaggtggggtcc
agcccacaacagtgtttttgcctggcccgtagtcttgaccccaaagaaacttcagtgtgtggacctccatgttatttccaatgacgtgtgtgcg
caagttcaccctcagaaggtgaccaagttcatgctgtgtgctggacgctggacaggggggcaaaagcacctgctcggtgagtcatccctac
tcccaagatcttgagggaaaggtgagtgggaccttaattctgggctggggtctagaagccaacaaggcgtctgcctcccctgctccccag
ctgtagccatgccacctccccgtgtctcatctcattcctccttccctcttctttgactccctcaaggcaataggttattcttacagcacaactcat
ctgttcctgcgttcagcacacggttactaggcacctgctatgcacccagcactgccctagagcctgggacatagcagtgaacagacagag
agcagcccctcccttctgtagcccccaagccagtgaggggcacaggcaggaacagggaccacaacacagaaaagctggagggtgtc
aggaggtgatcaggctctcggggagggagaaggggtggggagtgtgactgggaggagacatcctgcagaaggtgggagtgagcaa
acacctgcgcaggggaggggagggcctgcggcacctgggggagcagagggaacagcatctggccaggcctgggaggaggggcct
agagggcgtcaggagcagagaggaggttgcctggctggagtgaaggatcggggcagggtgcgagagggaacaaaggacccctcct
gcagggcctcacctgggccacaggaggacactgcttttcctctgaggagtcaggaactgtggatggtgctggacagaagcaggacagg
gcctggctcaggtgtccagaggctgcgctggcctcctatgggatcagactgcagggagggagggcagcagggatgtggagggagtg
atgatggggctgacctgggggtggctccaggcattgtccccacctgggcccttacccagcctccctcacaggctcctggccctcagtctct
cccctccactccattctccacctacccacagtgggtcattctgatcaccgaactgaccatgccagccctgccgatggtcctccatggctccc
tagtgccctggagaggaggtgtctagtcagagagtagtcctggaaggtggcctctgtgaggagccacggggacagcatcctgcagatg
gtcctggcccttgtcccaccgacctgtctacaaggactgtcctcgtggaccctcccctctgcacaggagctggaccctgaagtcccttccta
ccggccaggactggagcccctacccctctgttggaatccctgcccaccttcttctggaagtcggctctggagacatttctctcttcttccaaa
gctgggaactgctatctgttatctgcctgtccaggtctgaaagataggattgcccaggcagaaactgggactgacctatctcactctctccct
gcttttacccttagggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatcacgtcatggggcagtgaaccatgtgccctgc
ccgaaaggccttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacaccatcgtggccaacccctgagcacccctatca
agtccctattgtagtaaacttggaaccttggaaatgaccaggccaagactcaagcctccccagttctactgacctttgtccttaggtgtgagg
tccagggttgctaggaaaagaaatcagcagacacaggtgtagaccagagtgtttcttaaatggtgtaattttgtcctctctgtgtcctgggga
atactggccatgcctggagacatatcactcaatttctctgaggacacagttaggatggggtgtctgtgttatttgtgggatacagagatgaaa
gaggggtgggatcc (SEQ ID No: 28; GenBank Accession No. X14810). In another embodiment, the KLK3 protein is encoded by residues 401..446, 1688..1847, 3477..3763, 3907..4043, and 5413..5568 of SEQ ID No: 28. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 28. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 28. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 28. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ

ID No: 28.

[00139] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQ CVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSWVILITELTMPALPMVLH GSLVPWRGGV (SEQ ID No: 29; GenBank Accession No. NP_001025218) In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 29. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 29. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 29. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 29.

[00140] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

agccccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcaccctgtccgtgacgtggattggtgct gcacccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtggcagg gcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcatcaggaacaaaagcgtgatcttgctgggtcgg cacagcctgtttcatcctgaagacacaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcctgaaga atcgattcctcaggccaggtgatgactccagccacgacctcatgctgctccgcctgtcagagcctgccgagctcacggatgctgtgaagg tcatggacctgcccacccaggagccagcactggggaccacctgctacgcctcaggctggggcagcattgaaccagaggagttcttgac cccaaagaaacttcagtgtgtggacctccatgttatttccaatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcatgctgtg tgctggacgctggacaggggcaaaagcacctgctcgtgggtcattctgatcaccgaactgaccatgccagccctgccgatggtcctcc atggctccctagtgccctggagaggaggtgtctagtcagagagtagtcctggaaggtggcctctgtgaggagccacggggacagcatc ctgcagatggtcctggcccttgtcccaccgacctgtctacaaggactgtcctcgtggaccctcccctctgcacaggagctggaccctgaa gtcccttccccaccggccaggactggagcccctacccctctgttggaatccctgcccaccttcttctggaagtcggctctggagacatttct ctcttcttccaaagctgggaactgctatctgttatctgcctgtccaggtctgaaagataggattgcccaggcagaaactgggactgacctatc tcactctctccctgcttttacccttagggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatcacgtcatggggcagtgaac catgtgccctgcccgaaaggccttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacaccatcgtggccaaccccctga gcacccctatcaaccccctattgtagtaaacttggaaccttggaaatgaccaggccaagactcaagcctccccagttctactgacctttgtcc ttaggtgtgaggtccagggttgctaggaaaagaaatcagcagacacaggtgtagaccagagtgtttcttaaatggtgtaattttgtcctctct gtgtcctggggaatactggccatgcctggagacatatcactcaatttctctgaggacacagataggatggggtgtctgtgttatttgtggggt acagagatgaaagaggggtgggatccacactgagagagtggagagtgacatgtgctggacactgtccatgaagcactgagcagaagc tggaggcacaacgcaccagacactcacagcaaggatggagctgaaaacataacccactctgtcctggaggcactgggaagcctagag aaggctgtgagccaaggagggagggtcttcctttggcatgggatggggatgaagtaaggagagggactggacccccctggaagctgatt cactatgggggaggtgtattgaagtcctccagacaaccctcagatttgatgatttcctagtagaactcacagaaataaagagctgttatact gtg (SEQ ID No: 30; GenBank Accession No. NM_001030047). In another embodiment, the

KLK3 protein is encoded by residues 42-758 of SEQ ID No: 30. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 30. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 30. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 30. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 30.

[00141] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRK (SEQ ID No: 31; GenBank Accession No. NP_001025221). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 31. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 31. In another embodiment, the sequence of the KLK3 protein comprises SEQ ID No: 31. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 31. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 31.

[00142] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

agccccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcacccttccgtgacgtggattggtgctg
caccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtggcaggg
cagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcatcaggaagtgagtaggggcctggggtctgggg
agcaggtgtctgtgtcccagaggaataacagctgggcattttccccaggataacctctaaggccagccttgggactgggggagagaggg
aaagttctggttcaggtcacatggggaggcagggttggggctggaccaccctccccatggctgcctgggtctccatctgtgttcctctatgt
ctctttgtgtcgctttcattatgtctcttggtaactggcttcggttgtgtctctccgtgtgactattttgttctctctctccctctcttctctgtcttcagt

(SEQ ID No: 32). In another embodiment, the KLK3 protein is encoded by residues 42-758 of SEQ ID No: 32. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 32. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 32. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 32. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 32.

[00143] In another embodiment, the KLK3 protein that is the source of the KLK3 peptide has the sequence:

MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGD SGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 33). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 33. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 33. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 33. In another embodiment, the KLK3 protein is a

fragment of SEQ ID No: 33.

[00144] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

agccccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcaccctgtccgtgacgtggattggtgct
gcacccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtggcagg
gcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcatcaggaacaaaagcgtgatcttgctgggtcgg
cacagcctgtttcatcctgaagacacaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcctgaaga
atcgattcctcaggccaggtgatgactccagcattgaaccagaggagttcttgaccccaaagaaacttcagtgtgtggacctccatgttattt
ccaatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcatgctgtgtgctggacgctggacaggggcaaaagcacctgc
tcgggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatcacgtcatggggcagtgaaccatgtgccctgcccgaaaggc
cttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacaccatcgtggccaacccctgagcacccctatcaacccccctattg
tagtaaacttggaaccttggaaatgaccaggccaagactcaagcctccccagttctactgacctttgtccttaggtgtgaggtccagggttg
ctaggaaaagaaatcagcagacacaggtgtagaccagagtgtttcttaaatggtgtaattttgtcctctctgtgtcctggggaatactggcca
tgcctggagacatatcactcaatttctctgaggacacagataggatggggtgtctgtgttatttgtggggtacagagatgaaagaggggtgg
gatccacactgagagagtggagagtgacatgtgctggacactgtccatgaagcactgagcagaagctggaggcacaacgcaccagac
actcacagcaaggatggagctgaaaacataacccactctgtcctggaggcactgggaagcctagagaaggctgtgagccaaggaggg
agggtcttcctttggcatgggatggggatgaagtaaggagagggactggacccccctggaagctgattcactatgggggggaggtgtattga
agtcctccagacaaccctcagatttgatgatttcctagtagaactcacagaaataaagagctgttatactgtg (SEQ ID No: 34). In another embodiment, the KLK3 protein is encoded by residues 42-758 of SEQ ID No: 34. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 34. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 34. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 34. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 34.

[00145] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRKPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASG WGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDS GGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 35; GenBank Accession No. NP_001025219). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 35. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 35. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 35. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 35.

[00146] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

agccccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcaccctgtccgtgacgtggattggtgct

gcacccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtggcagg
gcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcatcaggaagccaggtgatgactccagccacga
cctcatgctgctccgcctgtcagagcctgccgagctcacggatgctgtgaaggtcatggacctgcccacccaggagccagcactgggg
accacctgctacgcctcaggctggggcagcattgaaccagaggagttcttgaccccaaagaaacttcagtgtgtggacctccatgttatttc
caatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcatgctgtgtgctggacgctggacaggggcaaaagcacctgct
cgggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatcacgtcatggggcagtgaaccatgtgccctgcccgaaaggcc
ttccctgtacaccaaggtggtgcattacccaaggacaccatcgtggccaacccctgagcacccctatcaacccccctattgtagtaaacttgg
aaccttggaaatgaccaggccaagactcaagcctccccagttctactgacctttgtccttaggtgtgaggtccagggttgctaggaaaaga
aatcagcagacacaggtgtagaccagagtgtttcttaaatggtgtaattttgtcctctctgtgtcctggggaatactggccatgcctggagac
atatcactcaatttctctgaggacacagataggatggggtgtctgtgttatttgtggggtacagagatgaaagaggggtgggatccacactg
agagagtggagagtgacatgtgctggacactgtccatgaagcactgagcagaagctggaggcacaacgcaccagacactcacagcaa
ggatggagctgaaaacataacccactctgtcctggaggcactgggaagcctagagaaggctgtgagccaaggagggagggtcttccttt
ggcatgggatggggatgaagtaaggagagggactggacccccctggaagctgattcactatgggggggaggtgtattgaagtcctccaga
caaccctcagatttgatgatttcctagtagaactcacagaaataaagagctgttatactgtg (SEQ ID No: 36; GenBank Accession No. NM_001030048). In another embodiment, the KLK3 protein is encoded by residues 42-758 of SEQ ID No: 36. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 36. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 36. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 36. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 36.

[00147] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQ CVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITS WGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 37; GenBank Accession No. NP_001639). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 37. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 37. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 37. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 37.

[00148] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

agccccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcaccctgtccgtgacgtggattggtgct
gcacccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtggcagg
gcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcatcaggaacaaaagcgtgatcttgctgggtcgg

cacagcctgtttcatcctgaagacacaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcctgaaga
atcgattcctcaggccaggtgatgactccagccacgacctcatgctgctccgcctgtcagagcctgccgagctcacggatgctgtgaagg
tcatggacctgcccacccaggagccagcactggggaccacctgctacgcctcaggctggggcagcattgaaccagaggagttcttgac
cccaaagaaacttcagtgtgtggacctccatgttatttccaatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcatgctgtg
tgctggacgctggacaggggggcaaaagcacctgctcgggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatcacgtca
tggggcagtgaaccatgtgccctgcccgaaaggccttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacaccatcgt
ggccaacccctgagcacccctatcaacccccctattgtagtaaacttggaaccttggaaatgaccaggccaagactcaagcctccccagttc
tactgacctttgtccttaggtgtgaggtccagggttgctaggaaaagaaatcagcagacacaggtgtagaccagagtgtttcttaaatggtgt
aattttgtcctctctgtgtcctggggaatactggccatgcctggagacatatcactcaatttctctgaggacacagataggatggggtgtctgt
gttatttgtggggtacagagatgaaagaggggtgggatccacactgagagagtggagagtgacatgtgctggacactgtccatgaagca
ctgagcagaagctggaggcacaacgcaccagacactcacagcaaggatggagctgaaaacataacccactctgtcctggaggcactg
ggaagcctagagaaggctgtgagccaaggagggagggtcttcctttggcatgggatggggatgaagtaaggagagggactggacccc
ctggaagctgattcactatgggggggaggtgtattgaagtcctccagacaaccctcagatttgatgatttcctagtagaactcacagaaataaa
gagctgttatactgtg (SEQ ID No: 38; GenBank Accession No. NM_001648). In another embodiment, the KLK3 protein is encoded by residues 42-827 of SEQ ID No: 38. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 38. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 38. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 38. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 38.

[00149] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQ CVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITS WGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 39 GenBank Accession No. AAX29407.1). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 39. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 39. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 39. In another embodiment, the sequence of the KLK3 protein comprises SEQ ID No: 39. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 39.

[00150] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

gggggagccccaagcttaccacctgcacccggagagctgtgtcaccatgtgggtcccggttgtcttcctcaccctgtccgtgacgtggatt
ggtgctgcacccctcatcctgtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtg
gcagggcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcatcaggaacaaaagcgtgatcttgctg

ggtcggcacagcctgtttcatcctgaagacacaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcc
tgaagaatcgattcctcaggccaggtgatgactccagccacgacctcatgctgctccgcctgtcagagcctgccgagctcacggatgctg
tgaaggtcatggacctgcccacccaggagccagcactggggaccacctgctacgcctcaggctggggcagcattgaaccagaggagtt
cttgaccccaaagaaacttcagtgtgtggacctccatgttatttccaatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcat
gctgtgtgctggacgctggacaggggcaaaagcacctgctcgggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatc
acgtcatggggcagtgaaccatgtgccctgcccgaaaggccttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacac
catcgtggccaacccctgagcacccctatcaactccctattgtagtaaacttggaaccttggaaatgaccaggccaagactcaggcctccc
cagttctactgacctttgtccttaggtgtgaggtccagggttgctaggaaaagaaatcagcagacacaggtgtagaccagagtgtttcttaaa
tggtgtaattttgtcctctctgtgtcctggggaatactggccatgcctggagacatatcactcaatttctctgaggacacagataggatggggt
gtctgtgttatttgtggggtacagagatgaaagaggggtgggatccacactgagagagtggagagtgacatgtgctggacactgtccatg
aagcactgagcagaagctggaggcacaacgcaccagacactcacagcaaggatggagctgaaaacataacccactctgtcctggagg
cactgggaagcctagagaaggctgtgagccaaggagggagggtcttcctttggcatgggatggggatgaagtagggagagggactgg
acccccctggaagctgattcactatgggggaggtgtattgaagtcctccagacaaccctcagatttgatgatttcctagtagaactcacaga
aataaagagctgttatactgcgaaaaaaaaaaaaaaaaaaaaaaaaa (SEQ ID No: 40; GenBank Accession No. BC056665). In another embodiment, the KLK3 protein is encoded by residues 47-832 of SEQ ID No: 40. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 40. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 40. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 40. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 40.

[00151] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGD SGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVA (SEQ ID No: 41; GenBank Accession No. AJ459782). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 41. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 41. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 41. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 41.

[00152] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQ CVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSVSHPYSQDLEGKGEWGP (SEQ ID No: 42, GenBank Accession No. AJ512346). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 42. In another embodiment, the KLK3 protein is a

variant of SEQ ID No: 42. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 42. In another embodiment, the sequence of the KLK3 protein comprises SEQ ID No: 42. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 42.

[00153] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGERGHGWGDAGEGASPDCQAEALSPPTQHPSPDRELGSFLSL PAPLQAHTPSPSILQQSSLPHQVPAPSHLPQNFLPIAQPAPCSQLLY (SEQ ID No: 43; GenBank Accession No. AJ459784). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 43. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 43. In another embodiment, the sequence of the KLK3 protein comprises SEQ ID No: 43. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 43. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 43.

[00154] In another embodiment, the KLK3 protein has the sequence: MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVH PQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQ CVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITS WGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID NO: 44 GenBank Accession No. AJ459783). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 44. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 44. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 44. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 44.

[00155] In another embodiment, the KLK3 protein is encoded by a nucleotide molecule having the sequence:

aagtttcccttctcccagtccaagaccccaaatcaccacaaaggacccaatccccagactcaagatatggtctgggcgctgtcttgtgtctc
ctaccctgatccctgggttcaactctgctcccagagcatgaagcctctccaccagcaccagccaccaacctgcaaacctagggaagattg
acagaattcccagcctttcccagctccccctgcccatgtcccaggactcccagccttggttctctgcccccgtgtcttttcaaacccacatcct
aaatccatctcctatccgagtcccccagttcctcctgtcaaccctgattcccctgatctagcacccccctctgcaggtgctgcacccctcatcct
gtctcggattgtgggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtagcctctcgtggcagggcagtctgcggcg
gtgttctggtgcacccccagtgggtcctcacagctacccactgcatcaggaacaaaagcgtgatcttgctgggtcggcacagcctgtttca
tcctgaagacacaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcctgaagaatcgattcctcagg
ccaggtgatgactccagccacgacctcatgctgctccgcctgtcagagcctgccgagctcacggatgctatgaaggtcatggacctgccc
acccaggagccagcactggggaccacctgctacgcctcaggctggggcagcattgaaccagaggagttcttgaccccaaagaaacttc
agtgtgtggacctccatgttatttccaatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcatgctgtgtgctggacgctgga
caggggcaaaagcacctgctcgggtgattctgggggcccacttgtctgtaatggtgtgcttcaaggtatcacgtcatggggcagtgaac
catgtgccctgcccgaaaggccttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacaccatcgtggccaacccctga

gcacccctatcaactccctattgtagtaaacttggaaccttggaaatgaccaggccaagactcaggcctccccagttctactgacctttgtcc ttaggtgtgaggtccagggttgctaggaaaagaaatcagcagacacaggtgtagaccagagtgtttcttaaatggtgtaattttgtcctctct gtgtcctggggaatactggccatgcctggagacatatcactcaatttctctgaggacacagataggatggggtgtctgtgttatttgtggggt acagagatgaaagaggggtgggatccacactgagagagtggagagtgacatgtgctggacactgtccatgaagcactgagcagaagc tggaggcacaacgcaccagacactcacagcaaggatggagctgaaaacataacccactctgtcctggaggcactgggaagcctagag aaggctgtgaaccaaggagggagggtcttcctttggcatgggatggggatgaagtaaggagagggactgacccctggaagctgattc actatgggggggaggtgtattgaagtcctccagacaaccctcagatttgatgatttcctagtagaactcacagaaataaagagctgttatactg tgaa (SEQ ID No: 45; GenBank Accession No. X07730). In another embodiment, the KLK3 protein is encoded by residues 67-1088 of SEQ ID No: 45. In another embodiment, the KLK3 protein is encoded by a homologue of SEQ ID No: 45. In another embodiment, the KLK3 protein is encoded by a variant of SEQ ID No: 45. In another embodiment, the KLK3 protein is encoded by an isomer of SEQ ID No: 45. In another embodiment, the KLK3 protein is encoded by a fragment of SEQ ID No: 45.

[00156] In another embodiment, the KLK3 protein has the sequence:

[00157] MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG GVLVHPQWVLTAAHCIRK (SEQ ID No: 46; GenBank Accession No. NM_001030050). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 46. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 46. In another embodiment, the sequence of the KLK3 protein comprises SEQ ID No: 46. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 46. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 46.

[00158] In another embodiment, the KLK3 protein that is the source of the KLK3 peptide has the sequence:

[00159] MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG GVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNR FLRPGDDSSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKST CSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 47; GenBank Accession No. NM_001064049). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 47. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 47. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 47. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 47.

[00160] In another embodiment, the KLK3 protein has the sequence:

[00161] MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG GVLVHPQWVLTAAHCIRKPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTT CYASGWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKST

CSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (SEQ ID No: 48; GenBank Accession No. NM_001030048). In another embodiment, the KLK3 protein is a homologue of SEQ ID No: 48. In another embodiment, the KLK3 protein is a variant of SEQ ID No: 48. In another embodiment, the KLK3 protein is an isomer of SEQ ID No: 48. In another embodiment, the KLK3 protein is a fragment of SEQ ID No: 48.

[00162] In another embodiment, the KLK3 protein is encoded by a sequence set forth in one of the following GenBank Accession Numbers: BC005307, AJ310938, AJ310937, AF335478, AF335477, M27274, and M26663. In another embodiment, the KLK3 protein is encoded by a sequence set forth in one of the above GenBank Accession Numbers.

[00163] In another embodiment, the KLK3 protein is encoded by a sequence set forth in one of the following GenBank Accession Numbers: NM_001030050, NM_001030049, NM_001030048, NM_001030047, NM_001648, AJ459782, AJ512346, or AJ459784. Each possibility represents a separate embodiment of the methods and compositions as disclosed herein . In one embodiment, the KLK3 protein is encoded by a variation of any of the sequences described herein wherein the sequence lacks MWVPVVFLTLSVTWIGAAPLILSR (SEQ ID NO: 49).

[00164] In another embodiment, the KLK3 protein has the sequence that comprises a sequence set forth in one of the following GenBank Accession Numbers: X13943, X13942, X13940, X13941, and X13944.

[00165] In another embodiment, the KLK3 protein is any other KLK3 protein known in the art.

[00166] In another embodiment, the KLK3 peptide is any other KLK3 peptide known in the art. In another embodiment, the KLK3 peptide is a fragment of any other KLK3 peptide known in the art.

[00167] "KLK3 peptide" refers, in another embodiment, to a full-length KLK3 protein. In another embodiment, the term refers to a fragment of a KLK3 protein. In another embodiment, the term refers to a fragment of a KLK3 protein that is lacking the KLK3 signal peptide. In another embodiment, the term refers to a KLK3 protein that contains the entire KLK3 sequence except the KLK3 signal peptide. "KLK3 signal sequence" refers, in another embodiment, to any signal sequence found in nature on a KLK3 protein. In another embodiment, a KLK3 protein of methods and compositions as disclosed herein does not contain any signal sequence.

[00168] In another embodiment, the kallikrein-related peptidase 3 (KLK3 protein) that is the source of a KLK3 peptide for use in the methods and compositions as disclosed herein is a PSA protein. In another embodiment, the KLK3 protein is a P-30 antigen protein. In another embodiment, the KLK3 protein is a gamma-seminoprotein protein. In another embodiment, the KLK3 protein is a kallikrein 3 protein. In another embodiment, the KLK3 protein is a

semenogelase protein. In another embodiment, the KLK3 protein is a seminin protein. In another embodiment, the KLK3 protein is any other type of KLK3 protein that is known in the art.

[00169] In another embodiment, the KLK3 protein is a splice variant 1 KLK3 protein. In another embodiment, the KLK3 protein is a splice variant 2 KLK3 protein. In another embodiment, the KLK3 protein is a splice variant 3 KLK3 protein. In another embodiment, the KLK3 protein is a transcript variant 1 KLK3 protein. In another embodiment, the KLK3 protein is a transcript variant 2 KLK3 protein. In another embodiment, the KLK3 protein is a transcript variant 3 KLK3 protein. In another embodiment, the KLK3 protein is a transcript variant 4 KLK3 protein. In another embodiment, the KLK3 protein is a transcript variant 5 KLK3 protein. In another embodiment, the KLK3 protein is a transcript variant 6 KLK3 protein. In another embodiment, the KLK3 protein is a splice variant RP5 KLK3 protein. In another embodiment, the KLK3 protein is any other splice variant KLK3 protein known in the art. In another embodiment, the KLK3 protein is any other transcript variant KLK3 protein known in the art.

[00170] In another embodiment, the KLK3 protein is a mature KLK3 protein. In another embodiment, the KLK3 protein is a pro-KLK3 protein. In another embodiment, the leader sequence has been removed from a mature KLK3 protein of methods and compositions as disclosed herein.

[00171] In another embodiment, the KLK3 protein that is the source of a KLK3 peptide of methods and compositions as disclosed herein is a human KLK3 protein. In another embodiment, the KLK3 protein is a primate KLK3 protein. In another embodiment, the KLK3 protein is a KLK3 protein of any other species known in the art. In another embodiment, one of the above KLK3 proteins is referred to in the art as a "KLK3 protein."

[00172] In one embodiment, a recombinant polypeptide disclosed herein comprising a truncated LLO fused to a PSA protein disclosed herein is encoded by a sequence comprising:

[00173] ATGAAAAAAATAATGCTAGTTTTTATTACACTTATATTAGTTAGTCTACCA
ATTGCGCAACAAACTGAAGCAAAGGATGCATCTGCATTCAATAAAGAAAATTCAA
TTTCATCCATGGCACCACCAGCATCTCCGCCTGCAAGTCCTAAGACGCCAATCGAA
AAGAAACACGCGGATGAAATCGATAAGTATATACAAGGATTGGATTACAATAAAA
ACAATGTATTAGTATACCACGGAGATGCAGTGACAAATGTGCCGCCAAGAAAAGG
TTACAAAGATGGAAATGAATATATTGTTGTGGAGAAAAAGAAGAAATCCATCAAT
CAAAATAATGCAGACATTCAAGTTGTGAATGCAATTTCGAGCCTAACCTATCCAGG
TGCTCTCGTAAAAGCGAATTCGGAATTAGTAGAAAATCAACCAGATGTTCTCCCTG
TAAAACGTGATTCATTAACACTCAGCATTGATTTGCCAGGTATGACTAATCAAGAC

AATAAAATAGTTGTAAAAAATGCCACTAAATCAAACGTTAACAACGCAGTAAATA
CATTAGTGGAAAGATGGAATGAAAAATATGCTCAAGCTTATCCAAATGTAAGTGC
AAAAATTGATTATGATGACGAAATGGCTTACAGTGAATCACAATTAATTGCGAAAT
TTGGTACAGCATTTAAAGCTGTAAATAATAGCTTGAATGTAAACTTCGGCGCAATC
AGTGAAGGGAAAATGCAAGAAGAAGTCATTAGTTTTAAACAAATTTACTATAACG
TGAATGTTAATGAACCTACAAGACCTTCCAGATTTTTCGGCAAAGCTGTTACTAAA
GAGCAGTTGCAAGCGCTTGGAGTGAATGCAGAAAATCCTCCTGCATATATCTCAAG
TGTGGCGTATGGCCGTCAAGTTTATTTGAAATTATCAACTAATTCCCATAGTACTAA
AGTAAAAGCTGCTTTTGATGCTGCCGTAAGCGGAAAATCTGTCTCAGGTGATGTAG
AACTAACAAATATCATCAAAAATTCTTCCTTCAAAGCCGTAATTTACGGAGGTTCC
GCAAAAGATGAAGTTCAAATCATCGACGGCAACCTCGGAGACTTACGCGATATTTT
GAAAAAAGGCGCTACTTTTAATCGAGAAACACCAGGAGTTCCCATTGCTTATACAA
CAAACTTCCTAAAAGACAATGAATTAGCTGTTATTAAAAACAACTCAGAATATATT
GAAACAACTTCAAAAGCTTATACAGATGGAAAAATTAACATCGATCACTCTGGAG
GATACGTTGCTCAATTCAACATTTCTTGGGATGAAGTAAATTATGAT**CTCGAG***attgtg*
ggaggctgggagtgcgagaagcattcccaaccctggcaggtgcttgtggcctctcgtggcagggcagtctgcggcggtgttctggtgc
acccccagtgggtcctcacagctgcccactgcatcaggaacaaaagcgtgatcttgctgggtcggcacagcctgtttcatcctgaagac
acaggccaggtatttcaggtcagccacagcttcccacacccgctctacgatatgagcctcctgaagaatcgattcctcaggccaggtga
tgactccagccacgacctcatgctgctccgcctgtcagagcctgccgagctcacggatgctgtgaaggtcatggacctgcccacccag
gagccagcactggggaccacctgctacgcctcaggctggggcagcattgaaccagaggagttcttgaccccaaagaaacttcagtgt
gtggacctccatgttatttccaatgacgtgtgtgcgcaagttcaccctcagaaggtgaccaagttcatgctgtgtgctggacgctggaca
gggggcaaaagcacctgctcgggtgattctgggggcccacttgtctgttatggtgtgcttcaaggtatcacgtcatggggcagtgaacc
atgtgccctgcccgaaaggccttccctgtacaccaaggtggtgcattaccggaagtggatcaaggacaccatcgtggccaacccc
(SEQ ID NO: 50). In another embodiment, the fusion protein is encoded by a homologue of SEQ ID No: 50. In another embodiment, the fusion protein is encoded by a variant of SEQ ID No: 50. In another embodiment, the fusion protein is encoded by an isomer of SEQ ID No: 50. In one embodiment, the "ctcgag" sequence within the fusion protein represents a Xho I restriction site used to ligate the tumor antigen to truncated LLO in the plasmid.

[00174] In another embodiment, a recombinant polypeptide disclosed herein comprising a truncated LLO fused to a PSA protein disclosed herein comprises the following sequence:

[00175] MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSMAPPASPPASPKTPIEK
KHADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNN
ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVK
NATKSNVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKA
VNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN

AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSFK AVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIKNN SEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYD**LE**IVGGWECEKHSQPWQVLV ASRGRAVCGGVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPL YDMSLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGW GSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGG PLVCYGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVANP (PSA sequence is underlined) (SEQ ID NO: 51). In another embodiment, the tLLO-PSA fusion protein is a homologue of SEQ ID NO: 51. In another embodiment, the tLLO-PSA fusion protein is a variant of SEQ ID NO: 51. In another embodiment, the tLLO-PSA fusion protein is an isomer of SEQ ID NO: 51. In another embodiment, the tLLO-PSA fusion protein is a fragment of SEQ ID NO: 51.

[00176] In one embodiment, the recombinant *Listeria* strain as disclosed herein comprises a nucleic acid molecule encoding a tumor associated antigen, wherein the antigen comprises an HPV-E7 protein. In one embodiment, the recombinant *Listeria* strain as disclosed herein comprises a nucleic acid molecule encoding HPV-E7 protein.

[00177] In one embodiment, either a whole E7 protein or a fragment thereof is fused to a LLO protein or truncation or peptide thereof, an ActA protein or truncation or peptide thereof, or a PEST-like sequence-containing peptide to generate a recombinant polypeptide or peptide of the composition and methods of the disclosure. The E7 protein that is utilized (either whole or as the source of the fragments) has, in another embodiment, the sequence

[00178] MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHY NIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP (SEQ ID No: 52). In another embodiment, the E7 protein is a homologue of SEQ ID No: 52. In another embodiment, the E7 protein is a variant of SEQ ID No: 52. In another embodiment, the E7 protein is an isomer of SEQ ID No: 52. In another embodiment, the E7 protein is a fragment of SEQ ID No: 52. In another embodiment, the E7 protein is a fragment of a homologue of SEQ ID No: 52. In another embodiment, the E7 protein is a fragment of a variant of SEQ ID No: 52. In another embodiment, the E7 protein is a fragment of an isomer of SEQ ID No: 52.

[00179] In another embodiment, the sequence of the E7 protein is:

[00180] MHGPKATLQDIVLHLEPQNEIPVDLLCHEQLSDSEEENDEIDGVNHQHLPARR AEPQRHTMLCMCCKCEARIELVVESSADDLRAFQQLFLNTLSFVCPWCASQQ (SEQ ID No: 53). In another embodiment, the E6 protein is a homologue of SEQ ID No: 53. In another embodiment, the E6 protein is a variant of SEQ ID No: 53. In another embodiment, the E6 protein is an isomer of SEQ ID No: 53. In another embodiment, the E6 protein is a fragment

of SEQ ID No: 53. In another embodiment, the E6 protein is a fragment of a homologue of SEQ ID No: 53. In another embodiment, the E6 protein is a fragment of a variant of SEQ ID No: 53. In another embodiment, the E6 protein is a fragment of an isomer of SEQ ID No: 53.

[00181] In another embodiment, the E7 protein has a sequence set forth in one of the following GenBank entries: M24215, NC_004500, V01116, X62843, or M14119. In another embodiment, the E7 protein is a homologue of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a variant of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is an isomer of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of a homologue of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of a variant of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of an isomer of a sequence from one of the above GenBank entries.

[00182] 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.

[00183] 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. In another embodiment, an HPV E6 antigen is utilized instead of or in addition to an E7 antigen in a composition or method of the disclosure for treating or ameliorating an HPV-mediated disease, disorder, or symptom. In another embodiment, an HPV-16 E6 and E7 is utilized instead of or in combination with an HPV-18 E6 and E7. In such an embodiment, the recombinant *Listeria* may express the HPV-16 E6 and E7 from the chromosome and the HPV-18 E6 and E7 from a plasmid, or vice versa. In another embodiment, the HPV-16 E6 and E7 antigens and the HPV-18 E6 and E7 antigens are expressed from a plasmid present in a recombinant *Listeria* disclosed herein . In another embodiment, the HPV-16 E6 and E7 antigens and the HPV-18 E6 and E7 antigens are expressed from the chromosome of a recombinant *Listeria* disclosed herein . In another embodiment, the HPV-16 E6 and E7 antigens and the HPV-18 E6 and E7 antigens are expressed in any combination of the above embodiments, including where each E6 and E7

antigen from each HPV strain is expressed from either the plasmid or the chromosome.

[00184] In another embodiment, either a whole E7 protein or a fragment thereof is fused to a LLO protein, ActA protein, or PEST amino acid sequence-containing peptide to generate a recombinant polypeptide disclosed herein. In one embodiment, the E7 protein that is utilized (either whole or as the source of the fragments) comprises the amino acid sequence set forth in SEQ ID NO: 54

[00185] H G D T P T L H E Y M L D L Q P E T T D L Y C Y E Q L N D S S E E E D E I D G P A G Q A E P D R A H Y N I V T F C C K C D S T L R L C V Q S T H V D I R T L E D L L M G T L G I V C P I C S Q K P (SEQ ID NO: 54). In another embodiment, the E7 protein is a homologue of SEQ ID No: 117. In another embodiment, the E7 protein is a variant of SEQ ID No: 54. In another embodiment, the E7 protein is an isomer of SEQ ID No: 54. In another embodiment, the E7 protein is a fragment of SEQ ID No: 54. In another embodiment, the E7 protein is a fragment of a homologue of SEQ ID No: 54. In another embodiment, the E7 protein is a fragment of a variant of SEQ ID No: 54. In another embodiment, the E7 protein is a fragment of an isomer of SEQ ID No: 54.

[00186] In another embodiment, the amino acid sequence of a truncated LLO fused to an E7 protein comprises the following amino acid sequence:

[00187] MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSMAPPASPPASPKTPIEK KHADEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNN ADIQVVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVK NATKSNVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKA VNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSFK AVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIKNN SEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYDLEHGDTPTLHEYMLDLQPETT DLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIRTL EDLLMGTLGIVCPICSQKP (SEQ ID NO: 55). In another embodiment, the fusion protein of tLLO-E7 is a homologue of SEQ ID No: 55. In another embodiment, the fusion protein is a variant of SEQ ID No: 55. In another embodiment, the tLLO-E7 fusion protein is an isomer of SEQ ID No: 55. In another embodiment, the tLLO-E7 fusion protein is a fragment of SEQ ID No: 55. In another embodiment, the tLLO-E7 fusion protein is a fragment of a homologue of SEQ ID No: 55. In another embodiment, the tLLO-E7 fusion protein is a fragment of a variant of SEQ ID No: 55. In another embodiment, the tLLO-E7 fusion protein is a fragment of an isomer of SEQ ID No: 55.

[00188] In one embodiment, the recombinant *Listeria* strain as disclosed herein comprises a

nucleic acid molecule encoding a tumor associated antigen, wherein the tumor associated antigen comprises an Her-2/neu peptide. In one embodiment, a tumor associated antigen comprises an Her-2/neu antigen. In one embodiment, the Her-2/neu peptide comprises a chimeric Her-2/neu antigen (cHer-2).

[00189] In one embodiment, the attenuated auxotrophic *Listeria* strain is based on a *Listeria* vector which is attenuated due to the deletion of virulence gene *actA* and retains the plasmid for Her2/neu expression *in vivo* and *in vitro* by complementation of *dal* gene. In one embodiment, the Listeria strain expresses and secretes a chimeric Her2/neu protein fused to the first 441 amino acids of listeriolysin O (LLO). In another embodiment, the *Listeria* is a *dal/dat/actA* *Listeria* having a mutation in the dal, dat and actA endogenous genes. In another embodiment, the mutation is a deletion, a truncation or an inactivation of the mutated genes. In another embodiment, *Listeria* strain exerts strong and antigen specific anti-tumor responses with ability to break tolerance toward HER2/neu in transgenic animals. In another embodiment, the dal/dat/actA strain is highly attenuated and has a better safety profile than previous *Listeria* generation, as it is more rapidly cleared from the spleens of the immunized mice. In another embodiment, the *Listeria* strain results in a longer delay of tumor onset in transgenic animals than *Lm*-LLO-ChHer2, the antibiotic resistant and more virulent version of this vaccine (see US Publication No. 2011/0142791, which is incorporated by reference herein in its entirety). In another embodiment, the *Listeria* strain causes a significant decrease in intra-tumoral T regulatory cells (Tregs). In another embodiment, the lower frequency of Tregs in tumors treated with *LmddA* vaccines result in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* vaccines. In one embodiment, the disclosure provides a recombinant polypeptide comprising an N-terminal fragment of an LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof. In one embodiment, the disclosure provides a recombinant polypeptide consisting of an N-terminal fragment of an LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof. In the embodiment, the heterologous antigen is a Her-2 chimeric protein or fragment thereof.

[00190] In another embodiment, the Her-2 chimeric protein of the methods and compositions of the disclosure is a human Her-2 chimeric protein. In another embodiment, the Her-2 protein is a mouse Her-2 chimeric protein. In another embodiment, the Her-2 protein is a rat Her-2 chimeric protein. In another embodiment, the Her-2 protein is a primate Her-2 chimeric protein. In another embodiment, the Her-2 protein is a Her-2 chimeric protein of human or any other animal species or combinations thereof known in the art. Each possibility represents a separate embodiment of the disclosure.

[00191] In another embodiment, a Her-2 protein is a protein referred to as "HER-2/neu," "Erbb2," "v-erb-b2," "c-erb-b2," "neu," or "cNeu."

[00192] In one embodiment, the Her2-neu chimeric protein, harbors two of the extracellular and one intracellular fragments of Her2/neu antigen showing clusters of MHC-class I epitopes of the oncogene, where, in another embodiment, the chimeric protein, harbors 3 H2Dq and at least 17 of the mapped human MHC-class I epitopes of the Her2/neu antigen (fragments EC1, EC2, and IC1) (See Fig. 21). In another embodiment, the chimeric protein harbors at least 13 of the mapped human MHC-class I epitopes (fragments EC2 and IC1). In another embodiment, the chimeric protein harbors at least 14 of the mapped human MHC-class I epitopes (fragments EC1 and IC1). In another embodiment, the chimeric protein harbors at least 9 of the mapped human MHC-class I epitopes (fragments EC1 and IC2). In another embodiment, the Her2-neu chimeric protein is fused to a non-hemolytic listeriolysin O (LLO). In another embodiment, the Her2-neu chimeric protein is fused to the first 441 amino acids of the *Listeria-monocytogenes* listeriolysin O (LLO) protein and expressed and secreted by the *Listeria monocytogenes* attenuated auxotrophic strain *LmddA*. In another embodiment, the expression and secretion of the fusion protein tLLO-ChHer2 from the attenuated auxotrophic strain disclosed herein that expresses a chimeric Her2/neu antigen/LLO fusion protein is comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (See Figure 21B).

[00193] In one embodiment, no CTL activity is detected in naïve animals or mice injected with an irrelevant *Listeria* (See Figure 22A). While in another embodiment, the attenuated auxotrophic strain disclosed herein is able to stimulate the secretion of IFN-γ by the splenocytes from wild type FVB/N mice (Figure 22B).

[00194] In one embodiment, the antigen is a chimeric Her2 antigen described in US patent application publication US2011/0142791, which is hereby incorporated by reference herein in its entirety.

[00195] In another embodiment, the Her-2 chimeric protein is encoded by the following nucleic acid sequence set forth in SEQ ID NO:56

[00196] gagacccacctggacatgctccgccacctctaccagggctgccaggtggtgcagggaaacctggaactcacctacctgcc
caccaatgccagcctgtccttcctgcaggatatccaggaggtgcagggctacgtgctcatcgctcacaaccaagtgaggcaggtcccact
gcagaggctgcggattgtgcgaggcacccagctctttgaggacaactatgccctggccgtgctagacaatggagacccgctgaacaata
ccacccctgtcacaggggcctccccaggaggcctgcgggagctgcagcttcgaagcctcacagagatcttgaaaggaggggtcttgat
ccagcggaacccccagctctgctaccaggacacgattttgtggaagaatatccaggagtttgctggctgcaagaagatctttgggagcct
ggcatttctgccggagagctttgatggggacccagcctccaacactgccccgctccagccagagcagctccaagtgtttgagactctgga
agagatcacaggttacctatacatctcagcatggccggacagcctgcctgacctcagcgtcttccagaacctgcaagtaatccggggacg

aattctgcacaatggcgcctactcgctgaccctgcaagggctgggcatcagctggctggggctgcgctcactgagggaactgggcagtg
gactggccctcatccaccataacacccacctctgcttcgtgcacacggtgccctgggaccagctctttcggaacccgcaccaagctctgct
ccacactgccaaccggccagaggacgagtgtgtgggcgagggcctggcctgccaccagctgtgcgcccgagggcagcagaagatcc
ggaagtacacgatgcggagactgctgcaggaaacggagctggtggagccgctgacacctagcggagcgatgcccaaccaggcgca
gatgcggatcctgaaagagacggagctgaggaaggtgaaggtgcttggatctggcgcttttggcacagtctacaagggcatctggatcc
ctgatggggagaatgtgaaaattccagtggccatcaaagtgttgagggaaaacacatcccccaaagccaacaaagaaatcttagacgaa
gcatacgtgatggctggtgtgggctccccatatgtctcccgccttctgggcatctgcctgacatccacggtgcagctggtgacacagcttat
gccctatggctgcctcttagactaa (SEQ ID NO: 56).

[00197] In another embodiment, the Her-2 chimeric protein has the sequence:

[00198] T H L D M L R H L Y Q G C Q V V Q G N L E L T Y L P T N A S
L S F L Q D I Q E V Q G Y V L I A H N Q V R Q V P L Q R L R I V R G
T Q L F E D N Y A L A V L D N G D P L N N T T P V T G A S P G G L
R E L Q L R S L T E I L K G G V L I Q R N P Q L C Y Q D T I L W K N
I Q E F A G C K K I F G S L A F L P E S F D G D P A S N T A P L Q P E
Q L Q V F E T L E E I T G Y L Y I S A W P D S L P D L S V F Q N L Q
V I R G R I L H N G A Y S L T L Q G L G I S W L G L R S L R E L G S
G L A L I H H N T H L C F V H T V P W D Q L F R N P H Q A L L H T
A N R P E D E C V G E G L A C H Q L C A R G Q Q K I R K Y T M R R
L L Q E T E L V E P L T P S G A M P N Q A Q M R I L K E T E L R K
V K V L G S G A F G T V Y K G I W I P D G E N V K I P V A I K V L R
E N T S P K A N K E I L D E A Y V M A G V G S P Y V S R L L G I C L T S
T V Q L V T Q L M P Y G C L L D (SEQ ID NO: 57).

[00199] In one embodiment, the Her2 chimeric protein or fragment thereof of the methods and compositions disclosed herein does not include a signal sequence thereof. In another embodiment, omission of the signal sequence enables the Her2 fragment to be successfully expressed in *Listeria*, due the high hydrophobicity of the signal sequence. Each possibility represents a separate embodiment of the disclosure.

[00200] In another embodiment, the fragment of a Her2 chimeric protein of methods and compositions of the disclosure does not include a transmembrane domain (TM) thereof. In one embodiment, omission of the TM enables the Her-2 fragment to be successfully expressed in *Listeria*, due the high hydrophobicity of the TM. In one embodiment, LmddA164 comprises a nucleic acid sequence comprising an open reading frame encoding tLLO fused to cHER2, wherein said nucleic acid sequence comprises SEQ ID NO: 58:

atgaaaaaaataatgctagtttttattacacttatattagttagtctaccaattgcgcaacaaactgaagcaaaggatgcatctgcattcaata
aagaaaattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaat

cgataagtatatacaaggattggattacaataaaaacaatgtattagtataccacggagatgcagtgacaaatgtgccgccaagaaaag
gttacaaagatggaaatgaatatattgttgtggagaaaaagaagaaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaat
ttcgagcctaacctatccaggtgctctcgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcat
taacactcagcattgatttgccaggtatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgttaacaacgcagt
aaatacattagtggaaagatggaatgaaaaatatgctcaagcttatccaaatgtaagtgcaaaaattgattatgatgacgaaatggcttac
agtgaatcacaattaattgcgaaatttggtacagcatttaaagctgtaaataatagcttgaatgtaaacttcggcgcaatcagtgaaggga
aaatgcaagaagaagtcattagttttaaacaaatttactataacgtgaatgttaatgaacctacaagaccttccagatttttcggcaaagctg
ttactaaagagcagttgcaagcgcttggagtgaatgcagaaaatcctcctgcatatatctcaagtgtggcgtatggccgtcaagtttatttg
aaattatcaactaattcccatagtactaaagtaaaagctgcttttgatgctgccgtaagcggaaaatctgtctcaggtgatgtagaactaac
aaatatcatcaaaaattcttccttcaaagccgtaatttacggaggttccgcaaaagatgaagttcaaatcatcgacggcaacctcggaga
cttacgcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcccattgcttatacaacaaacttcctaaaagacaatg
aattagctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacagatggaaaaattaacatcgatcactctggagga
tacgttgctcaattcaacatttcttgggatgaagtaaattatgatctcgagACCCACCTGGACATGCTCCGCCACC
TCTACCAGGGCTGCCAGGTGGTGCAGGGAAACCTGGAACTCACCTACCTGCCCAC
CAATGCCAGCCTGTCCTTCCTGCAGGATATCCAGGAGGTGCAGGGCTACGTGCTC
ATCGCTCACAACCAAGTGAGGCAGGTCCCACTGCAGAGGCTGCGGATTGTGCGA
GGCACCCAGCTCTTTGAGGACAACTATGCCCTGGCCGTGCTAGACAATGGAGACC
CGCTGAACAATACCACCCCTGTCACAGGGGCCTCCCCAGGAGGCCTGCGGGAGCT
GCAGCTTCGAAGCCTCACAGAGATCTTGAAAGGAGGGGTCTTGATCCAGCGGAA
CCCCCAGCTCTGCTACCAGGACACGATTTTGTGGAAGAATATCCAGGAGTTTGCT
GGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTCTGCCGGAGAGCTTTGATGGGG
ACCCAGCCTCCAACACTGCCCCGCTCCAGCCAGAGCAGCTCCAAGTGTTTGAGAC
TCTGGAAGAGATCACAGGTTACCTATACATCTCAGCATGGCCGGACAGCCTGCCT
GACCTCAGCGTCTTCCAGAACCTGCAAGTAATCCGGGGACGAATTCTGCACAATG
GCGCCTACTCGCTGACCCTGCAAGGGCTGGGCATCAGCTGGCTGGGGCTGCGCTC
ACTGAGGGAACTGGGCAGTGGACTGGCCCTCATCCACCATAACACCCACCTCTGC
TTCGTGCACACGGTGCCCTGGGACCAGCTCTTTCGGAACCCGCACCAAGCTCTGC
TCCACACTGCCAACCGGCCAGAGGACGAGTGTGTGGGCGAGGGCCTGGCCTGCC
ACCAGCTGTGCGCCCGAGGGCAGCAGAAGATCCGGAAGTACACGATGCGGAGAC
TGCTGCAGGAAACGGAGCTGGTGGAGCCGCTGACACCTAGCGGAGCGATGCCCA
ACCAGGCGCAGATGCGGATCCTGAAAGAGACGGAGCTGAGGAAGGTGAAGGTGC
TTGGATCTGGCGCTTTTGGCACAGTCTACAAGGGCATCTGGATCCCTGATGGGGA
GAATGTGAAAATTCCAGTGGCCATCAAAGTGTTGAGGGAAAACACATCCCCCAA
AGCCAACAAAGAAATCTTAGACGAAGCATACGTGATGGCTGGTGTGGGCTCCCC
ATATGTCTCCCGCCTTCTGGGCATCTGCCTGACATCCACGGTGCAGCTGGTGACA

CAGCTTATGCCCTATGGCTGCCTCTTAGAC (SEQ ID NO: 58), wherein the UPPERCASE sequences encode cHER2, the lowercase sequences encode tLLO and the underlined "ctcgag" sequence represents the Xho I restriction site used to ligate the tumor antigen to truncated LLO in the plasmid. In another embodiment, plasmid pAdv168 comprises SEQ ID NO: 58. In one embodiment, the truncated LLO-cHER2 fusion is a homolog of SEQ ID NO: 58. In another embodiment, the truncated LLO-cHER2 fusion is a variant of SEQ ID NO: 58. In another embodiment, the truncated LLO-cHER2 fusion is an isomer of SEQ ID NO: 58.

[00201] In one embodiment, an amino acid sequence of a recombinant protein comprising tLLO fused to a cHER2 comprises SEQ ID NO: 59:

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSMAPPASPPASPKTPIEKKHADE
IDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQ
VVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVKNA
TKSNVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKAV
NNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN
AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSF
KAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK
NNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYDLETHLDMLRHLYQGCQV
VQGNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNY
ALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWK
NIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSL
PDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFV
HTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLCARGQQKIRKYTMRRLLQE
TELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPDGENVKIPV
AIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLL
D (SEQ ID NO: 59). In one embodiment, the truncated LLO-cHER2 fusion is a homolog of SEQ ID NO: 59. In another embodiment, the truncated LLO-cHER2 fusion is a variant of SEQ ID NO: 59. In another embodiment, the truncated LLO-cHER2 fusion is an isomer of SEQ ID NO: 59.

[00202] In one embodiment, the nucleic acid sequence of human-Her2/neu gene is:

[00203] ATGGAGCTGGCGGCCTTGTGCCGCTGGGGGCTCCTCCTCGCCCTCTTGCCC
CCCGGAGCCGCGAGCACCCAAGTGTGCACCGGCACAGACATGAAGCTGCGGCTCC
CTGCCAGTCCCGAGACCCACCTGGACATGCTCCGCCACCTCTACCAGGGCTGCCA
GGTGGTGCAGGGAAACCTGGAACTCACCTACCTGCCCACCAATGCCAGCCTGTCC

TTCCTGCAGGATATCCAGGAGGTGCAGGGCTACGTGCTCATCGCTCACAACCAAG
TGAGGCAGGTCCCACTGCAGAGGCTGCGGATTGTGCGAGGCACCCAGCTCTTTGA
GGACAACTATGCCCTGGCCGTGCTAGACAATGGAGACCCGCTGAACAATACCACC
CCTGTCACAGGGGCCTCCCCAGGAGGCCTGCGGGAGCTGCAGCTTCGAAGCCTCA
CAGAGATCTTGAAAGGAGGGGTCTTGATCCAGCGGAACCCCCAGCTCTGCTACCA
GGACACGATTTTGTGGAAGGACATCTTCCACAAGAACAACCAGCTGGCTCTCACA
CTGATAGACACCAACCGCTCTCGGGCCTGCCACCCCTGTTCTCCGATGTGTAAGGG
CTCCCGCTGCTGGGGAGAGAGTTCTGAGGATTGTCAGAGCCTGACGCGCACTGTC
TGTGCCGGTGGCTGTGCCCGCTGCAAGGGGCCACTGCCCACTGACTGCTGCCATG
AGCAGTGTGCTGCCGGCTGCACGGGCCCCAAGCACTCTGACTGCCTGGCCTGCCT
CCACTTCAACCACAGTGGCATCTGTGAGCTGCACTGCCCAGCCCTGGTCACCTACA
ACACAGACACGTTTGAGTCCATGCCCAATCCCGAGGGCCGGTATACATTCGGCGC
CAGCTGTGTGACTGCCTGTCCCTACAACTACCTTTCTACGGACGTGGGATCCTGCA
CCCTCGTCTGCCCCCTGCACAACCAAGAGGTGACAGCAGAGGATGGAACACAGCG
GTGTGAGAAGTGCAGCAAGCCCTGTGCCCGAGTGTGCTATGGTCTGGGCATGGAG
CACTTGCGAGAGGTGAGGGCAGTTACCAGTGCCAATATCCAGGAGTTTGCTGGCT
GCAAGAAGATCTTTGGGAGCCTGGCATTTCTGCCGGAGAGCTTTGATGGGGACCC
AGCCTCCAACACTGCCCCGCTCCAGCCAGAGCAGCTCCAAGTGTTTGAGACTCTG
GAAGAGATCACAGGTTACCTATACATCTCAGCATGGCCGGACAGCCTGCCTGACC
TCAGCGTCTTCCAGAACCTGCAAGTAATCCGGGGACGAATTCTGCACAATGGCGC
CTACTCGCTGACCCTGCAAGGGCTGGGCATCAGCTGGCTGGGGCTGCGCTCACTG
AGGGAACTGGGCAGTGGACTGGCCCTCATCCACCATAACACCCACCTCTGCTTCG
TGCACACGGTGCCCTGGGACCAGCTCTTTCGGAACCCGCACCAAGCTCTGCTCCAC
ACTGCCAACCGGCCAGAGGACGAGTGTGTGGGCGAGGGCCTGGCCTGCCACCAG
CTGTGCGCCCGAGGGCACTGCTGGGGTCCAGGGCCCACCCAGTGTGTCAACTGCA
GCCAGTTCCTTCGGGGCCAGGAGTGCGTGGAGGAATGCCGAGTACTGCAGGGGCT
CCCCAGGGAGTATGTGAATGCCAGGCACTGTTTGCCGTGCCACCCTGAGTGTCAG
CCCCAGAATGGCTCAGTGACCTGTTTTGGACCGGAGGCTGACCAGTGTGTGGCCT
GTGCCCACTATAAGGACCCTCCCTTCTGCGTGGCCCGCTGCCCCAGCGGTGTGAAA
CCTGACCTCTCCTACATGCCCATCTGGAAGTTTCCAGATGAGGAGGGCGCATGCC
AGCCTTGCCCCATCAACTGCACCCACTCCTGTGTGGACCTGGATGACAAGGGCTG
CCCCGCCGAGCAGAGAGCCAGCCCTCTGACGTCCATCGTCTCTGCGGTGGTTGGC
ATTCTGCTGGTCGTGGTCTTGGGGGTGGTCTTTGGGATCCTCATCAAGCGACGGCA
GCAGAAGATCCGGAAGTACACGATGCGGAGACTGCTGCAGGAAACGGAGCTGGT
GGAGCCGCTGACACCTAGCGGAGCGATGCCCAACCAGGCGCAGATGCGGATCCT

GAAAGAGACGGAGCTGAGGAAGGTGAAGGTGCTTGGATCTGGCGCTTTTGGCAC
AGTCTACAAGGGCATCTGGATCCCTGATGGGGAGAATGTGAAAATTCCAGTGGCC
ATCAAAGTGTTGAGGGAAAACACATCCCCCAAAGCCAACAAAGAAATCTTAGAC
GAAGCATACGTGATGGCTGGTGTGGGCTCCCCATATGTCTCCCGCCTTCTGGGCAT
CTGCCTGACATCCACGGTGCAGCTGGTGACACAGCTTATGCCCTATGGCTGCCTCT
TAGACCATGTCCGGGAAAACCGCGGACGCCTGGGCTCCCAGGACCTGCTGAACTG
GTGTATGCAGATTGCCAAGGGGATGAGCTACCTGGAGGATGTGCGGCTCGTACAC
AGGGACTTGGCCGCTCGGAACGTGCTGGTCAAGAGTCCCAACCATGTCAAAATTA
CAGACTTCGGGCTGGCTCGGCTGCTGGACATTGACGAGACAGAGTACCATGCAGA
TGGGGGCAAGGTGCCCATCAAGTGGATGGCGCTGGAGTCCATTCTCCGCCGGCGG
TTCACCCACCAGAGTGATGTGTGGAGTTATGGTGTGACTGTGTGGGAGCTGATGA
CTTTTGGGGCCAAACCTTACGATGGGATCCCAGCCCGGGAGATCCCTGACCTGCT
GGAAAAGGGGGAGCGGCTGCCCCAGCCCCCCATCTGCACCATTGATGTCTACATG
ATCATGGTCAAATGTTGGATGATTGACTCTGAATGTCGGCCAAGATTCCGGGAGTT
GGTGTCTGAATTCTCCCGCATGGCCAGGGACCCCCAGCGCTTTGTGGTCATCCAGA
ATGAGGACTTGGGCCCAGCCAGTCCCTTGGACAGCACCTTCTACCGCTCACTGCTG
GAGGACGATGACATGGGGGACCTGGTGGATGCTGAGGAGTATCTGGTACCCCAGC
AGGGCTTCTTCTGTCCAGACCCTGCCCCGGGCGCTGGGGGCATGGTCCACCACAG
GCACCGCAGCTCATCTACCAGGAGTGGCGGTGGGGACCTGACACTAGGGCTGGAG
CCCTCTGAAGAGGAGGCCCCCAGGTCTCCACTGGCACCCTCCGAAGGGGCTGGCT
CCGATGTATTTGATGGTGACCTGGGAATGGGGGCAGCCAAGGGGCTGCAAAGCCT
CCCCACACATGACCCCAGCCCTCTACAGCGGTACAGTGAGGACCCCACAGTACCC
CTGCCCTCTGAGACTGATGGCTACGTTGCCCCCCTGACCTGCAGCCCCCAGCCTGA
ATATGTGAACCAGCCAGATGTTCGGCCCCAGCCCCCTTCGCCCCGAGAGGGCCCT
CTGCCTGCTGCCCGACCTGCTGGTGCCACTCTGGAAAGGGCCAAGACTCTCTCCCC
AGGGAAGAATGGGGTCGTCAAAGACGTTTTTGCCTTTGGGGGTGCCGTGGAGAAC
CCCGAGTACTTGACACCCCAGGGAGGAGCTGCCCCTCAGCCCCACCCTCCTCCTGC
CTTCAGCCCAGCCTTCGACAACCTCTATTACTGGGACCAGGACCCACCAGAGCGG
GGGGCTCCACCCAGCACCTTCAAAGGGACACCTACGGCAGAGAACCCAGAGTAC
CTGGGTCTGGACGTGCCAGTGTGAACCAGAAGGCCAAGTCCGCAGAAGCCCTGA
(SEQ ID NO: 60).

[00204] In another embodiment, the nucleic acid sequence encoding the human her2/neu EC1 fragment implemented into the chimera spans from 120-510 bp of the human EC1 region and is set forth in (SEQ ID NO: 61).

[00205] GAGACCCACCTGGACATGCTCCGCCACCTCTACCAGGGCTGCCAGGTGGT

GCAGGGAAACCTGGAACTCACCTACCTGCCCACCAATGCCAGCCTGTCCTTCCTGC
AGGATATCCAGGAGGTGCAGGGCTACGTGCTCATCGCTCACAACCAAGTGAGGCA
GGTCCCACTGCAGAGGCTGCGGATTGTGCGAGGCACCCAGCTCTTTGAGGACAAC
TATGCCCTGGCCGTGCTAGACAATGGAGACCCGCTGAACAATACCACCCCTGTCA
CAGGGGCCTCCCCAGGAGGCCTGCGGGAGCTGCAGCTTCGAAGCCTCACAGAGAT
CTTGAAAGGAGGGGTCTTGATCCAGCGGAACCCCCAGCTCTGCTACCAGGACACG
ATTTTGTGGAAG (SEQ ID NO: 61).

[00206] In one embodiment, the complete EC1 human her2/neu fragment spans from (58-979 bp of the human her2/neu gene and is set forth in (SEQ ID NO: 62).

[00207] GCCGCGAGCACCCAAGTGTGCACCGGCACAGACATGAAGCTGCGGCTCCC
TGCCAGTCCCGAGACCCACCTGGACATGCTCCGCCACCTCTACCAGGGCTGCCAG
GTGGTGCAGGGAAACCTGGAACTCACCTACCTGCCCACCAATGCCAGCCTGTCCT
TCCTGCAGGATATCCAGGAGGTGCAGGGCTACGTGCTCATCGCTCACAACCAAGT
GAGGCAGGTCCCACTGCAGAGGCTGCGGATTGTGCGAGGCACCCAGCTCTTTGAG
GACAACTATGCCCTGGCCGTGCTAGACAATGGAGACCCGCTGAACAATACCACCC
CTGTCACAGGGGCCTCCCCAGGAGGCCTGCGGGAGCTGCAGCTTCGAAGCCTCAC
AGAGATCTTGAAAGGAGGGGTCTTGATCCAGCGGAACCCCCAGCTCTGCTACCAG
GACACGATTTTGTGGAAGGACATCTTCCACAAGAACAACCAGCTGGCTCTCACAC
TGATAGACACCAACCGCTCTCGGGCCTGCCACCCCTGTTCTCCGATGTGTAAGGGC
TCCCGCTGCTGGGGAGAGAGTTCTGAGGATTGTCAGAGCCTGACGCGCACTGTCT
GTGCCGGTGGCTGTGCCCGCTGCAAGGGGCCACTGCCCACTGACTGCTGCCATGA
GCAGTGTGCTGCCGGCTGCACGGGCCCCAAGCACTCTGACTGCCTGGCCTGCCTCC
ACTTCAACCACAGTGGCATCTGTGAGCTGCACTGCCCAGCCCTGGTCACCTACAAC
ACAGACACGTTTGAGTCCATGCCCAATCCCGAGGGCCGGTATACATTCGGCGCCA
GCTGTGTGACTGCCTGTCCCTACAACTACCTTTCTACGGACGTGGGATCCTGCACC
CTCGTCTGCCCCCTGCACAACCAAGAGGTGACAGCAGAGGAT (SEQ ID NO: 62).

[00208] In another embodiment, the nucleic acid sequence encoding the human her2/neu EC2 fragment implemented into the chimera spans from 1077-1554 bp of the human her2/neu EC2 fragment and includes a 50 bp extension, and is set forth in (SEQ ID NO: 63).

[00209] AATATCCAGGAGTTTGCTGGCTGCAAGAAGATCTTTGGGAGCCTGGCATT
TCTGCCGGAGAGCTTTGATGGGGACCCAGCCTCCAACACTGCCCCGCTCCAGCCA
GAGCAGCTCCAAGTGTTTGAGACTCTGGAAGAGATCACAGGTTACCTATACATCT
CAGCATGGCCGGACAGCCTGCCTGACCTCAGCGTCTTCCAGAACCTGCAAGTAAT
CCGGGGACGAATTCTGCACAATGGCGCCTACTCGCTGACCCTGCAAGGGCTGGGC
ATCAGCTGGCTGGGGCTGCGCTCACTGAGGGAACTGGGCAGTGGACTGGCCCTCA

TCCACCATAACACCCACCTCTGCTTCGTGCACACGGTGCCCTGGGACCAGCTCTTT
CGGAACCCGCACCAAGCTCTGCTCCACACTGCCAACCGGCCAGAGGACGAGTGTG
TGGGCGAGGGCCTGGCCTGCCACCAGCTGTGCGCCCGAGGG (SEQ ID NO: 63).

[00210] In one embodiment, complete EC2 human her2/neu fragment spans from 907-1504 bp of the human her2/neu gene and is set forth in (SEQ ID NO: 64).

[00211] TACCTTTCTACGGACGTGGGATCCTGCACCCTCGTCTGCCCCCTGCACAAC
CAAGAGGTGACAGCAGAGGATGGAACACAGCGGTGTGAGAAGTGCAGCAAGCCC
TGTGCCCGAGTGTGCTATGGTCTGGGCATGGAGCACTTGCGAGAGGTGAGGGCAG
TTACCAGTGCCAATATCCAGGAGTTTGCTGGCTGCAAGAAGATCTTTGGGAGCCT
GGCATTTCTGCCGGAGAGCTTTGATGGGGACCCAGCCTCCAACACTGCCCCGCTCC
AGCCAGAGCAGCTCCAAGTGTTTGAGACTCTGGAAGAGATCACAGGTTACCTATA
CATCTCAGCATGGCCGGACAGCCTGCCTGACCTCAGCGTCTTCCAGAACCTGCAA
GTAATCCGGGGACGAATTCTGCACAATGGCGCCTACTCGCTGACCCTGCAAGGGC
TGGGCATCAGCTGGCTGGGGCTGCGCTCACTGAGGGAACTGGGCAGTGGACTGGC
CCTCATCCACCATAACACCCACCTCTGCTTCGTGCACACGGTGCCCTGGGACCAGC
TCTTTCGGAACCCGCACCAAGCTCTGCTCCACACTGCCAACCGGCCAGAG (SEQ ID NO: 64).

[00212] In another embodiment, the nucleic acid sequence encoding the human her2/neu IC1 fragment implemented into the chimera is set forth in (SEQ ID NO: 65).

[00213] CAGCAGAAGATCCGGAAGTACACGATGCGGAGACTGCTGCAGGAAACGG
AGCTGGTGGAGCCGCTGACACCTAGCGGAGCGATGCCCAACCAGGCGCAGATGC
GGATCCTGAAAGAGACGGAGCTGAGGAAGGTGAAGGTGCTTGGATCTGGCGCTTT
TGGCACAGTCTACAAGGGCATCTGGATCCCTGATGGGGAGAATGTGAAAATTCCA
GTGGCCATCAAAGTGTTGAGGGAAAACACATCCCCCAAAGCCAACAAAGAAATC
TTAGACGAAGCATACGTGATGGCTGGTGTGGGCTCCCCATATGTCTCCCGCCTTCT
GGGCATCTGCCTGACATCCACGGTGCAGCTGGTGACACAGCTTATGCCCTATGGCT
GCCTCTTAGACT (SEQ ID NO: 65).

[00214] In another embodiment, the nucleic acid sequence encoding the complete human her2/neu IC1 fragment spans from 2034-3243 of the human her2/neu gene and is set forth in (SEQ ID NO: 66).

[00215] CAGCAGAAGATCCGGAAGTACACGATGCGGAGACTGCTGCAGGAAACGG
AGCTGGTGGAGCCGCTGACACCTAGCGGAGCGATGCCCAACCAGGCGCAGATGC
GGATCCTGAAAGAGACGGAGCTGAGGAAGGTGAAGGTGCTTGGATCTGGCGCTTT
TGGCACAGTCTACAAGGGCATCTGGATCCCTGATGGGGAGAATGTGAAAATTCCA
GTGGCCATCAAAGTGTTGAGGGAAAACACATCCCCCAAAGCCAACAAAGAAATC

TTAGACGAAGCATACGTGATGGCTGGTGTGGGCTCCCCATATGTCTCCCGCCTTCT
GGGCATCTGCCTGACATCCACGGTGCAGCTGGTGACACAGCTTATGCCCTATGGCT
GCCTCTTAGACCATGTCCGGGAAAACCGCGGACGCCTGGGCTCCCAGGACCTGCT
GAACTGGTGTATGCAGATTGCCAAGGGGATGAGCTACCTGGAGGATGTGCGGCTC
GTACACAGGGACTTGGCCGCTCGGAACGTGCTGGTCAAGAGTCCCAACCATGTCA
AAATTACAGACTTCGGGCTGGCTCGGCTGCTGGACATTGACGAGACAGAGTACCA
TGCAGATGGGGGCAAGGTGCCCATCAAGTGGATGGCGCTGGAGTCCATTCTCCGC
CGGCGGTTCACCCACCAGAGTGATGTGTGGAGTTATGGTGTGACTGTGTGGGAGC
TGATGACTTTTGGGGCCAAACCTTACGATGGGATCCCAGCCCGGGAGATCCCTGA
CCTGCTGGAAAAGGGGGAGCGGCTGCCCCAGCCCCCCATCTGCACCATTGATGTC
TACATGATCATGGTCAAATGTTGGATGATTGACTCTGAATGTCGGCCAAGATTCCG
GGAGTTGGTGTCTGAATTCTCCCGCATGGCCAGGGACCCCCAGCGCTTTGTGGTCA
TCCAGAATGAGGACTTGGGCCCAGCCAGTCCCTTGGACAGCACCTTCTACCGCTC
ACTGCTGGAGGACGATGACATGGGGGACCTGGTGGATGCTGAGGAGTATCTGGTA
CCCCAGCAGGGCTTCTTCTGTCCAGACCCTGCCCCGGGCGCTGGGGGCATGGTCC
ACCACAGGCACCGCAGCTCATCTACCAGGAGTGGCGGTGGGGACCTGACACTAGG
GCTGGAGCCCTCTGAAGAGGAGGCCCCCAGGTCTCCACTGGCACCCTCCGAAGGG
GCT (SEQ ID NO: 66).

[00216] Point mutations or amino-acid deletions in the oncogenic protein Her2/neu, have been reported to mediate treatment of resistant tumor cells, when these tumors have been targeted by small fragment *Listeria*-based vaccines or trastuzumab (a monoclonal antibody against an epitope located at the extracellular domain of the Her2/neu antigen). Described herein is a chimeric Her2/neu based composition which harbors two of the extracellular and one intracellular fragments of Her2/neu antigen showing clusters of MHC-class I epitopes of the oncogene. This chimeric protein, which harbors 3 H2Dq and at least 17 of the mapped human MHC-class I epitopes of the Her2/neu antigen was fused to the first 441 amino acids of the *Listeria-monocytogenes* listeriolysin O protein and expressed and secreted by the *Listeria monocytogenes* attenuated strain *LmddA*.

[00217] In another embodiment, the antigen of interest is a KLK9 polypeptide.

[00218] 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. 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, SCCE, WT-1, HIV-1 Gag, Proteinase 3, Tyrosinase related protein 2, PSA (prostate-specific antigen). In another embodiment, the antigen is a tumor-associated antigen. In another embodiment, the antigen is an infectious disease antigen.

[00219] 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 angiogeneic vasculature, which in another embodiment, is associated with neovascularization *in vivo*. In another embodiment, the angiogenic antigen is HMW-MAA. In another embodiment, the angiogenic antigen is one known in the art and are provided in WO2010/102140, which is incorporated by reference herein.

[00220] In other embodiments, the antigen is 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.

[00221] 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 erthematosus, 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 circumsporozite protein, microbial antigens, viral antigens, autoantigens, and lesteriosis.

[00222] In another embodiment, the heterologous antigen disclosed herein is a tumor-associated antigen, which in one embodiment, is one of the following tumor antigens: a MAGE (Melanoma-Associated Antigen E) protein, e.g. MAGE 1, MAGE 2, MAGE 3, MAGE 4, a tyrosinase; a mutant ras protein; a mutant p53 protein; p97 melanoma antigen, a ras peptide or p53 peptide associated with advanced cancers; the HPV 16/18 antigens associated with cervical cancers, KLH antigen associated with breast carcinoma, CEA (carcinoembryonic antigen) associated with colorectal cancer, gp100, a MART1 antigen associated with melanoma, or the PSA antigen associated with prostate cancer. In another embodiment, the antigen for the compositions and methods 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. In another embodiment, the tumor associated antigen is an angiogenic antigen.

[00223] In another embodiment, the heterologous antigen is an infectious disease antigen. In one embodiment, the antigen is an auto antigen or a self-antigen.

[00224] In another embodiment, the heterologous antigen is 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, human papilloma virus antigens E1 and E2 from type HPV-16, -18, -31, -33, -35 or -45 human papilloma viruses, or a combination thereof.

[00225] In another embodiments, the heterologous 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 cough3 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 erthematosus, 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 circumsporozite protein, microbial antigens, viral antigens, autoantigens, and lesteriosis.

[00226] In another embodiment of the methods and compositions as disclosed herein, “nucleic acids” or “nucleotide” refers to a string of at least two base-sugar-phosphate combinations. The term includes, in one embodiment, DNA and RNA. “Nucleotides” refers, in one embodiment, to the monomeric units of nucleic acid polymers. RNA may be, in one embodiment, in the form of a tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, small inhibitory RNA (siRNA), micro RNA (miRNA) and ribozymes. The use of siRNA and miRNA has been described (Caudy AA et al, Genes & Devel 16: 2491-96 and references cited therein). DNA may be in form of plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition, these forms of DNA and RNA may be single, double, triple, or quadruple stranded. The term also includes, in another embodiment, artificial nucleic acids that may contain other types of backbones but the same bases. In one embodiment, the artificial nucleic acid is a PNA (peptide nucleic acid). PNA contain peptide backbones and nucleotide bases and are able to bind, in one embodiment, to both DNA and RNA molecules. In another embodiment, the nucleotide is oxetane modified. In another embodiment, the nucleotide is modified by replacement of one or more phosphodiester bonds with a phosphorothioate bond. In another embodiment, the artificial nucleic acid contains any other variant of the phosphate backbone of native nucleic acids known in the art. The use of phosphothiorate nucleic acids and PNA are known to those skilled in the art, and are described in, for example, Neilsen PE, Curr Opin Struct Biol 9:353-57; and Raz NK et al Biochem Biophys Res Commun. 297:1075-84. The production and use of nucleic acids is known to those skilled in art and is described, for example, in Molecular Cloning, (2001), Sambrook and

Russell, eds. and Methods in Enzymology: Methods for molecular cloning in eukaryotic cells (2003) Purchio and G. C. Fareed.

[00227] In one embodiment, the term "oligonucleotide" is interchangeable with the term "nucleic acid", and may refer to a molecule, which may include, but is not limited to, prokaryotic sequences, eukaryotic mRNA, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. The term also refers to sequences that include any of the known base analogs of DNA and RNA.

[00228] 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.

[00229] In another embodiment, the construct or nucleic acid molecule disclosed herein 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.

[00230] 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.

[00231] In another embodiment, the construct or nucleic acid molecule is integrated into the

Listerial chromosome using phage integration sites (Lauer P, Chow MY et al, Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J Bacteriol 2002; 184(15): 4177-86). In certain embodiments of this method, an integrase gene and attachment site of a bacteriophage (e.g. U153 or PSA listeriophage) is used to insert the heterologous gene into the corresponding attachment site, which may be any appropriate site in the genome (e.g. comK or the 3' end of the arg tRNA gene). In another embodiment, endogenous prophages are cured from the attachment site utilized prior to integration of the construct or heterologous gene. In another embodiment, this method results in single-copy integrants. In another embodiment, the disclosure further comprises a phage based chromosomal integration system for clinical applications, where a host strain that is auxotrophic for essential enzymes, including, but not limited to, d-alanine racemase can be used, for example *Lm*dal(-)dat(-). In another embodiment, in order to avoid a "phage curing step," a phage integration system based on PSA is used. This requires, in another embodiment, continuous selection by antibiotics to maintain the integrated gene. Thus, in another embodiment, the current disclosure enables the establishment of a phage based chromosomal integration system that does not require selection with antibiotics. Instead, an auxotrophic host strain can be complemented.

[00232] In one embodiment of the methods and compositions as disclosed herein, the term "recombination site" or "site-specific recombination site" refers to a sequence of bases in a nucleic acid molecule that is recognized by a recombinase (along with associated proteins, in some cases) that mediates exchange or excision of the nucleic acid segments flanking the recombination sites. The recombinases and associated proteins are collectively referred to as "recombination proteins" see, e.g., Landy, A., (Current Opinion in Genetics & Development) 3:699-707; 1993).

[00233] A "phage expression vector" or "phagemid" refers to any phage-based recombinant expression system for the purpose of expressing a nucleic acid sequence of the methods and compositions as disclosed herein *in vitro* or *in vivo*, constitutively or inducibly, in any cell, including prokaryotic, yeast, fungal, plant, insect or mammalian cell. A phage expression vector typically can both reproduce in a bacterial cell and, under proper conditions, produce phage particles. The term includes linear or circular expression systems and encompasses both phage-based expression vectors that remain episomal or integrate into the host cell genome.

[00234] In one embodiment, the term "operably linked" as used herein means that the transcriptional and translational regulatory nucleic acid, is positioned relative to any coding sequences in such a manner that transcription is initiated. Generally, this will mean that the promoter and transcriptional initiation or start sequences are positioned 5' to the coding region.

[00235] In one embodiment, an "open reading frame" or "ORF" is a portion of an organism's genome which contains a sequence of bases that could potentially encode a protein. In another embodiment, the start and stop ends of the ORF are not equivalent to the ends of the mRNA, but they are usually contained within the mRNA. In one embodiment, ORFs are located between the start-code sequence (initiation codon) and the stop-codon sequence (termination codon) of a gene. Thus, in one embodiment, a nucleic acid molecule operably integrated into a genome as an open reading frame with an endogenous polypeptide is a nucleic acid molecule that has integrated into a genome in the same open reading frame as an endogenous polypeptide.

[00236] In one embodiment, the disclosure provides a fusion polypeptide comprising a linker sequence. In one embodiment, a "linker sequence" refers to an amino acid sequence that joins two heterologous polypeptides, or fragments or domains thereof. In general, as used herein, a linker is an amino acid sequence that covalently links the polypeptides to form a fusion polypeptide. A linker typically includes the amino acids translated from the remaining recombination signal after removal of a reporter gene from a display vector to create a fusion protein comprising an amino acid sequence encoded by an open reading frame and the display protein. As appreciated by one of skill in the art, the linker can comprise additional amino acids, such as glycine and other small neutral amino acids.

[00237] In one embodiment, "endogenous" as used herein describes an item that has developed or originated within the reference organism or arisen from causes within the reference organism. In another embodiment, endogenous refers to native.

[00238] "Stably maintained" refers, in another embodiment, to maintenance of a nucleic acid molecule or plasmid in the absence of selection (e.g. antibiotic selection) for 10 generations, without detectable loss. In another embodiment, the period is 15 generations. In another embodiment, the period is 20 generations. In another embodiment, the period is 25 generations. In another embodiment, the period is 30 generations. In another embodiment, the period is 40 generations. In another embodiment, the period is 50 generations. In another embodiment, the period is 60 generations. In another embodiment, the period is 80 generations. In another embodiment, the period is 100 generations. In another embodiment, the period is 150 generations. In another embodiment, the period is 200 generations. In another embodiment, the period is 300 generations. In another embodiment, the period is 500 generations. In another embodiment, the period is more than generations. In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vitro* (e.g. in culture). In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vivo*. In another embodiment, the nucleic acid molecule or plasmid is maintained stably both *in vitro* and *in vitro*. Each possibility

represents a separate embodiment of the disclosure.

[00239] In another embodiment, a recombinant *Listeria* strain of the methods and compositions as disclosed herein comprise a nucleic acid molecule operably integrated into the *Listeria* genome as an open reading frame with an endogenous ActA sequence. In another embodiment, a recombinant *Listeria* strain of the methods and compositions as disclosed herein comprise an episomal expression vector comprising a nucleic acid molecule encoding fusion protein comprising an antigen fused to an ActA or a truncated ActA. In one embodiment, the expression and secretion of the antigen is under the control of an *actA* promoter and ActA signal sequence and it is expressed as fusion to 1-233 amino acids of ActA (truncated ActA or tActA). In another embodiment, the truncated ActA consists of the first 390 amino acids of the wild type ActA protein as described in US Patent Serial No. 7,655,238, which is incorporated by reference herein in its entirety. In another embodiment, the truncated ActA is an ActA-N100 or a modified version thereof (referred to as ActA-N100*) in which a PEST motif has been deleted and containing the nonconservative QDNKR substitution as described in US Patent Publication Serial No. 2014/0186387.

[00240] In another embodiment, a "functional fragment" is an immunogenic fragment and elicits an immune response when administered to a subject alone or in a vaccine composition disclosed herein. In another embodiment, a functional fragment has biological activity as will be understood by a skilled artisan and as further disclosed herein.

[00241] The recombinant *Listeria* strain of methods and compositions of the disclosure is, in another embodiment, a recombinant *Listeria monocytogenes* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria seeligeri* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria grayi* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria ivanovii* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria murrayi* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria welshimeri* strain. In another embodiment, the *Listeria* strain is a recombinant strain of any other *Listeria* species known in the art.

[00242] In another embodiment, a recombinant *Listeria* strain of the disclosure has been passaged through an animal host. In another embodiment, the passaging maximizes efficacy of the strain as a vaccine vector. In another embodiment, the passaging stabilizes the immunogenicity of the *Listeria* strain. In another embodiment, the passaging stabilizes the virulence of the *Listeria* strain. In another embodiment, the passaging increases the immunogenicity of the *Listeria* strain. In another embodiment, the passaging increases the virulence of the *Listeria* strain. In another embodiment, the passaging removes unstable sub-strains of the *Listeria* strain. In another embodiment, the passaging reduces the prevalence of

unstable sub-strains of the *Listeria* strain. In another embodiment, the *Listeria* strain contains a genomic insertion of the gene encoding the antigen-containing recombinant peptide. In another embodiment, the *Listeria* strain carries a plasmid comprising the gene encoding the antigen-containing recombinant peptide. In another embodiment, the passaging is performed as described herein. In another embodiment, the passaging is performed by any other method known in the art.

[00243] In another embodiment, a recombinant nucleic acid of the disclosure is operably linked to a promoter/regulatory sequence that drives expression of the encoded peptide in the *Listeria* strain. Promoter/regulatory sequences useful for driving constitutive expression of a gene are well known in the art and include, but are not limited to, for example, the P_{hlyA}, P_{ActA}, and p60 promoters of *Listeria*, the *Streptococcus* bac promoter, the *Streptomyces griseus* sgiA promoter, and the *B. thuringiensis* phaZ promoter.

[00244] In another embodiment, inducible and tissue specific expression of the nucleic acid encoding a peptide of the disclosure is accomplished by placing the nucleic acid encoding the peptide under the control of an inducible or tissue specific promoter/regulatory sequence. Examples of tissue specific or inducible promoter/regulatory sequences which are useful for his purpose include, but are not limited to the MMTV LTR inducible promoter, and the SV40 late enhancer/promoter. In another embodiment, a promoter that is induced in response to inducing agents such as metals, glucocorticoids, and the like, is utilized. Thus, it will be appreciated that the disclosure includes the use of any promoter/regulatory sequence, which is either known or unknown, and which is capable of driving expression of the desired protein operably linked thereto. It will be appreciated by a skilled artisan that the term "heterologous" encompasses a nucleic acid, amino acid, peptide, polypeptide, or protein derived from a different species than the reference species. Thus, for example, a *Listeria* strain expressing a heterologous polypeptide, in one embodiment, would express a polypeptide that is not native or endogenous to the *Listeria* strain, or in another embodiment, a polypeptide that is not normally expressed by the *Listeria* strain, or in another embodiment, a polypeptide from a source other than the *Listeria* strain. In another embodiment, heterologous may be used to describe something derived from a different organism within the same species. In another embodiment, the heterologous antigen is expressed by a recombinant strain of *Listeria*, and is processed and presented to cytotoxic T-cells upon infection of mammalian cells by the recombinant strain. In another embodiment, the heterologous antigen expressed by *Listeria* species need not precisely match the corresponding unmodified antigen or protein in the tumor cell or infectious agent so long as it results in a T-cell response that recognizes the unmodified antigen or protein which is naturally expressed in the mammal. The term heterologous antigen may be referred to herein as

"antigenic polypeptide", "heterologous protein", "heterologous protein antigen", "protein antigen", "antigen", and the like.

[00245] It will be appreciated by the skilled artisan that the term "episomal expression vector" encompasses a nucleic acid vector which may be linear or circular, and which is usually double-stranded in form and is extrachromosomal in that it is present in the cytoplasm of a host bacteria or cell as opposed to being integrated into the bacteria's or cell's genome. In one embodiment, an episomal expression vector comprises a gene of interest. In another embodiment, episomal vectors persist in multiple copies in the bacterial cytoplasm, resulting in amplification of the gene of interest, and, in another embodiment, viral trans-acting factors are supplied when necessary. In another embodiment, the episomal expression vector may be referred to as a plasmid herein. In another embodiment, an "integrative plasmid" comprises sequences that target its insertion or the insertion of the gene of interest carried within into a host genome. In another embodiment, an inserted gene of interest is not interrupted or subjected to regulatory constraints which often occur from integration into cellular DNA. In another embodiment, the presence of the inserted heterologous gene does not lead to rearrangement or interruption of the cell's own important regions. In another embodiment, in stable transfection procedures, the use of episomal vectors often results in higher transfection efficiency than the use of chromosome-integrating plasmids (Belt, P.B.G.M., et al (1991) Efficient cDNA cloning by direct phenotypic correction of a mutant human cell line (HPRT2) using an Epstein-Barr virus-derived cDNA expression vector. Nucleic Acids Res. 19, 4861-4866; Mazda, O., et al. (1997) Extremely efficient gene transfection into lympho-hematopoietic cell lines by Epstein-Barr virus-based vectors. J. Immunol. Methods 204, 143-151). In one embodiment, the episomal expression vectors of the methods and compositions as disclosed herein may be delivered to cells in vivo, ex vivo, or in vitro by any of a variety of the methods employed to deliver DNA molecules to cells. The vectors may also be delivered alone or in the form of a pharmaceutical composition that enhances delivery to cells of a subject.

[00246] In one embodiment, the term "fused" refers to operable linkage by covalent bonding. In one embodiment, the term includes recombinant fusion (of nucleic acid sequences or open reading frames thereof). In another embodiment, the term includes chemical conjugation.

[00247] "Transforming," in one embodiment, 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.

[00248] 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).

[00249] In one embodiment, the term "attenuation," refers to a diminution in the ability of the bacterium to cause disease in an animal. In other words, the pathogenic characteristics of the attenuated *Listeria* strain have been lessened compared with wild-type *Listeria*, although the attenuated *Listeria* is capable of growth and maintenance in culture. Using as an example the intravenous inoculation of Balb/c mice with an attenuated *Listeria*, the lethal dose at which 50% of inoculated animals survive (LD_{50}) is preferably increased above the LD_{50} of wild-type *Listeria* by at least about 10-fold, more preferably by at least about 100-fold, more preferably at least about 1,000 fold, even more preferably at least about 10,000 fold, and most preferably at least about 100,000-fold. An attenuated strain of *Listeria* is thus one which does not kill an animal to which it is administered, or is one which kills the animal only when the number of bacteria administered is vastly greater than the number of wild type non-attenuated bacteria which would be required to kill the same animal. An attenuated bacterium should also be construed to mean one which is incapable of replication in the general environment because the nutrient required for its growth is not present therein. Thus, the bacterium is limited to replication in a controlled environment wherein the required nutrient is provided. The attenuated strains of the disclosure are therefore environmentally safe in that they are incapable of uncontrolled replication.

Compositions

[00250] In one embodiment, compositions of the disclosure are immunogenic compositions. In one embodiment, compositions of the disclosure induce a strong innate stimulation of interferon-gamma, which in one embodiment, has anti-angiogenic properties. In one embodiment, a *Listeria* of the disclosure induces a strong innate stimulation of 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.

[00251] In another embodiment, administration of compositions of the 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 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. 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.

[00252] 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.

[00253] Compositions disclosed herein may be used in methods disclosed herein in order to elicit an enhanced anti-tumor T cell response in a subject, in order to inhibit tumor –medicated immunosuppression in a subject, or for increasing the ratio or T effector cells to regulatory T cells (Tregs) in the spleen and tumor of a subject, or any combination thereof.

[00254] In another embodiment, a composition comprising a Listeria strain of the disclosure further comprises an adjuvant. In one embodiment, a composition of the disclosure further comprises an adjuvant. The adjuvant utilized in methods and compositions of the 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.

[00255] In one embodiment, an immunogenic composition disclosed herein comprises a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof. In one embodiment, an immunogenic composition disclosed herein comprises a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, said composition further comprising an additional active agent. In one embodiment said additional active agent comprises an oncolytic virus. In another embodiment, the additional active agent comprises a T cell receptor engineered T cell (Receptor engineered T cells). In another embodiment, the additional active agent comprises a chimeric antigen receptor engineered cells (CAR T cells). In another embodiment, the additional active agent comprises a therapeutic or immunomodulating monoclonal antibody. In another embodiment, the additional active agent comprises a targeting thymidine kinase inhibitor (TKI). In another embodiment, the additional active agent comprises an adoptively transferred cell incorporating engineered T cell receptors. In another embodiment, an additional active agent disclosed herein comprises an attenuated oncolytic virus, a T cell receptor engineered T cell (Receptor engineered T cells), a chimeric antigen receptor engineered T cell (CAR T cells), a therapeutic or immunomodulating monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or an adoptively transferred cells incorporating engineered T cell receptors, or any combination thereof.

[00256] In one embodiment, the Receptor engineered T cells comprises a receptor engineered to have a selected specificity. In another embodiment, both polypeptides of the engineered receptor have been recombinantly engineered to have a selected specificity. In one embodiment, selected specificity is to cell-surface tumor ligands.

[00257] In one embodiment, the Receptor engineered T cells are autologous. In another embodiment, the Receptor engineered T cells are allogeneic.

[00258] In one embodiment, the CAR T cell is an autologous CAR T cell. In another embodiment, the CAR T cell is allogeneic. In another embodiment, the CART T cell is a single-source CAR T cell. In another embodiment, the CAR T cell is an autologous HLA masked CAR T cell. In another embodiment, the CAR T cell is an autologous HLA deleted CAR T cell. In

another embodiment, the CAR T cell is a single-source HLA masked CAR T cell. In another embodiment, the CART T cell is a single-source HLA deleted CAR T cell.

[00259] In one embodiment, an additional active agent is an oncolytic virus. The term "oncolytic virus" refers in one embodiment to a genetically engineered virus capable of selectively replicating in and slowing the growth of or inducing the death of a cancerous or hyperproliferative cell, either *in vitro* or *in vivo*, while having no or minimal effect on normal cells.

[00260] In one embodiment, the oncolytic virus is selected from the group comprising a vesicular stomatitis virus (VSV), a newcastle disease virus (NDV), a retrovirus, a reovirus, a measles virus, a sinbis virus, an influenza virus, a herpes simplex virus, a vaccinia virus, and an adenovirus. In one embodiment, the oncolytic virus infects tumor cells. In some embodiments, the oncolytic virus infects prostate tumor cells. In other embodiments, the oncolytic virus infects cervical cancer tumor cells. In one embodiment, an oncolytic virus comprises a nucleic acid sequence encoding a heterologous antigen. In one embodiment, the heterologous antigen is a tumor associated antigen or fragment thereof. In one embodiment, the heterologous antigen is a PSA antigen or fragment thereof, a HPV antigen or a fragment thereof or a chimeric Her-2/neu antigen or fragment thereof. In another embodiment, the heterologous antigen is a programmed cell death receptor (PD-1) binding agonist or antagonist.

[00261] In one embodiment, the therapeutic or immunomodulating monoclonal antibody recognizes an epitope of said heterologous antigen present on the surface of a cancer cell. In one embodiment, the heterologous antigen is a tumor associated antigen. In one embodiment, the heterologous antigen is a PSA antigen, a HPV antigen, or a chimeric Her-2/neu antigen. In one embodiment, the monoclonal antibody recognizes a PSA epitope. In one embodiment, the monoclonal antibody recognizes an HPV epitope. In one embodiment, the monoclonal antibody recognizes a Her-2/neu epitope. In another embodiment, the monoclonal antibody recognizing a Her-2/neu epitope comprises trastuzuman (trademarked as Heceptin®), panitumumab, or any other known in the art to recognize a Her-2/neu epitope. In another embodiment, the therapeutic or immunomodulating monoclonal antibody recognizes an epitope that is not present on said heterologous antigen.

[00262] In some embodiments, the term "antibody" refers to intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv that are capable of specifically interacting with a desired target as described herein, for example, binding to phagocytic cells. In some embodiments, the antibody fragments comprise:

[00263] (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, which can be produced by digestion of whole antibody with the enzyme

papain to yield an intact light chain and a portion of one heavy chain;

[00264] (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

[00265] (3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bonds;

[00266] (4) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

[00267] (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

[00268] Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

[00269] In some embodiments, the antibody fragments may be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment.

[00270] Antibody fragments can, in some embodiments, be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R., Biochem. J., 73: 119-126, 1959. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

[00271] Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar *et al.*, Proc. Nat'l Acad. Sci. USA 69:2659-62, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL

chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow and Filpula, Methods, 2: 97-105, 1991; Bird *et al.*, Science 242:423-426, 1988; Pack *et al.*, Bio/Technology 11:1271-77, 1993; and Ladner *et al.*, U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

[00272] Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry, Methods, 2: 106-10, 1991.

[00273] In some embodiments, the antibodies or fragments described herein comprise "humanized forms" of antibodies. In some embodiments, the term "humanized forms of antibodies" refers to non-human (e.g. murine) antibodies, which are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

[00274] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which

is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature 332:323-327 (1988); Verhoeyen *et al.*, Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[00275] Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks *et al.*, J. Mol. Biol., 222:581 (1991)]. The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, J. Immunol., 147(1):86-95 (1991)]. Similarly, human can be made by introducing of human immunoglobulin loci into transgenic animals, e.g. mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, Bio/Technology 10, 779-783 (1992); Lonberg *et al.*, Nature 368 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild *et al.*, Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14, 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13 65-93 (1995).

[00276] The term "epitope" or antigenic determinant" refers to a site on an antigen to which an immunoglobulin or antibody, or fragment thereof, specifically binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from continuous aminio acis are typically retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance.

[00277] In one embodiment, compositions disclosed herein comprise a therapeutic or

immunomodulating monoclonal antibody. In another embodiment, a composition disclosed herein comprises an Lm strain and a therapeutic or immunomodulating monoclonal antibody. In another embodiment, a composition disclosed herein comprises a therapeutic or immunomodulating monoclonal antibody, wherein the composition does not include a *Listeria* strain disclosed herein.

[00278] In one embodiment, the thymidine kinase inhibitor comprises imatinib mesylate (IM), dasatinib (D), nilotinib (N) bosutinib (B) or INNO 406, or any combination thereof.

[00279] In one embodiment, compositions disclosed herein comprise a targeting thymidine kinase inhibitor (TKI). In another embodiment, a composition disclosed herein comprises an Lm strain and a targeting thymidine kinase inhibitor (TKI). In another embodiment, a composition disclosed herein comprises a targeting thymidine kinase inhibitor (TKI), wherein the composition does not include a *Listeria* strain disclosed herein.

[00280] In one embodiment, the disease disclosed herein is a cancer or a tumor. In one embodiment, the cancer treated by a method of the disclosure is breast cancer. In another embodiment, the cancer is a cervical 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 multiform. 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. In another embodiment, the cancer is oropharyngeal cancer. In another embodiment, the cancer is lung cancer. In another embodiment, the cancer is anal cancer. In another embodiment, the cancer is colorectal cancer. In another embodiment, the cancer is esophageal cancer. In another embodiment, the cancer is mesothelioma.

[00281] In one embodiment the heterologous antigen is PD-1 or an immunogenic fragment thereof. In another embodiment, the heterologous antigen is a PD-1 antagonist. In one embodiment, the PD-1 antagonist is selected from the group comprising an antibody or fragment

thereof, an PD-1 antagonist or a fragment thereof, or a PD-1 partial antagonist or fragment thereof, or any combination thereof.

[00282] In another embodiment, the antigen is HPV-E7. In another embodiment, the antigen is HPV-E6. In another embodiment, the antigen is Her-2/neu. In another embodiment, the antigen is NY-ESO-1. In another embodiment, the antigen is telomerase (TERT). In another embodiment, the antigen is SCCE. In another embodiment, the antigen is CEA. In another embodiment, the antigen is LMP-1. In another embodiment, the antigen is p53. In another embodiment, the antigen is carboxic anhydrase IX (CAIX). In another embodiment, the antigen is PSMA. In another embodiment, the antigen is prostate stem cell antigen (PSCA). In another embodiment, the antigen is HMW-MAA. In another embodiment, the antigen is WT-1. In another embodiment, the antigen is HIV-1 Gag. In another embodiment, the antigen is Proteinase 3. In another embodiment, the antigen is Tyrosinase related protein 2. In another embodiment, the antigen is PSA (prostate-specific antigen). In another embodiment, the antigen is selected from HPV-E7, HPV-E6, Her-2, NY-ESO-1, telomerase (TERT), SCCE, HMW-MAA, EGFR-III, survivin, baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), WT-1, HIV-1 Gag, CEA, LMP-1, p53, PSMA, PSCA, Proteinase 3, Tyrosinase related protein 2, Muc1, PSA (prostate-specific antigen), or a combination thereof.

[00283] In another embodiment, an "immunogenic fragment" is one that elicits an immune response when administered to a subject alone or in a vaccine composition disclosed herein. Such a fragment contains, in another embodiment, the necessary epitopes in order to elicit either a humoral immune response, and/or an adaptive immune response.

[00284] In one embodiments, the heterologous antigen expressed in an oncolytic virus comprises the same or nearly the same heterologous antigen or fragment thereof expressed in a Lm strain disclosed herein. For example, an *Lm* strain disclosed herein comprising a fusion polypeptide comprising a heterologous antigen or fragment thereof, may comprise the same heterologous antigen or fragment thereof as is expressed from an oncolytic virus disclosed herein. In another embodiment, while the heterologous antigen may be the same, the fragment of the heterologous antigen may be different or may include different domains of the heterologous antigen. For example, the Lm strain might express an N-terminal region of an antigen, while the oncolytic virus expresses the C-terminal region of the same antigen, or vice versa.

[00285] In one embodiment, the oncolytic virus infects a tumor cell.

[00286] In one embodiment, an oncolytic virus infects a PSA overexpressing tumor cell. In one embodiment, an oncolytic virus infects a prostate tumor cell. In another embodiment, an oncolytic virus infects a precursor of a prostate tumor cell. In another embodiment, an oncolytic virus infects an HPV overexpressing tumor cell. In another embodiment, an oncolytic virus

infects a cervical cancer tumor cell. In another embodiment, an oncolytic virus infects a precursor of a cervical cancer cell. In yet another embodiment, an oncolytic virus infects an Her2/neu over-expressing tumor cell. In yet another embodiment, an oncolytic virus infects a osteosarcoma or Ewing's sarcoma (ES) cell.

[00287] In one embodiment, an oncolytic virus disclosed herein expresses a programmed cell death receptor (PD-1) binding agonist or antagonist. In one embodiment, a PD-1 antagonist is selected from the group comprising an antibody or a fragment thereof, a PD-1 antagonist, or a PD-1 partial antagonist, or any combination thereof. It will be well appreciated by the skilled artisan that the term "PD-1" is an acronym for the Programmed Cell Death 1 protein, a 50-55 kDa type I transmembrane receptor originally identified by subtractive hybridization of a mouse T cell line undergoing apoptosis (Ishida et al., 1992, Embo J. 1 1 :3887-95). PD-1 is expressed on activated T, B, and myeloid lineage cells (Greenwald ef al. , 2005, Annu. Rev. Immunol. 23:515-48; Sharpe et al., 2007, Nat. Immunol. 8:239-45). The amino acid sequence of human PD-1 is GenBank Accession No. NP_005009.2. The amino acid sequence of murine PD-1 is GenBank Accession No. AAI 19180.1.

[00288] In one embodiment, oncolytic viruses that target tumor cells lead to tumor cell death.

[00289] In one embodiment, compositions disclosed herein comprise an oncolytic virus. In another embodiment, a composition disclosed herein comprises an Lm strain and an oncolytic virus. In another embodiment, a composition disclosed herein comprises an oncolytic virus, wherein the composition does not include a *Listeria* strain disclosed herein.

[00290] In one embodiment, an additional active agent of the compositions disclosed herein is a T cell receptor engineered T cell (Receptor engineered T cells). In another embodiment, T cells are transduced to express a receptor engineered for selected specificity. The receptors of Receptor engineered T cells are molecules that exhibit a specific tumor specificity. In one embodiment, the genetically modified receptors of Receptor engineered T cells have selected specificity to an human HPV tumor ligand, a PSA tumor ligand or a Her-2/neu tumor ligand.

[00291] The Receptor engineered cells of the disclosure are genetically modified to stably express a desired genetically modified T cell receptor. In one embodiment, the T cell is genetically modified to stably express a modified receptor on its surface, conferring novel tumor specificity.

[00292] In one embodiment, Receptor engineered T cells comprise a nucleic acid that encodes a receptor that would recognize a tumor cell-surface ligand. In one embodiment, Receptor engineered T cells express a receptor that recognizes a tumor cell-surface ligand. In one embodiment, Receptor engineered T cells express a receptor that binds to a tumor cell-surface ligand. In one embodiment, a tumor cell-surface ligand comprises an HPV tumor cell-surface

ligand, or a portion thereof. In another embodiment, a tumor cell-surface ligand comprises a PSA tumor cell-surface ligand, or a portion thereof. In another embodiment, a tumor cell-surface ligand comprises a Her-2/neu tumor cell-surface ligand or a portion thereof.

[00293] In one embodiment, the genetically modified receptor of a Receptor engineered T cell binds to a prostate specific antigen (PSA) cell-surface ligand domain or a fragment thereof, a human papilloma virus (HPV) cell-surface ligand domain or a fragment thereof, or a chimeric Her2/neu cell-surface ligand domain or a fragment thereof. In another embodiment, the genetically modified receptor of a Receptor engineered T cell has selective binding specificity to the same or nearly the same tumor cell-surface ligand or fragment thereof expressed in a *Lm* strain disclosed herein as a heterologous antigen. For example, an *Lm* strain disclosed herein comprising a fusion polypeptide comprising a heterologous antigen or fragment thereof, may comprise the same heterologous antigen or fragment thereof as is specifically recognized by Receptor engineered T cells disclosed herein. While the heterologous antigen of an Lm strain disclosed herein may comprise the same the antigen recognized by Receptor engineered T cells disclosed herein, the actual binding specificity recognized by the receptors of Receptor engineered T cells may not be included within the heterologous antigen expressed from the *Lm* strain. For example, the Lm strain might express an N-terminal region of an antigen, while the Receptor engineered T cells have selective specificity to the C-terminal region of the same antigen, or vice versa.

[00294] In one embodiment, compositions disclosed herein comprise Receptor engineered T cells. In one embodiment, a composition disclosed herein comprises an *Lm* strain and Receptor engineered T cells. In another embodiment, a composition disclosed herein comprises Receptor engineered T cells, wherein the composition does not include a *Listeria* strain as described herein.

[00295] In one embodiment, a composition disclosed herein comprises a recombinant *Listeria monocytogenes* (*Lm*) strain.

[00296] In one embodiment, an immunogenic composition comprises Receptor engineered T cells disclosed herein, and a recombinant attenuated *Listeria* strain disclosed herein. In another embodiment, each component of the immunogenic compositions disclosed herein is administered prior to, concurrently with, of after another component of the immunogenic compositions disclosed herein. In one embodiment, even when administered concurrently, an composition comprising Lm and a composition comprising Receptor engineered T cells may be administered as two separate compositions. In another embodiment, even when administered concurrently, an Lm composition and a Receptor engineered T cells composition may be administered as two separate compositions. In yet another embodiment, an Lm composition comprises Receptor

engineered T cells.

[00297] In another embodiment, an additional active agent of the compositions disclosed herein is a chimeric antigen receptor engineered T cells (CAR T cells). In one embodiment, T cells are transduced 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.

[00298] The CAR T cells of the 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.

[00299] In one embodiment, the antigen recognized by CAR T cells is a tumor associated antigen. In one embodiment, the antigen specificity is for a PSA antigen or an immunogenic fragment thereof. In another embodiment, the antigen specificity is for a HPV antigen or an immunogenic fragment thereof. In another embodiment, the antigen specificity is for a tumor-associated antigen as disclosed herein or an immunogenic fragment thereof.

[00300] In yet another embodiment, the antigen specificity is for a chimeric Her-2/neu antigen or fragment thereof. In another embodiment, CAR T cells comprise a nucleic acid encoding a polypeptide that specifically recognizes the tumor associated antigen. In another embodiment, the polypeptide comprises an antigen binding domain. In another embodiment, the antigen binding domain comprises an antibody or an antigen binding domain thereof.

[00301] The term "antibody fragment" refers to a portion of an intact antibody that is capable of specifically binding to an antigen. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

[00302] An "antibody heavy chain," as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

[00303] An "antibody light chain," as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations, κ and λ light chains refer to the two major antibody light chain isotypes.

[00304] By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody,

wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

[00305] In one embodiment, CAR T cells comprise a nucleic acid that encodes an antigen binding region. In one embodiment, CAR T cells express an antigen binding region. In one embodiment, an antigen binding regions is an antibody or an antigen-binding domain thereof. In one embodiment, the antigen-binding domain thereof is a Fab or a scFv.

[00306] It will be appreciated by a skilled artisan that the term "specifically binds," with respect to an antibody, encompasses an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species, but, such cross-species reactivity does not itself alter the classification of an antibody as specific, in another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than a specific amino acid sequence. I

[00307] In one embodiment, the antigen binding domain of a CAR binds to a prostate specific antigen (PSA) domain or a fragment thereof, a human papilloma virus (HPV) antigen domain or a fragment thereof, or a chimeric Her2/neu antigen domain or a fragment thereof. In another embodiment, the antigen binding domain of a CAR specifically recognizes the same or nearly the same heterologous antigen or fragment thereof expressed in a *Lm* strain disclosed herein. For example, an *Lm* strain disclosed herein comprising a fusion polypeptide comprising a heterologous antigen or fragment thereof, may comprise the same heterologous antigen or fragment thereof as is specifically recognized by CAR T cells disclosed herein. While the heterologous antigen of an Lm strain disclosed herein may comprise the same the antigen recognized by CAR T cells disclosed herein, the actual antigen epitope recognized by the CAR T cells may not be included within the heterologous antigen expressed from the *Lm* strain. For example, the Lm strain might express an N-terminal region of an antigen, while the CAR T cells specifically recognize an epitope within the C-terminal region of the same antigen, or vice versa.

[00308] In one embodiment, compositions disclosed herein comprise CAR T cells. In one embodiment, a composition disclosed herein comprises an *Lm* strain and CAR T cells. In another embodiment, a composition disclosed herein comprises CAR T cells, wherein the composition

does not include a *Listeria* strain as described herein.

[00309] In one embodiment, a composition disclosed herein comprises a recombinant *Listeria monocytogenes* (*Lm*) strain.

[00310] In one embodiment, an immunogenic composition comprises an oncolytic virus disclosed herein and/or a chimeric antigen receptor engineered cells (CAR T cells) disclosed herein, and a recombinant attenuated *Listeria* disclosed herein. In another embodiment, each component of the immunogenic compositions disclosed herein is administered prior to, concurrently with, of after another component of the immunogenic compositions disclosed herein. In one embodiment, even when administered concurrently, an Lm composition and CAR T cells may be administered as two separate compositions. In another embodiment, even when administered concurrently, an Lm composition and CAR T cells may be administered as two separate compositions. Alternately, in another embodiment, an Lm composition may comprise an CAR T cells. In yet another embodiment, an Lm composition comprises CAR T cells.

[00311] The compositions disclosed herein, 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-peritonealy, intra-ventricularly, intra-cranially, intra-vaginally or intra-tumorally.

[00312] 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 the like. In another embodiment of the disclosure, the active ingredient is formulated in a capsule. In accordance with this embodiment, the compositions of the disclosure comprise, in addition to the active compound and the inert carrier or diluent, a hard gelating capsule.

[00313] In another embodiment, compositions are administered by intravenous, intra-arterial, or intra-muscular injection of a liquid preparation. Suitable liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are administered intravenously and are thus formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions are administered intra-arterially and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions are administered intra-muscularly and are thus formulated in a form suitable for intra-muscular administration.

[00314] In some embodiments, when the oncolytic virus is administered separately from a composition comprising a recombinant Lm strain, the oncolytic viruses may be injected intravenously, subcutaneously, or directly into the tumor or tumor bed. In one embodiment, a

composition comprising an oncolytic virus is injected into the space left after a tumor has been surgically removed, e.g., the space in a prostate gland following removal of a prostate tumor.

[00315] In some embodiments, when the Receptor engineered T cells are administered separately from a composition comprising a recombinant Lm strain, the Receptor engineered T cells may be injected intravenously, subcutaneously, or directly into the tumor or tumor bed. In one embodiment, a composition comprising Receptor engineered T cells is injected into the space left after a tumor has been surgically removed, e.g., the space in a prostate gland following removal of a prostate tumor.

[00316] In some embodiments, when the CAR T cells are administered separately from a composition comprising a recombinant Lm strain, 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, e.g., the space in a prostate gland following removal of a prostate tumor.

[00317] In some embodiments, when the monoclonal antibodies are administered separately from a composition comprising a recombinant Lm strain, the monoclonal antibodies may be injected intravenously, subcutaneously, or directly into the tumor or tumor bed. In one embodiment, a composition comprising monoclonal antibodies is injected into the space left after a tumor has been surgically removed, e.g., the space in a prostate gland following removal of a prostate tumor.

[00318] In some embodiments, when the TKI is administered separately from a composition comprising a recombinant Lm strain, the TKI may be injected intravenously, subcutaneously, or directly into the tumor or tumor bed. In one embodiment, a composition comprising TKI is injected into the space left after a tumor has been surgically removed, e.g., the space in a prostate gland following removal of a prostate tumor.

[00319] In one embodiment, the term "immunogenic composition" encompasses the recombinant *Listeria* disclosed herein, and an adjuvant, an oncolytic virus, a chimeric antigen receptor engineered cells (CAR T cells), a therapeutic or immunomodulatory monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), 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. 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.

[00320] In one embodiment, this disclosure provides methods of use which comprise administering a composition comprising the described *Listeria* strains, and further comprising

additional agents such as oncolytic viruses, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or Receptor engineered T cells. In one embodiment, the term "pharmaceutical composition" encompasses a therapeutically effective amount of the active ingredient or ingredients including the *Listeria* strain, the oncolytic viruses, the CAR T cells), the therapeutic or immunomodulatory monoclonal antibody, the targeting thymidine kinase inhibitor (TKI), or Receptor engineered T cells , 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.

[00321] It will be understood by the skilled artisan that the term “administering” encompasses bringing a subject in contact with a composition of the 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 disclosure encompasses administering the Listeria strains and compositions thereof of the disclosure to a subject.

[00322] 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%. It is to be understood by the skilled artisan that the term "subject" can encompass a mammal including an adult human or a human child, teenager or adolescent in need of therapy for, or susceptible to, a condition or its sequelae, and also may include non-human mammals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. It will also be appreciated that the term may encompass livestock. The term "subject" does not exclude an individual that is normal in all respects.

[00323] 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.

Combination Therapies and Methods of Use Thereof

[00324] In one embodiment, this disclosure provides a method of eliciting an enhanced anti-tumor T cell response in a subject, the method comprising the step of administering to the subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein (a) said composition further comprises an additional active agent; (b) said method further comprises a step of administering an effective amount of a composition comprising an additional active agent to said subject; or (c) said method further comprises a step of administering a targeted radiation therapy to said subject; or any combination thereof of (a)-(c).

[00325] In one embodiment, any composition comprising a *Listeria* strain described herein may be used in the methods disclosed herein. In one embodiment, any composition comprising a Listeria strain and an additional active agent, for example an oncolytic virus, chimeric antigen receptor engineered cells (CAR T cells), a therapeutic or immunomodulatory monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or adoptively transferred cells incorporating engineered T cell receptors, or any combination thereof, described herein may be used in the methods disclosed herein. In one embodiment, any composition comprising an additional agent described herein may be used in the methods disclosed herein. Compositions comprising *Listeria* strains with and without additional agents have been described in detail above. Compositions with additional agents have also been described in detail above. In some embodiment, in a method disclosed herein a composition comprising an additional active agent, for example an oncolytic virus, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or adoptively transferred cells incorporating engineered T cell receptors may be administered prior to, concurrent with or following administration of a composition comprising a *Listeria* strain.

[00326] Radiation therapy (RT) is a local therapy and does not demonstrate the systemic toxicity and immunosuppressive effects of chemotherapy. There are sufficient pre-clinical evidence showing that RT induces an immunogenic cell death by increasing tumor neo-antigen presentation in MHC I on tumor cell surface, by increasing translocation of calreticulin, and by inducing the secretion of HMGB1.

[00327] In one embodiment, "radiation therapy" is a term commonly used in the art to refer to multiple types of radiation therapy including internal and external radiation therapy,

radioimmunotherapy, and the use of various types of radiation including X-rays, gamma rays, alpha particles, beta particles, photons, electrons, neutrons, radioisotopes, implants of radioactive isotopes and other forms of ionizing radiation. Recent experimental therapy employs monoclonal antibodies specific to the malignant tumor to deliver radioactive isotopes directly to the site of the tumor, termed radioimmunotherapy. The most common type of radiation treatment is radiation directed to the body area containing the neoplastic tumor, which is known as regional or local radiation therapy.

[00328] In one embodiment, administration of radiation therapy includes methods well known in the art, such as internal and external radiation therapy. In another embodiment, external therapy includes the administration of radiation via high-energy external beam radiation, administered either regionally (locally) to the tumor site or whole body irradiation. In another embodiment, examples of internal radiation (brachytherapy) include the implantation of radioactive isotopes in permanent, temporary, sealed, unsealed, intracavity or interstitial implants. In one embodiment, the choice of implant is determined by the characteristics of the neoplasia, including the location and extent of the tumor. In another embodiment, the choice between external or internal radiation treatment and type of external radiation treatment is also determined by the characteristics of the neoplasia and can be determined by those skilled in the art.

[00329] In one embodiment, "radiation therapy" or "radiotherapy" refers to the medical use of ionizing radiation as part of cancer treatment to control malignant cells. Radiotherapy may be used for curative, adjuvant, or palliative treatment. Suitable types of radiotherapy include conventional external beam radiotherapy, stereotactic radiation therapy (e.g., Axesse, Cyberknife, Gamma Knife, Novalis, Primatom, Synergy, X-Knife, TomoTherapy or Trilogy), Intensity-Modulated Radiation Therapy, particle therapy (e.g., proton therapy), brachytherapy, delivery of radioisotopes, etc. This list is not meant to be limiting. In one embodiment, radiation therapy comprises a targeted radiation therapy wherein the procedure uses computers to create a 3-dimensional picture of the tumor in order to target the tumor as accurately as possible and give it the highest possible dose of radiation while sparing normal tissue as much as possible. It is also known as 3-D conformal (or conformational). The radiation used for cancer treatment may come from a machine outside the body, or it may come from radioactive material placed in the body near tumor cells or injected into the bloodstream. In one embodiment, targeted radiation therapy comprises a method wherein a radioactive material is targeted to a particular place in the body, for example near the tumor cells in order to target and limit the killing of cells to cancer cells, while sparing normal tissue.

[00330] In one embodiment, targeted radiation therapy is Internal Radiation Therapy, also

known as brachytherapy, which is radiation delivered from radiation sources (radioactive materials) placed inside or on the body. Several brachytherapy techniques are used in cancer treatment. Interstitial brachytherapy uses a radiation source placed within tumor tissue, such as within a prostate tumor. Intracavitary brachytherapy uses a source placed within a surgical cavity or a body cavity, such as the chest cavity, near a tumor. Episcleral brachytherapy, which is used to treat melanoma inside the eye, uses a source that is attached to the eye.

[00331] In brachytherapy, radioactive isotopes are sealed in tiny pellets or "seeds." These seeds are placed in patients using delivery devices, such as needles, catheters, or some other type of carrier. As the isotopes decay naturally, they give off radiation that damages nearby cancer cells. If left in place, after a few weeks or months, the isotopes decay completely and no longer give off radiation. The seeds will not cause harm if they are left in the body (see permanent brachytherapy, described below).

[00332] Brachytherapy may be able to deliver higher doses of radiation to some cancers than external-beam radiation therapy while causing less damage to normal tissue.

[00333] Brachytherapy can be given as a low-dose-rate or a high-dose-rate treatment:

[00334] In low-dose-rate treatment, cancer cells receive continuous low-dose radiation from the source over a period of several days. In high-dose-rate treatment, a robotic machine attached to delivery tubes placed inside the body guides one or more radioactive sources into or near a tumor, and then removes the sources at the end of each treatment session. High-dose-rate treatment can be given in one or more treatment sessions. In one embodiment, administration of a targeted radiation therapy comprises the placement of brachytherapy sources in or near a tumor. In one embodiment the placement of the source is temporary. In another embodiment, the placement of the source is permanent.

[00335] For permanent brachytherapy, the sources are surgically sealed within the body and left there, even after all of the radiation has been given off. The remaining material (in which the radioactive isotopes were sealed) does not cause any discomfort or harm to the patient. Permanent brachytherapy is a type of low-dose-rate brachytherapy. For temporary brachytherapy, tubes (catheters) or other carriers are used to deliver the radiation sources, and both the carriers and the radiation sources are removed after treatment. Temporary brachytherapy can be either low-dose-rate or high-dose-rate treatment.

[00336] In one embodiment, a patient may receive radiation therapy before, during, or after administration of a composition disclosed herein, depending on the type of cancer being treated. In one embodiment, methods disclosed herein comprise administering a composition disclosed herein comprising a recombinant *Listeria* and administering a targeted radiation therapy. In one embodiment, methods disclosed herein comprise administering a composition disclosed herein

comprising a recombinant *Listeria* and an additional active agent, and administering a targeted radiation therapy. In another embodiment, methods disclosed herein comprise administering a composition disclosed herein comprising a recombinant *Listeria*, administering a composition comprising an additional active agent, and administering a targeted radiation therapy. In one embodiment, an additional active agent is an oncolytic virus. In another embodiment, an additional active agent is CAR T cells. In another embodiment, an additional active agent is a therapeutic or immunomodulatory monoclonal antibody. In another embodiment, an additional active agent is a targeting thymidine kinase inhibitor (TKI). In another embodiment, an additional active agent is an adoptively transferred cell incorporating engineered T cell receptors.

[00337] In one embodiment, the amount/course of physical energy administered to the individual is determined by the clinician(s) administering the therapy. In another embodiment, the amount/course of physical energy administered to the individual during radiation therapy is determined by the characteristics of the individual's disease, the method of delivery and the weight, age, general health and response of the individual. For radiation therapy in particular, the location of the tumor is a determining factor in the administration, as the radio-sensitivity of the tumor and surrounding tissue are variable according to tissue type, oxygen supply and other factors. In another embodiment, the amount of radiation administered is the dosage known in the art to be effective given the characteristics of the individual and the disease. In other embodiments, the amount of physical energy administered is about 2X, about 5X, about 10X, or about 15X less than that known in the art to be effective for the particular individual and characteristics of the disease. In another embodiment, the amount of physical energy administered is about 20X, about 50X, about 100X or about 1000X less than that known in the art to be effective for the particular individual and characteristics of the disease. In another embodiment the dosage is a sub-lethal or sub-toxic dosage.

[00338] In one embodiment, the radiation dose administered to a subject disclosed herein is from about 1.0 to 10 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 11 to 20 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 21 to 30 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 31 to 40 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 41 to 50 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 61 to 70 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 71 to 80 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about

81 to 90 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 91 to 100 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 100 to 150 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 151 to 200 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 200 to 500 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 501 to 1,000 cGy/min. In another embodiment, the radiation dose administered to a subject disclosed herein is from about 1,001 to 10,000 cGy/min.

[00339] In another embodiment, the radiation dose administered to a subject disclosed herein is a total fraction ranging from about 11 to 20 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 21 to 30 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 31 to 40 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 41 to 50 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 51 to 60 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 61 to 70 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 71 to 80 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 81 to 90 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 91 to 100 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 101 to 200 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 201 to 500 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 501 to 1,000 cGy. In another embodiment, the radiation dose administered to a subject ranges from about 1,001 to 10,000 cGy.

[00340] In one embodiment, repeat doses may be undertaken immediately following the first course of treatment or after an interval of days, weeks or months to achieve tumor regression. In another embodiment, repeat doses may be undertaken immediately following the first course of treatment or after an interval of days, weeks or months to achieve suppression of tumor growth. A particular course of treatment according to the above-described methods, for example, combined *Listeria* and physical energy treatment, may later be followed by a course of combined chemotherapy and *Listeria* treatment. Assessment may be determined by any of the techniques known in the art, including diagnostic methods such as imaging techniques, analysis of serum tumor markers, biopsy, or the presence, absence or amelioration of tumor associated symptoms.

[00341] In one embodiment, radiation treatment entails the administration of a radiosensitizing

agent or radioprotectant to facilitate the treatment. Recent evidence suggests that the antineoplastic agent TAXOL™ (paclitaxel) may function as a radiosensitizer. Liebmann et al., J. National Cancer Inst. 86:441, 1994. Similar evidence has been found for TAXOTERE™ (docetaxel). Creane et al., Int. J. Radiat. Biol. 75:731, 1999; Sikov et al., Front. Biosci. May 1: 221, 1997. Other radiation sensitizers include E2F-1, anti-ras single chain antibody, p53, GM-CSF, and cytosine deaminase. A tumor specific adenovirus may further comprise a radiation sensitizer, such as p53 for example, or a chemo sensitizer.

[00342] In one embodiment, combination treatment with compositions comprising a recombinant *Listeria* plus or minus an additional active agent and radiation therapy are used as components of a combined modality treatment, and the choice of additional active agent(s) and type and course of radiation therapy treatment is generally governed by the characteristics of the individual cancer and the response of the individual. While target cell-specific *Listeria* strains can be used with either radiation therapy or with additional active agents such as oncolytic viruses, CAR T cells, therapeutic or immunomodulatory monoclonal antibodies, targeting thymidine kinase inhibitors (TKI) and/or Receptor engineered T cells, as separate courses of treatment, they can also be combined with both methods of treatment in the same course of therapy. Accordingly, the disclosure encompasses combinations of the methods discussed above.

[00343] Accordingly, the disclosure includes methods for suppressing tumor growth in an individual comprising the following steps, in any order: a) administering to the individual an effective amount of a composition comprising a target cell-specific *Listeria* strain and optionally, at least one additional active agent; and b) administering an effective amount of an appropriate course of radiation therapy to the individual.

[00344] In one embodiment, the method may further comprise the step of: c) administering to the individual an additional dose of the *Listeria* and optionally a composition comprising an additional active agent, such as an oncolytic virus, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, TKI, or Receptor engineered T cells s, and the radiation therapy as necessary to treat the individual's neoplasia.

[00345] In another embodiment, the method may further comprise time delays after any one of steps a), b) and c). A time delay interval may be hours, days, weeks or months.

[00346] In one embodiment, the above-described methods include administration of the *Listeria* strains, and radiation therapy, and optionally additional active agent(s) such as oncolytic viruses, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, TKI, or Receptor engineered T cells, in any order and may include sequential administration or simultaneous administration of all or some of the components (i.e. simultaneous administration of physical energy and *Listeria* strain followed sequentially by radiation therapy, or sequential

administration of *Listeria* strain first, radiation therapy second and thirdly, an oncolytic virus, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, TKI, and/or Receptor engineered T cells, etc.).

[00347] In one embodiment, disclosed herein are methods and compositions for preventing, treating and vaccinating against a heterologous antigen-expressing tumor and inducing an immune response against sub-dominant epitopes of the heterologous antigen, while preventing an escape mutation of the tumor.

[00348] In one embodiment, the methods and compositions for preventing, treating and vaccinating against a heterologous antigen-expressing tumor comprise the use of a Listeriolysin (LLO) adjuvant. In another embodiment, the methods and compositions disclosed herein comprise a recombinant *Listeria* strain overexpressing LLO. In one embodiment, the LLO is expressed from the chromosome of the *Listeria* strain. In another embodiment, the LLO is expressed from a plasmid within the *Listeria* strain.

[00349] In another embodiment, disclosed herein is a method of inhibiting tumor-mediated immunosuppression in a subject, the method comprising the step of administering to the subject an immunogenic composition comprising a programmed cell death receptor-1 (PD-1) signaling pathway inhibitor, and a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[00350] In another embodiment, disclosed herein is a method of preventing or treating a tumor growth or cancer in a subject, the method comprising the step of administering to the subject an immunogenic composition comprising a programmed cell death receptor-1 (PD-1) signaling pathway inhibitor, and a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof.

[00351] In one embodiment, the term "treating" refers to curing a disease. In another embodiment, "treating" refers to preventing a disease. In another embodiment, "treating" refers to reducing the incidence of a disease. In another embodiment, "treating" refers to ameliorating symptoms of a disease. In another embodiment, "treating" refers to increasing performance free survival or overall survival of a patient. In another embodiment, "treating" refers to stabilizing the progression of a disease. In another embodiment, "treating" refers to inducing remission. In another embodiment, "treating" refers to slowing the progression of a disease. The terms "reducing", "suppressing" and "inhibiting" refer in another embodiment to lessening or

decreasing.

[00352] In one embodiment, disclosed herein is a method of increasing a ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor microenvironments of a subject, comprising administering the immunogenic composition disclosed herein. In another embodiment, increasing a ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor microenvironments in a subject allows for a more profound anti-tumor response in the subject.

[00353] In another embodiment, the T effector cells comprise CD4+FoxP3- T cells. In another embodiment, the T effector cells are CD4+FoxP3- T cells. In another embodiment, the T effector cells comprise CD4+FoxP3- T cells and CD8+ T cells. In another embodiment, the T effector cells are CD4+FoxP3- T cells and CD8+ T cells. In another embodiment, the regulatory T cells is a CD4+FoxP3+ T cell.

[00354] In one embodiment, the disclosure provides methods of treating, protecting against, and inducing an immune response against a tumor or a cancer, comprising the step of administering to a subject the immunogenic composition disclosed herein.

[00355] In one embodiment, the disclosure provides a method of preventing or treating a tumor or cancer in a human subject, comprising the step of administering to the subject the immunogenic composition strain disclosed herein, the recombinant *Listeria* strain comprising a recombinant polypeptide comprising an N-terminal fragment of an LLO protein and tumor-associated antigen, whereby the recombinant *Listeria* strain induces an immune response against the tumor-associated antigen, thereby treating a tumor or cancer in a human subject. In another embodiment, the immune response is an T-cell response. In another embodiment, the T-cell response is a CD4+FoxP3- T cell response. In another embodiment, the T-cell response is a CD8+ T cell response. In another embodiment, the T-cell response is a CD4+FoxP3- and CD8+ T cell response. In another embodiment, the disclosure provides a method of protecting a subject against a tumor or cancer, comprising the step of administering to the subject the immunogenic composition disclosed herein. In another embodiment, the disclosure provides a method of inducing regression of a tumor in a subject, comprising the step of administering to the subject the immunogenic composition disclosed herein. In another embodiment, the disclosure provides a method of reducing the incidence or relapse of a tumor or cancer, comprising the step of administering to the subject the immunogenic composition disclosed herein. In another embodiment, the disclosure provides a method of suppressing the formation of a tumor in a subject, comprising the step of administering to the subject the immunogenic composition disclosed herein. In another embodiment, the disclosure provides a method of inducing a remission of a cancer in a subject, comprising the step of administering to the subject

the immunogenic composition disclosed herein. In one embodiment, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide is integrated into the *Listeria* genome. In another embodiment, the nucleic acid is in a plasmid in the recombinant *Listeria* strain. In another embodiment, the nucleic acid molecule is in a bacterial artificial chromosome in the recombinant *Listeria* strain.

[00356] In one embodiment, the method comprises the step of co-administering the recombinant *Listeria* with an additional therapy. In another embodiment, the additional therapy is surgery, chemotherapy, an immunotherapy, a radiation therapy, CAR T cell therapy, oncolytic virus based therapy, a therapeutic or immunomodulatory monoclonal antibody therapy, targeting TKI therapy, or Receptor engineered T cell therapy, or a combination thereof. In another embodiment, the additional therapy precedes administration of the recombinant *Listeria*. In another embodiment, the additional therapy follows administration of the recombinant *Listeria*. In another embodiment, the additional therapy is an antibody therapy. In another embodiment, the antibody therapy is an anti-PD1, anti-CTLA4. In another embodiment, the recombinant *Listeria* is administered in increasing doses in order to increase the T-effector cell to regulatory T cell ration and generate a more potent anti-tumor immune response. It will be appreciated by a skilled artisan that the anti-tumor immune response can be further strengthened by providing the subject having a tumor with cytokines including, but not limited to IFN-γ, TNF-α, and other cytokines known in the art to enhance cellular immune response, some of which can be found in US Patent Serial No. 6,991,785, incorporated by reference herein.

[00357] In one embodiment, the methods disclosed herein further comprise the step of co-administering an immunogenic composition disclosed herein with an oncolytic virus that enhances an anti-tumor immune response in said subject.

[00358] In one embodiment, the methods disclosed herein further comprise the step of co-administering an immunogenic composition disclosed herein with a indoleamine 2,3-dioxygenase (IDO) pathway inhibitor. IDO pathway inhibitors for use in the disclosure include any IDO pathway inhibitor known in the art, including but not limited to, 1-methyltryptophan (1MT), 1-methyltryptophan (1MT), Necrostatin-1, Pyridoxal Isonicotinoyl Hydrazone, Ebselen, 5-Methylindole-3-carboxaldehyde, CAY10581, an anti-IDO antibody or a small molecule IDO inhibitor. In another embodiment, the compositions and methods disclosed herein are also used in conjunction with, prior to, or following a chemotherapeutic or radiotherapeutic regiment. In another embodiment, IDO inhibition enhances the efficiency of chemotherapeutic agents.

[00359] In some embodiments, selecting a dosage regimen (also referred to herein as an administration regimen) for a combination therapy disclosed herein depends on several factors,

including the serum or tissue turnover rate of the entity, the level of symptoms, the immunogenicity of the entity, and the accessibility of the target cells, tissue or organ in the individual being treated. Preferably, a dosage regimen maximizes the amount of each therapeutic agent delivered to the patient consistent with an acceptable level of side effects. Accordingly, the dose amount and dosing frequency of each biotherapeutic and chemotherapeutic agent in the combination depends in part on the particular therapeutic agent, the severity of the cancer being treated, and patient characteristics. Guidance in selecting appropriate doses of antibodies, cytokines, and small molecules are available. *See, e.g.*, Wawrzynczak (1996) *Antibody Therapy*, Bios Scientific Pub. Ltd, Oxfordshire, UK; Kresina (ed.) (1991) *Monoclonal Antibodies, Cytokines and Arthritis*, Marcel Dekker, New York, NY; Bach (ed.) (1993) *Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases*, Marcel Dekker, New York, NY; Baert *et al.* (2003) *New Engl. J. Med.* 348:601-608; Milgrom *et al.* (1999) *New Engl. J. Med.* 341:1966-1973; Slamon *et al.* (2001) *New Engl. J. Med.* 344:783-792; Beniaminovitz *et al.* (2000) *New Engl. J. Med.* 342:613-619; Ghosh *et al.* (2003) *New Engl. J. Med.* 348:24-32; Lipsky *et al.* (2000) *New Engl. J. Med.* 343:1594-1602; Physicians' Desk Reference 2003 (Physicians' Desk Reference, 57th Ed); Medical Economics Company; ISBN: 1563634457; 57th edition (November 2002). Determination of the appropriate dosage regimen may be made by the clinician, *e.g.*, using parameters or factors known or suspected in the art to affect treatment or predicted to affect treatment, and will depend, for example, the patient's clinical history (e.g., previous therapy), the type and stage of the cancer to be treated and biomarkers of response to one or more of the therapeutic agents in the combination therapy.

[00360] It will be appreciated by a skilled artisan that the terms “synergy” or “synergistic” may encompass an immune response as a result of the combination of the two or more compositions disclosed herein that is more potent than the sum of each composition’s individual immune response as a result of their individual administration. More specifically, in the in vitro setting one measure of synergy is known as “Bliss synergy.” Bliss synergy may encompass “excess over Bliss independence,” as determined by the Bliss value defined above. When the Bliss value is greater than zero (0), or more preferably greater than 0.2, it is considered indicative of synergy. Of course, the use of “synergy” herein also encompasses in vitro synergy as measured by additional and/or alternate methods. References herein to a combination’s in vitro biological effects, including but not limited to anti-cancer effects, being greater than, or equal to, the sum of the combination’s components individually, may be correlated to Bliss values. Again, the use of “synergy” herein, including whether a combination of components demonstrates activity equal to or greater than the sum of the components individually, may be measured by additional and/or alternate methods and are known, or will be apparent, to those skilled in this art.

[00361] In one embodiment, a combination therapy disclosed is used to treat a tumor that is large enough to be found by palpation or by imaging techniques well known in the art, such as MRI, ultrasound, or CAT scan. In some embodiments, a combination therapy disclosed herein is used to treat an advanced stage tumor having dimensions of at least about 200 mm^3, 300 mm^3, 400 mm^3, 500 mm^3, 750 mm^3, or up to 1000 mm^3.

[00362] In one embodiment, a disclosed combination therapy is administered to a patient diagnosed with a cancer that tests positive for PD-L1 expression. In some embodiments, PD-L1 expression is detected using a diagnostic anti-human PD-L1 antibody, or antigen binding fragment thereof, in an IHC assay on an FFPE or frozen tissue section of a tumor sample removed from the patient. Typically, the patient's physician would order a diagnostic test to determine PD-L1 expression in a tumor tissue sample removed from the patient prior to initiation of treatment with the PD-1 antagonist or PD-L1 antagonist and the live-attenuated *Listeria* strains provided for herein, but it is envisioned that the physician could order the first or subsequent diagnostic tests at any time after initiation of treatment, such as for example after completion of a treatment cycle.

[00363] In one embodiment, a disclosed combination therapy is administered to a patient diagnosed with a cancer that tests positive for CTLA-4 expression. In some embodiments, CTLA-4 expression is detected using a diagnostic anti-human CTLA-4 antibody, or antigen binding fragment thereof, in an IHC assay on an FFPE or frozen tissue section of a tumor sample removed from the patient. Typically, the patient's physician would order a diagnostic test to determine CTLA-4 expression in a tumor tissue sample removed from the patient prior to initiation of treatment with the CTLA-4 antagonist and the live-attenuated *Listeria* strains provided for herein, but it is envisioned that the physician could order the first or subsequent diagnostic tests at any time after initiation of treatment, such as for example after completion of a treatment cycle.

[00364] In some embodiments that employ one or more disclosed immune-modulating antibody in the combination therapy, the dosing regimen will comprise administering said one or more immune-modulating antibodies at a flat dose of 100 to 500 mg or a weight-based dose of 1 to 10 mg/kg at intervals of about 14 days (± 2 days) or about 21 days (± 2 days) or about 30 days (± 2 days) throughout the course of treatment.

[00365] In other embodiments that employ an anti-human PD-1 mAb as the PD-1 antagonist in the combination therapy, the dosing regimen will comprise administering the immune-modulating antibody at a dose of from about 0.005 mg/kg to about 10 mg/kg, with intra-patient dose escalation. In other escalating dose embodiments, the interval between doses will be progressively shortened, e.g., about 30 days (± 2 days) between the first and second dose, about

14 days (± 2 days) between the second and third doses. In certain embodiments, the dosing interval will be about 14 days (± 2 days), for doses subsequent to the second dose.

[00366] In one embodiment, the terms "treatment regimen", "dosing protocol" and "dosing regimen" are used interchangeably herein and encompass the dose and timing of administration of each therapeutic agent in a combination therapy disclosed herein.

[00367] In certain embodiments, a subject will be administered an intravenous (IV) infusion of a medicament comprising any of the immune-modulating antibodies described herein.

[00368] In one embodiment, an immune-modulating antibody in the combination therapy is an antagonist antibody. In another embodiment, an immune-modulating antibody in the combination therapy is an antagonist antibody.

[00369] In another embodiment, an immune-modulating antibody is administered intravenously at a dose selected from the group consisting of: 1 mg/kg, one dose every 2 weeks (Q2W), 2 mg/kg Q2W, 3 mg/kg Q2W, 5 mg/kg Q2W, 10 mg Q2W, 1 mg/kg one dose every three weeks (Q3W), 2 mg/kg Q3W, 3 mg/kg Q3W, 5 mg/kg Q3W, and 10 mg Q3W.

[00370] In another embodiment, the immune-modulating antibody in the combination therapy is administered in a liquid medicament at a dose selected from the group consisting of 200 mg Q3W, 1 mg/kg Q2W, 2 mg/kg Q2W, 3 mg/kg Q2W, 5 mg/kg Q2W, 10 mg Q2W, 1 mg/kg Q3W, 2 mg/kg Q3W, 3 mg/kg Q3W, 5 mg/kg Q3W, and 10 mg Q3W or equivalents of any of these doses (e.g., a PK model of an immune-modulating antibody estimates that the fixed dose of 200 mg Q3W provides exposures that are consistent with those obtained with 2 mg/kg Q3W). In some embodiments, an immune-modulating antibody is administered as a liquid medicament which comprises 25 mg/ml the antibody, 7% (w/v) sucrose, 0.02% (w/v) polysorbate 80 in 10 mM histidine buffer pH 5.5, and the selected dose of the medicament is administered by IV infusion over a time period of 30 minutes +/-10 min.

[00371] In another embodiment, the attenuated bacterial or attenuated *Listeria* in the combination therapy is a live-attenuated *Listeria* strain disclosed herein, which is administered in a liquid medicament at a dose selected from the group consisting of 1×10^9, 5×10^9 and 1×10^{10} CFU. In some embodiments, a dose ranges from about 1×10^9 CFU up to 3.31×10^{10} CFU, from about 5×10^8 CFU up to 5×10^{10} CFU, from about 7×10^8 CFU up to 5×10^{10} CFU, from about 1×10^9 CFU up to 5×10^{10} CFU, from about 2×10^9 CFU up to 5×10^{10} CFU, from about 3×10^9 CFU up to 5×10^{10} CFU, from about 5×10^9 CFU up to 5×10^{10} CFU, from about 7×10^9 CFU up to 5×10^{10} CFU, from about 1×10^{10} CFU up to 5×10^{10} CFU, from about 1.5×10^9 CFU up to 5×10^{10} CFU, from about 5×10^8 CFU - up to 3×10^{10} CFU, from about 5×10^8 CFU up to 2×10^{10} CFU, from about 5×10^8 CFU up to 1.5×10^9 CFU, from about 5×10^8 CFU up to 1×10^{10} CFU, from about 5×10^8 CFU up to 7×10^9 CFU, from about 5×10^8 CFU up to 5×10^9

CFU, from about 5 x 10^8 CFU up to 3 x 10^9 CFU, from about 5 x 10^8 CFU up to 2 x 10^9 CFU, from about 1 x 10^9 CFU up to 3 x 10^{10} CFU, from about 1 x 10^9 CFU up to 2x 10^{10}CFU, from about 2 x 10^9 CFU up to 3 x 10^{10} CFU, from about 1 x 10^9 CFU up to 1x 10^{10} CFU, from about 2 x 10^9 CFU up to 1 x 10^{10} CFU, from about 3 x 10^9 CFU up to 1 x 10^{10} CFU, from about 2 x 10^9 CFU up to 7 x 10^9 CFU, from about 2 x 10^9 CFU up to 5 x 10^9 CFU.

[00372] In other embodiments, a dose of recombinant *Listeria* ranges from about 1 x 10^7 organisms to about1.5 x 10^8 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 1 x 10^8 organisms to about 1.5 x 10^9 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 1 x 10^9 organisms to about 2 x 10^9 organisms. -, In another embodiment, a dose of recombinant *Listeria* ranges from about 2 x 10^9 organisms to about 5 x 10^9 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 2 x 10^9 organisms to about 1 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 3 x 10^9 organisms to about 1 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 4 x 10^9 organisms to about 1 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 1 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 6 x 10^9 organisms to about 1 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 7 x 10^9 organisms to about 1 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 1 x 10^9 organisms to about 5 x 10^9 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 1 x 10^9 organisms to about 4 x 10^9 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 1 x 10^9 organisms to about 3 x 10^9 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 8 x 10^9 organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 1.5 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 2 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 2.5 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 3 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 3.5 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 4 x 10^{10} organisms. In another embodiment, a dose of recombinant *Listeria* ranges from about 5 x 10^9 organisms to about 5 x 10^{10} organisms. In another embodiment, the dose ranges from 1 x 10^7 organisms-5 x 10^{10} organisms.

[00373] The optimal dose for a combination therapy comprising a disclosed immune-

modulating antibody in combination with a disclosed live-attenuated *Listeria* strain is identified by dose escalation of one or both of these agents. In another embodiment, the optimal dose for a composition comprising either the immune-modulating antibody disclosed herein or the live-attenuated *Listeria* strain disclosed herein is identified by dose escalation of one or both of these agents.

[00374] In one embodiment, a patient is treated with the combination therapy disclosed herein on day 1 of weeks 1, 4 and 7 in a 12 week cycle, starting with at least one immune-modulating antibody that is administered at a starting dose of 50, 100, 150, or 200 mg, and a live-attenuated *Listeria* strain disclosed herein at a starting dose of ranging from about 1 x 10^7 CFU to about 3.5 x 10^{10} CFU.

[00375] In an embodiment, the immune-modulating antibody infusion is administered first, followed by a NSAIDS, e.g., naproxen or ibuprofen, and oral antiemetic medication within a predetermined amount of time prior to administration of a live-attenuated *Listeria* strain provided herein. In another embodiment, the predetermined amount of time is 5-10 min, 11-20 min, 21-40 min, 41-60 min. In another embodiment, the predetermined amount of time is at least one hour. In another embodiment, the predetermined amount of time is 1-2 hours, 2-4 hours, 4-6 hours, 6-10 hours. In another embodiment, administrations of a NSAIDS, e.g., naproxen or ibuprofen, and oral antiemetic medication is repeated on a need basis to the subject, prior to administration of a live-attenuated *Listeria* strain disclosed herein.

[00376] In another embodiment, at least one immune-modulating antibody is administered at a starting dose of 50, 100, 150 or 200 mg Q3W and a live-attenuated *Listeria* strain disclosed herein is administered Q3W at a starting dose of between 1 x 10^7 and 3.5 x 10^{10} CFU.

[00377] In one embodiment, a live-attenuated *Listeria* strain disclosed herein is administered in combination with at least one immune-modulating antibody. In one embodiment, a live-attenuated *Listeria* strain disclosed herein is administered in combination with an agonist antibody. In another embodiment, a live-attenuated *Listeria* strain disclosed herein is administered in combination with an immune checkpoint inhibitor antibody. In another embodiment, a live-attenuated *Listeria* strain disclosed herein is administered in combination with two types of immune-modulating antibodies. In another embodiment, a live-attenuated *Listeria* strain disclosed herein is administered in combination with an agonist and an immune checkpoint inhibitor antibody. In another embodiment, a live-attenuated *Listeria* strain disclosed herein is administered at a starting dose of 5 x 10^9 Q3W and at least one immune-modulating antibody is administered at a starting dose of 200 mg Q3W, and if the starting dose of the combination is not tolerated by the patient, then the dose of the live-attenuated *Listeria* strain is reduced to 1 x 10^9 cfu Q3W or the dose of least one immune-modulating antibody is reduced to

150 mg Q3W. It is to be understood by a skilled artisan that the doses of any of the components of a combination therapy provided herein may be incrementally adjusted to a lower or higher dose based on a subjects response to the combination therapy.

[00378] In some embodiments, dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed, as determined by those skilled in the art.

[00379] In some embodiments, a treatment cycle begins with the first day of combination treatment and lasts for at least 12 weeks, 24 weeks or 48 weeks. On any day of a treatment cycle that the drugs are co-administered, the timing between the separate IV infusions of an anti-PD1 antibody and a live-attenuated *Listeria* strain disclosed herein is between about 15 minutes to about 45 minutes. The disclosure contemplates that an immune-modulating antibody and a live-attenuated *Listeria* strain disclosed herein may be administered in either order or by simultaneous IV infusion.

[00380] In some embodiments, the combination therapy is administered for at least 2 to 4 weeks after the patient achieves a complete remission (CR).

[00381] In some embodiments, a patient selected for treatment with the combination therapy of the disclosure has been diagnosed with a metastatic cancer and the patient has progressed or become resistant to no more than 2 prior systemic treatment regimens. In some embodiments, a patient selected for treatment with the combination therapy of the disclosure has been diagnosed with a metastatic cancer and the patient has progressed or become resistant to no more than 3 prior systemic treatment regimens.

[00382] The present disclosure also provides a medicament which comprises at least one immune-modulating antibody herein and a pharmaceutically acceptable excipient. When an immune-modulating antibody disclosed herein is a biotherapeutic agent, e.g., a mAb, the antibody may be produced in a producing cell line known in the art, such as, but not limited to CHO cells using conventional cell culture and recovery/purification technologies.

[00383] In some embodiments, a medicament comprising an immune-modulating antibody disclosed herein may be provided as a liquid formulation or prepared by reconstituting a lyophilized powder with sterile water for injection prior to use. WO 2012/135408 describes the preparation of liquid and lyophilized medicaments comprising an anti-PD-1 antibody that are suitable for use in the disclosure.

[00384] The present disclosure also provides a medicament which comprises a live-attenuated *Listeria* strain disclosed herein and a pharmaceutically acceptable excipient.

[00385] An immune-modulating antibody medicament and a live-attenuated *Listeria* strain disclosed herein medicament may be provided as a kit which comprises a first container and a

second container and a package insert. The first container contains at least one dose of a medicament comprising at least one immune-modulating antibody, the second container contains at least one dose of a medicament comprising a live-attenuated *Listeria* strain disclosed herein, and the package insert, or label, which comprises instructions for treating a patient for a cancer using the medicaments. The first and second containers may be comprised of the same or different shape (e.g., vials, syringes and bottles) and/or material (e.g., plastic or glass). The kit may further comprise other materials that may be useful in administering the medicaments, such as diluents, filters, IV bags and lines, needles and syringes. In some embodiments of a kit disclosed herein, the immune checkpoint inhibitor antibody and the instructions state that the medicaments are intended for use in treating a patient having a cancer that tests positive for expression of a target disclosed herein (e.g. CTLA-4, PD-L1) by an IHC assay.

[00386] In another embodiment, a method of disclosure further comprises the step of boosting the subject with a recombinant *Listeria* strain, an oncolytic virus, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, TKI, or Receptor engineered T cells, as disclosed herein. In another embodiment, the recombinant Listeria strain used in the booster inoculation is the same as the strain used in the initial "priming" inoculation. In another embodiment, the booster strain is different from the priming strain. In another embodiment, the recombinant immune checkpoint inhibitor used in the booster inoculation is the same as the inhibitor used in the initial "priming" inoculation. In another embodiment, the booster inhibitor is different from the priming inhibitor. In another embodiment, the same doses are used in the priming and boosting inoculations. In another embodiment, a larger dose is used in the booster. In another embodiment, a smaller dose is used in the booster. In another embodiment, the methods of the 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 strain is experimentally determined by the skilled artisan. In another embodiment, the period between a prime and a boost strain 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 strain is administered 8-10 weeks after the prime strain.

[00387] In another embodiment, a method of the disclosure further comprises boosting the subject with a immunogenic composition comprising an attenuated Listeria strain disclosed

herein. In another embodiment, a method of the disclosure comprises the step of administering a booster dose of the immunogenic composition comprising the attenuated Listeria strain disclosed herein . In another embodiment, the booster dose is an alternate form of said immunogenic composition. In another embodiment, the methods of the disclosure further comprise the step of administering to the subject a booster immunogenic composition. In one embodiment, the booster dose follows a single priming dose of said immunogenic composition. In another embodiment, a single booster dose is administered after the priming dose. In another embodiment, two booster doses are administered after the priming dose. In another embodiment, three booster doses are administered after the priming dose. In one embodiment, the period between a prime and a boost dose of an immunogenic composition comprising the attenuated *Listeria* disclosed herein is experimentally determined by the skilled artisan. In another embodiment, the dose is experimentally determined by a skilled artisan. In another embodiment, the period between a prime and a boost dose 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 dose is administered 8-10 weeks after the prime dose of the immunogenic composition.

[00388] Heterologous "prime boost" strategies have been effective for enhancing immune responses and protection against numerous pathogens. Schneider et al., Immunol. Rev. 170:29-38 (1999); Robinson, H. L., Nat. Rev. Immunol. 2:239-50 (2002); Gonzalo, R. M. et al., Strain 20:1226-31 (2002); Tanghe, A., Infect. Immun. 69:3041-7 (2001). Providing antigen in different forms in the prime and the boost injections appears to maximize the immune response to the antigen. DNA strain priming followed by boosting with protein in adjuvant or by viral vector delivery of DNA encoding antigen appears to be the most effective way of improving antigen specific antibody and CD4+ T-cell responses or CD8+ T-cell responses respectively. Shiver J. W. et al., Nature 415: 331-5 (2002); Gilbert, S. C. et al., Strain 20:1039-45 (2002); Billaut-Mulot, O. et al., Strain 19:95-102 (2000); Sin, J. I. et al., DNA Cell Biol. 18:771-9 (1999). Recent data from monkey vaccination studies suggests that adding CRL1005 poloxamer (12 kDa, 5% POE), to DNA encoding the HIV gag antigen enhances T-cell responses when monkeys are vaccinated with an HIV gag DNA prime followed by a boost with an adenoviral vector expressing HIV gag (Ad5-gag). The cellular immune responses for a DNA/poloxamer prime followed by an Ad5-gag boost were greater than the responses induced with a DNA (without poloxamer) prime followed by Ad5-gag boost or for Ad5-gag only. Shiver, J. W. et al. Nature 415:331-5 (2002). U.S. Patent Appl. Publication No. US 2002/0165172 A1 describes simultaneous administration of a vector construct encoding an immunogenic portion of an antigen and a protein comprising the immunogenic portion of an antigen such that an immune response is generated. The document is

limited to hepatitis B antigens and HIV antigens. Moreover, U.S. Pat. No. 6,500,432 is directed to methods of enhancing an immune response of nucleic acid vaccination by simultaneous administration of a polynucleotide and polypeptide of interest. According to the patent, simultaneous administration means administration of the polynucleotide and the polypeptide during the same immune response, preferably within 0-10 or 3-7 days of each other. The antigens contemplated by the patent include, among others, those of Hepatitis (all forms), HSV, HIV, CMV, EBV, RSV, VZV, HPV, polio, influenza, parasites (e.g., from the genus Plasmodium), and pathogenic bacteria (including but not limited to M. tuberculosis, M. leprae, Chlamydia, Shigella, B. burgdorferi, enterotoxigenic E. coli, S. typhosa, H. pylori, V. cholerae, B. pertussis, etc.). All of the above references are herein incorporated by reference in their entireties.

[00389] In one embodiment, a treatment protocol of the disclosure is therapeutic. In another embodiment, the protocol is prophylactic. In another embodiment, the compositions of the 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 after debulking of tumor growth by surgery, conventional chemotherapy or radiation treatment. Following such treatments, the vaccines of the 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 disclosure are used to effect the growth of previously established tumors and to kill existing tumor cells. Each possibility represents a separate embodiment of the disclosure.

[00390] In some embodiments, the term "comprise" or grammatical forms thereof, refers to the inclusion of the indicated active agent, such as the *Lm* strains disclosed herein, as well as inclusion of other active agents, such as oncolytic viruses, CAR T cells, a therapeutic or immunomodulatory monoclonal antibody, TKI, or adoptively transferred cells incorporating engineered T cell receptors, and pharmaceutically acceptable carriers, excipients, emollients, stabilizers, etc., as are known in the pharmaceutical industry. In some embodiments, the term "consisting essentially of" refers to a composition, whose only active ingredient is the indicated active ingredient, however, other compounds may be included which are for stabilizing, preserving, etc. the formulation, but are not involved directly in the therapeutic effect of the indicated active ingredient. In some embodiments, the term "consisting essentially of" may refer to components, which exert a therapeutic effect via a mechanism distinct from that of the indicated active ingredient. In some embodiments, the term "consisting essentially of" may refer to components, which exert a therapeutic effect and belong to a class of compounds distinct from that of the indicated active ingredient. In some embodiments, the term "consisting essentially of"

may refer to components, which exert a therapeutic effect and may be distinct from that of the indicated active ingredient, by acting via a different mechanism of action, for example. In some embodiments, the term "consisting essentially of" may refer to components which facilitate the release of the active ingredient. In some embodiments, the term "consisting" refers to a composition, which contains the active ingredient and a pharmaceutically acceptable carrier or excipient.

[00391] As used herein, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

[00392] Throughout this application, various embodiments disclosed herein may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosure. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[00393] Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals there between.

[00394] As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

[00395] In the following examples, 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 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 disclosure.

EXAMPLES

[00396] **Materials and Methods (EXAMPLES 1-8):**

[00397] 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.

[00398] **Table 1. Plasmids and strains**

Plasmids	Features
pGG55	pAM401/pGB354 shuttle plasmid with gram(-) and gram(+) *cm* resistance, LLO-E7 expression cassette and a copy of *Lm prfA* gene
pTV3	Derived from pGG55 by deleting *cm* genes and inserting the *Lm dal* gene
pADV119	Derived from pTV3 by deleting the *prfA* gene
pADV134	Derived from pADV119 by replacing the *Lm dal* gene by the *Bacillus dal* gene
pADV142	Derived from pADV134 by replacing HPV16 *e7* with *klk3*
pADV168	Derived from pADV134 by replacing HPV16 *e7* with $hmw\text{-}maa_{2160\text{-}2258}$
Strains	Genotype
10403S	Wild-type *Listeria monocytogenes*:: *str*
XFL-7	10403S $prfA^{(-)}$
Lmdd	10403S $dal^{(-)}$ $dat^{(-)}$
LmddA	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$
LmddA-134	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *pADV134*
LmddA-142	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *pADV142*
Lmdd-143	10403S $dal^{(-)}$ $dat^{(-)}$ *with klk3 fused to the hly gene in the chromosome*
LmddA-143	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *with klk3 fused to the hly gene in the chromosome*
LmddA-168	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *pADV168*
Lmdd-143/134	*Lmdd-143 pADV134*
LmddA-143/134	*LmddA-143 pADV134*
Lmdd-143/168	*Lmdd-143 pADV168*
LmddA-	*LmddA-143 pADV168*

[00399] The sequence of the plasmid pAdv142 (6523 bp) was as follows:

[00400] cggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcacc
ggtgcgtcagcagaatatgtgatacaggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaat
ggcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgtttttccat
aggctccgccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgacaggactataaagataccaggcgtttc
cccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcattccacgcctga
cactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaacccccgttcagtccgaccgctgcgccttatccggtaactatc
gtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagttagtcttgaagtcatgcgc
cggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctcggttcaaagagttggtagctcagagaacctt
cgaaaaaccgccctgcaaggcggtttttcgttttcagagcaagagattacgcgcagaccaaaacgatctcaagaagatcatcttattaatcag
ataaaatatttctagccctcctttgattagtatattcctatcttaaagttacttttatgtggaggcattaacatttgttaatgacgtcaaaaggatagca
agactagaataaagctataaagcaagcatataatattgcgtttcatctttagaagcgaatttcgccaatattataattatcaaaagagaggggtg
gcaaacggtatttggcattattaggttaaaaaatgtagaaggagagtgaaacccatgaaaaaaataatgctagtttttattacacttatattagtta
gtctaccaattgcgcaacaaactgaagcaaaggatgcatctgcattcaataaagaaaattcaatttcatccatggcaccaccagcatctccgc
ctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcgataagtatatacaaggattggattacaataaaaacaatgtatta
gtataccacggagatgcagtgacaaatgtgccgccaagaaaaggttacaaagatggaaatgaatatattgttgtggagaaaaagaagaaat
ccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttcgagcctaacctatccaggtgctctcgtaaaagcgaattcggaattagta
gaaaatcaaccagatgttctccctgtaaaacgtgattcattaacactcagcattgatttgccaggtatgactaatcaagacaataaaatagttgta
aaaaatgccactaaatcaaacgttaacaacgcagtaaatacattagtggaaagatggaatgaaaaatatgctcaagcttatccaaatgtaagtg
caaaaattgattatgatgacgaaatggcttacagtgaatcacaattaattgcgaaatttggtacagcatttaaagctgtaaataatagcttgaatgt
aaacttcggcgcaatcagtgaagggaaaatgcaagaagaagtcattagttttaaacaaatttactataacgtgaatgttaatgaacctacaaga
ccttccagatttttcggcaaagctgttactaaagagcagttgcaagcgcttggagtgaatgcagaaaatcctcctgcatatatctcaagtgtggc
gtatggccgtcaagtttatttgaaattatcaactaattcccatagtactaaagtaaaagctgcttttgatgctgccgtaagcggaaaatctgtctca
ggtgatgtagaactaacaaatatcatcaaaaattcttccttcaaagccgtaatttacggaggttccgcaaaagatgaagttcaaatcatcgacg
gcaacctcggagacttacgcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcccattgcttatacaacaaacttccta
aaagacaatgaattagctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacagatggaaaaattaacatcgatcactct
ggaggatacgttgctcaattcaacatttcttgggatgaagtaaattatgatctcgagattgtgggaggctgggagtgcgagaagcattcccaa
ccctggcaggtgcttgtggcctctcgtggcagggcagtctgcggcggtgttctggtgcacccccagtgggtcctcacagctgcccactgcat
caggaacaaaagcgtgatcttgctgggtcggcacagcctgtttcatcctgaagacacaggccaggtatttcaggtcagccacagcttcccac
acccgctctacgatatgagcctcctgaagaatcgattcctcaggccaggtgatgactccagccacgacctcatgctgctccgcctgtcagag
cctgccgagctcacggatgctgtgaaggtcatggacctgcccacccaggagccagcactggggaccacctgctacgcctcaggctgggg
cagcattgaaccagaggagttcttgaccccaaagaaacttcagtgtgtggacctccatgttatttccaatgacgtgtgtgcgcaagttcaccct
cagaaggtgaccaagttcatgctgtgtgctggacgctggacaggggcaaaagcacctgctcgggtgattctgggggcccacttgtctgtt

atggtgtgcttcaaggtatcacgtcatggggcagtgaaccatgtgccctgcccgaaaggccttccctgtacaccaaggtggtgcattaccgg
aagtggatcaaggacaccatcgtggccaaccccTAAcccgggccactaactcaacgctagtagtggatttaatcccaaatgagccaaca
gaaccagaaccagaaacagaacaagtaacattggagttagaaatggaagaagaaaaaagcaatgatttcgtgtgaataatgcacgaaatca
ttgcttatttttttaaaaagcgatatactagatataacgaaacaacgaactgaataaagaatacaaaaaaagagccacgaccagttaaagcctg
agaaactttaactgcgagccttaattgattaccaccaatcaattaaagaagtcgagacccaaaatttggtaaagtatttaattactttattaatcag
atacttaaatatctgtaaacccattatatcgggtttttgaggggatttcaagtctttaagaagataccaggcaatcaattaagaaaaacttagttgat
tgccttttttgttgtgattcaactttgatcgtagcttctaactaattaattttcgtaagaaaggagaacagctgaatgaatatccctttttgttgtagaaa
ctgtgcttcatgacggcttgttaaagtacaaatttaaaaatagtaaaattcgctcaatcactaccaagccaggtaaaagtaaaggggctatttttg
cgtatcgctcaaaaaaaagcatgattggcggacgtggcgttgttctgacttccgaagaagcgattcacgaaaatcaagatacatttacgcattg
gacaccaaacgtttatcgttatggtacgtatgcagacgaaaaccgttcatacactaaaggacattctgaaaacaatttaagacaaatcaatacct
tctttattgattttgatattcacacggaaaaagaaactatttcagcaagcgatattttaacaacagctattgatttaggttttatgcctacgttaattatc
aaatctgataaaggttatcaagcatattttgttttagaaacgccagtctatgtgacttcaaaatcagaatttaaatctgtcaaagcagccaaaataa
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tctgtaaaaaggttttgtgctcatggaaaactcctctctttttttcagaaaatcccagtacgtaattaagtatttgagaattaattttatattgattaatac taagtttacccagttttcacctaaaaaacaaatgatgagataatagctccaaaggctaaagaggactataccaactatttgttaattaa (SEQ ID NO: 67). This plasmid was sequenced at Genewiz facility from the E. coli strain on 2-20-08.

EXAMPLE 1: Construction of attenuated *Listeria* strain-LmddΔ*actA* and insertion of the human *klk3* gene in frame to the *hly* gene in the *Lmdd* and *Lmdda* strains.

[00401] The strain Lm *dal dat* (Lmdd) was attenuated by the irreversible deletion of the virulence factor, ActA. An in-frame deletion of *actA* in the Lm*daldat* (Lmdd) background was constructed to avoid any polar effects on the expression of downstream genes. The Lm *dal dat* Δ*actA* contains the first 19 amino acids at the N-terminal and 28 amino acid residues of the C-terminal with a deletion of 591 amino acids of ActA.

[00402] The actA deletion mutant was produced by amplifying the chromosomal region corresponding to the upstream (657 bp-oligo's Adv 271/272) and downstream (625 bp- oligo's Adv 273/274) portions of *actA* and joining by PCR. The sequence of the primers used for this amplification is given in the Table 2. The upstream and downstream DNA regions of *actA* were cloned in the pNEB193 at the EcoRI/PstI restriction site and from this plasmid, the EcoRI/PstI was further cloned in the temperature sensitive plasmid pKSV7, resulting in ΔactA/pKSV7 (pAdv120).

[00403] **Table 2: Sequence of primers that was used for the amplification of DNA sequences upstream and downstream of *actA***

Primer	Sequence	SEQ ID NO:
Adv271-actAF1	cg GAATTCGGATCCgcgccaaatcattggttgattg	68
Adv272-actAR1	gcgaGTCGACgtcggggttaatcgtaatgcaattggc	69
Adv273-actAF2	gcgaGTCGACccatacgacgttaattcttgcaatg	70
Adv274-actAR2	gataCTGCAGGGATCCttcccttctcggtaatcagtcac	71

[00404] The deletion of the gene from its chromosomal location was verified using primers that bind externally to the *actA* deletion region, which are shown in **Figure 1** as primer 3 (Adv 305-tgggatggccaagaaattc, SEQ ID NO: 72) and primer 4 (Adv304-ctaccatgtcttccgttgcttg; SEQ ID NO: 73) . The PCR analysis was performed on the chromosomal DNA isolated from Lmdd and LmddΔ*actA*. The sizes of the DNA fragments after amplification with two different sets of primer pairs 1/2 and 3/4 in Lmdd chromosomal DNA was expected to be 3.0 Kb and 3.4 Kb. On the other hand, the expected sizes of PCR using the primer pairs 1/2 and 3/4 for the LmddΔ*actA* was 1.2 Kb and 1.6 Kb. Thus, PCR analysis in **Figure 1** confirms that the 1.8 kb region of *actA* was deleted in the LmddΔ*actA* strain. DNA sequencing was also performed on PCR products to

confirm the deletion of *actA* containing region in the strain, LmddΔ*actA*.

EXAMPLE 2: Construction of the antibiotic-independent episomal expression system for antigen delivery by *Lm* vectors.

[00405] The antibiotic-independent episomal expression system for antigen delivery by *Lm* vectors (pAdv142) is the next generation of the antibiotic-free plasmid pTV3 (Verch et al., Infect Immun, 2004. **72**(11):6418-25, incorporated herein by reference). The gene for virulence gene transcription activator, *prfA* was deleted from pTV3 since *Listeria* strain Lmdd contains a copy of *prfA* gene in the chromosome. Additionally, the cassette for p60-*Listeria dal* at the NheI/PacI restriction site was replaced by p60-*Bacillus subtilis dal* resulting in plasmid pAdv134 (**Figure 2A**). The similarity of the *Listeria* and *Bacillus dal* genes is ~30%, virtually eliminating the chance of recombination between the plasmid and the remaining fragment of the *dal* gene in the *Lmdd* chromosome. The plasmid pAdv134 contained the antigen expression cassette tLLO-E7. The *LmddA* strain was transformed with the pADV134 plasmid and expression of the LLO-E7 protein from selected clones confirmed by Western blot (**Figure 2B**). The *Lmdd* system derived from the 10403S wild-type strain lacks antibiotic resistance markers, except for the *Lmdd* streptomycin resistance.

[00406] Further, pAdv134 was restricted with XhoI/XmaI to clone human PSA, *klk3* resulting in the plasmid, pAdv142. The new plasmid, pAdv142 (**Figure 2C, Table 1**) contains *Bacillus dal* (B-Dal) under the control of *Listeria* p60 promoter. The shuttle plasmid, pAdv142 complemented the growth of both *E. coli ala drx* MB2159 as well as *Listeria monocytogenes* strain Lmdd in the absence of exogenous D-alanine. The antigen expression cassette in the plasmid pAdv142 consists of *hly* promoter and LLO-PSA fusion protein (**Figure 2C**).

[00407] The plasmid pAdv142 was transformed to the *Listeria* background strains, Lmdd*actA* strain resulting in Lm-ddA-LLO-PSA. The expression and secretion of LLO-PSA fusion protein by the strain, Lm-ddA-LLO-PSA was confirmed by Western Blot using anti-LLO and anti-PSA antibody (**Figure 2D**). There was stable expression and secretion of LLO-PSA fusion protein by the strain, Lm-ddA-LLO-PSA after two *in vivo* passages.

EXAMPLE 3: *In vitro* and *in vivo* stability of the strain LmddA-LLO-PSA

[00408] The *in vitro* stability of the plasmid was examined by culturing the LmddA-LLO-PSA *Listeria* strain in the presence or absence of selective pressure for eight days. The selective pressure for the strain LmddA-LLO-PSA is D-alanine. Therefore, the strain LmddA-LLO-PSA was passaged in Brain-Heart Infusion (BHI) and BHI+ 100 μg/ml D-alanine. CFUs were determined for each day after plating on selective (BHI) and non-selective (BHI+D-alanine) medium. It was expected that a loss of plasmid will result in higher CFU after plating on non-selective medium (BHI+D-alanine). As depicted in **Figure 3A**, there was no difference between

the number of CFU in selective and non-selective medium. This suggests that the plasmid pAdv142 was stable for at least 50 generations, when the experiment was terminated.

[00409] Plasmid maintenance *in vivo* was determined by intravenous injection of 5 x 10^7 CFU LmddA-LLO-PSA, in C57BL/6 mice. Viable bacteria were isolated from spleens homogenized in PBS at 24 h and 48 h. CFUs for each sample were determined at each time point on BHI plates and BHI + 100 mg/ml D-alanine. After plating the splenocytes on selective and non-selective medium, the colonies were recovered after 24 h. Since this strain is highly attenuated, the bacterial load is cleared *in vivo* in 24 h. No significant differences of CFUs were detected on selective and non-selective plates, indicating the stable presence of the recombinant plasmid in all isolated bacteria (**Figure 3B**).

EXAMPLE 4: *In vivo* passaging, virulence and clearance of the strain LmddA-142 (LmddA-LLO-PSA)

[00410] *LmddA*-142 is a recombinant *Listeria* strain that secretes the episomally expressed tLLO-PSA fusion protein. To determine a safe dose, mice were immunized with LmddA-LLO-PSA at various doses and toxic effects were determined. LmddA-LLO-PSA caused minimum toxic effects (data not shown). The results suggested that a dose of 10^8 CFU of LmddA-LLO-PSA was well tolerated by mice. Virulence studies indicate that the strain LmddA-LLO-PSA was highly attenuated.

[00411] The *in vivo* clearance of LmddA-LLO-PSA after administration of the safe dose, 10^8 CFU intraperitoneally in C57BL/6 mice, was determined. There were no detectable colonies in the liver and spleen of mice immunized with LmddA-LLO-PSA after day 2. Since this strain is highly attenuated, it was completely cleared *in vivo* at 48 h (**Figure 4A**).

[00412] To determine if the attenuation of LmddA-LLO-PSA attenuated the ability of the strain LmddA-LLO-PSA to infect macrophages and grow intracellularly, a cell infection assay was performed. Mouse macrophage-like cell line such as J774A.1, were infected *in vitro* with *Listeria* constructs and intracellular growth was quantified. The positive control strain, wild type *Listeria* strain 10403S grows intracellularly, and the negative control XFL7, a *prfA* mutant, cannot escape the phagolysosome and thus does not grow in J774 cells. The intracytoplasmic growth of LmddA-LLO-PSA was slower than 10403S due to the loss of the ability of this strain to spread from cell to cell (**Figure 4B**). The results indicate that LmddA-LLO-PSA has the ability to infect macrophages and grow intracytoplasmically.

EXAMPLE 5: Immunogenicity of the strain-LmddA-LLO-PSA in C57BL/6 mice

[00413] The PSA-specific immune responses elicited by the construct LmddA-LLO-PSA in C57BL/6 mice were determined using PSA tetramer staining. Mice were immunized twice with LmddA-LLO-PSA at one week intervals and the splenocytes were stained for PSA tetramer on

day 6 after the boost. Staining of splenocytes with the PSA-specific tetramer showed that LmddA-LLO-PSA elicited 23% of PSA tetramer$^+$CD8$^+$CD62L^{low} cells (**Figure 5A**).

[00414] The functional ability of the PSA-specific T cells to secrete IFN-γ after stimulation with PSA peptide for 5 h was examined using intracellular cytokine staining. There was a 200-fold increase in the percentage of CD8$^+$CD62L^{low}IFN-γ secreting cells stimulated with PSA peptide in the LmddA-LLO-PSA group compared to the naïve mice (**Figure 5B**), indicating that the LmddA-LLO-PSA strain is very immunogenic and primes high levels of functionally active PSA CD8$^+$ T cell responses against PSA in the spleen.

[00415] To determine the functional activity of cytotoxic T cells generated against PSA after immunizing mice with LmddA-LLO-PSA, we tested the ability of PSA-specific CTLs to lyse cells EL4 cells pulsed with H-2D^b peptide in an *in vitro* assay. A FACS-based caspase assay (**Figure 5C**) and Europium release (**Figure 5D**) were used to measure cell lysis. Splenocytes of mice immunized with LmddA-LLO-PSA contained CTLs with high cytolytic activity for the cells that display PSA peptide as a target antigen.

[00416] Elispot was performed to determine the functional ability of effector T cells to secrete IFN-γ after 24 h stimulation with antigen. Using ELISpot, a 20-fold increase in the number of spots for IFN-γ in splenocytes from mice immunized with LmddA-LLO-PSA stimulated with specific peptide when compared to the splenocytes of the naïve mice was observed (**Figure 5E**).

EXAMPLE 6: Immunization with the *LmddA*-142 strains induces regression of a tumor expressing PSA and infiltration of the tumor by PSA-specific CTLs.

[00417] The therapeutic efficacy of the construct *LmddA*-142 (LmddA-LLO-PSA) was determined using a prostrate adenocarcinoma cell line engineered to express PSA (Tramp-C1-PSA (TPSA); Shahabi et al., 2008). Mice were subcutaneously implanted with 2 x 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 (**Figure 6A**). 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 6B**). 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 6C**). 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.

[00418] 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 6B**), compared to none in the untreated group (**Figure 6A**). The *LmddA*-142 was constructed using a highly attenuated vector (*LmddA*) and the plasmid pADV142 (**Table 1**).

[00419] 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.

[00420] 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 7A). Interestingly, the population of $CD8^{+}CD62L^{low}$ $PSA^{tetramer+}$ cells in spleen was 7.5 fold less than in tumor (**Figure 7A**).

[00421] In addition, the presence of $CD4^{+}/CD25^{+}/Foxp3^{+}$ T regulatory cells (regs) 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 $CD4^{+}$ $CD25^{+}FoxP3^{+}$ T-regs in tumor but not in spleen (**Figure 7B**). However, the construct LmddA-LLO-PSA had a stronger impact in decreasing the frequency of $CD4^{+}$ $CD25^{+}FoxP3^{+}$ T-regs in tumors when compared to the naïve and Lm-LLO-E7 immunized group (**Figure 7B**).

[00422] Thus, the *LmddA*-142 vaccine can induce PSA-specific $CD8^{+}$ T cells that are able to infiltrate the tumor site (**Figure 7A**). Interestingly, immunization with *LmddA*-142 was associated with a decreased number of regulatory T cells in the tumor (**Figure 7B**), probably creating a more favorable environment for an efficient anti-tumor CTL activity.

EXAMPLE 7: *Lmdd*-143 and *LmddA*-143 secretes a functional LLO despite the PSA fusion.

[00423] The *Lmdd*-143 and *LmddA*-143 contain the full-length human *klk3* gene, which encodes the PSA protein, inserted by homologous recombination downstream and in frame with the *hly* gene in the chromosome. These constructs were made by homologous recombination using the pKSV7 plasmid (Smith and Youngman, Biochimie. 1992; 74 (7-8) p705-711), which has a temperature-sensitive replicon, carrying the *hly-klk3-mpl* recombination cassette. Because of the plasmid excision after the second recombination event, the antibiotic resistance marker used for integration selection is lost. Additionally, the *actA* gene is deleted in the *LmddA*-143 strain (**Figure 8A**). The insertion of *klk3* in frame with *hly* into the chromosome was verified by PCR

(**Figure 8B**) and sequencing (data not shown) in both constructs.

[00424] One important aspect of these chromosomal constructs is that the production of LLO-PSA would not completely abolish the function of LLO, which is required for escape of *Listeria* from the phagosome, cytosol invasion and efficient immunity generated by *L. monocytogenes*. Western-blot analysis of secreted proteins from *Lmdd*-143 and *LmddA*-143 culture supernatants revealed an ~81 kDa band corresponding to the LLO-PSA fusion protein and an ~60 kDa band, which is the expected size of LLO (**Figure 9A**), indicating that LLO is either cleaved from the LLO-PSA fusion or still produced as a single protein by *L. monocytogenes*, despite the fusion gene in the chromosome. The LLO secreted by *Lmdd*-143 and *LmddA*-143 retained 50% of the hemolytic activity, as compared to the wild-type *L. monocytogenes* 10403S (**Figure 9B**). In agreement with these results, both *Lmdd*-143 and *LmddA*-143 were able to replicate intracellularly in the macrophage-like J774 cell line (**Figure 9C**).

EXAMPLE 8: Both *Lmdd*-143 and *LmddA*-143 elicit cell-mediated immune responses against the PSA antigen.

[00425] After showing that both *Lmdd*-143 and *LmddA*-143 were able to secrete PSA fused to LLO, the question of if these strains could elicit PSA-specific immune responses *in vivo* was investigated. C57Bl/6 mice were either left untreated or immunized twice with the *Lmdd*-143, *LmddA*-143 or *LmddA*-142. PSA-specific $CD8^+$ T cell responses were measured by stimulating splenocytes with the PSA_{65-74} peptide and intracellular staining for IFN-γ. As shown in **Figure 10**, the immune response induced by the chromosomal and the plasmid-based vectors is similar.

Materials and Methods (EXAMPLES 9-13)

MDSC and Treg Function

[00426] Tumors were implanted in mice on the flank or a physiological site depending on the tumor model. After 7 days, mice were then vaccinated, the initial vaccination day depends on the tumor model being used. The mice were then administered a booster vaccine one week after the vaccine was given.

[00427] Mice were then sacrificed and tumors and spleen were harvested 1 week after the boost or, in the case of an aggressive tumor model, 3-4 days after the boost. Five days before harvesting the tumor, non-tumor bearing mice were vaccinated to use for responder T cells. Splenocytes were prepared using standard methodology.

[00428] 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 with the tumor dissociation kit.

[00429] MDSCs or Tregs were purified from tumors and spleens using a Miltenyi kit and columns or the autoMACs separator. Cells were then counted.

[00430] Single cell suspension was prepared and the red blood cells were lysed. Responder T cells were then labeled with CFSE.

[00431] Cells were plated together at a 2:1 ratio of responder T cells (from all division cycle stages) to MDSCs or Tregs at a density of $1x10^5$ T cells per well in 96 well plates. Responder T cells were then stimulated with either the appropriate peptide (PSA OR CA9) or non-specifically with PMA/ionomycin. Cells were incubated in the dark for 2 days at 37°C with 5% CO_2. Two days later, the cells were stained for FACS and analyzed on a FACS machine.

Analysis of T-cell responses

[00432] 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.

[00433] 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.

[00434] Briefly, IFN-γ was detected using the mAb R46-A2 at 5 mg/ml and polyclonal rabbit anti- IFN-γ used at an optimal dilution (kindly provided by Dr. Phillip Scott, University of Pennsylvania, Philadelphia, PA). The levels of IFN-γ were calculated by comparison with a standard curve using murine rIFN-γ (Life Technologies, Gaithersburg, MD). Plates were developed using a peroxidase-conjugated goat anti-rabbit IgG Ab (IFN-γ). Plates were then read at 405 nm. The lower limit of detection for the assays was 30 pg/ml.

RESULTS

EXAMPLE 9: SUPPRESSOR CELL FUNCTION AFTER *LISTERIA* TREATMENT

[00435] At day 0 tumors were implanted in mice. At day 7 mice were vaccinated with Lmdda-E7 or LmddA-PSA. At day 14 tumors were harvested and the number and percentages of

infiltrating MDSCs and Treg were measured for vaccinated and naïve groups. It was found that there is a decrease in the percentages of both MDSC and Tregs in the tumors of Listeria-treated mice, and the absolute number of MDSC, whereas the same effect is not observed in the spleens or the draining lymph nodes (TLDN) (Fig. 11).

[00436] Isolated splenocytes and tumor-infiltrating lymphocytes (TILs) extracted from tumor bearing mice in the above experiment were pooled and stained for CD3, and CD8 to elucidate the effect of immunization with Lm-LLO-E7, *Lm*–LLO–PSA and *Lm*-LLO- CA9, Lm-LLO-Her2 (Fig. 12-14) on the presence of MDSCs and Tregs (both splenic and tumoral MDSCs and Tregs) in the tumor. Each column represents the % of T cell population at a particular cell division stage and is subgrouped under a particular treatment group (naïve, peptide –CA9 or PSA- treated, no MDSC/Treg, and no MDSC + PMA/ionomycin) (see Figs 12-14).

[00437] 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 10: MDSCs FROM TPSA23 TUMORS BUT NOT SPLEEN ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[00438] Suppressor assays were carried out using monocytic and granulocytic MDSCs isolated from TPSA23 tumors with non-specifically activated naïve murine cells, and specifically activated cells (PSA, CA9, PMA/ionomycyn). Results demonstrated that the MDSCs isolated from tumors from the Lm vaccinated groups have a diminished capacity to suppress the division of activated T cells as compared to MDSC from the tumors of naïve mice. (see Lm-LLO-PSA and Lm-LLO-treated Groups in Figs. 12 & 14, right-hand panel in figures represents pooled cell division data from left-hand panel). In addition, T responder cells from untreated mice where no MDSCs were present and where the cells were unstimulated/activated, remained in their parental (resting) state (Fig. 12 & 14), whereas T cells stimulated with PMA or ionomycin were observed to replicate (Fig. 12 & 14). Further, it was observed that both, the Gr+Ly6G+ and the GrdimLy6G- MDSCs are less suppressive after treatment with *Listeria*s. 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.

[00439] Moreover, suppressor assays carried out using MDSCs isolated from TPSA23 tumors with non-specifically activated naïve murine cells demonstrated that the MDSCs isolated from tumors from the Lm vaccinated groups have a diminished capacity to suppress the division of activated T cells as compared to MDSC from the tumors of naïve mice (see Figs. 12 & 14).

[00440] In addition, the observations discussed immediately above relating to Figures 12 and 18

were not observed when using splenic MDSCs. In the latter, splenocytes/ T cells from the naïve group, the Listeria-treated group (PSA, CA9), and the PMA/ionomycin stimulated group (positive control) all demonstrated the same level of replication (Fig. 13 & 15). 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 11: TUMOR T REGULATORY CELLS' REDUCED SUPPRESSION

[00441] Suppressor assays were carried out using Tregs isolated from TPSA23 tumors after Listeria treatment. It was observed that after treatment with *Listeria* there is a reduction of the suppressive ability of Tregs from tumors (Fig. 16), however, it was found that splenic Tregs are still suppressive (Fig. 17).

[00442] As a control conventional CD4+ T cells were used in place of MDSCs or Tregs and were found not to have an effect on cell division (Fig. 18).

EXAMPLE 12: MDSCs AND TREGS FROM 4T1 TUMORS BUT NOT SPLEEN ARE LESS SUPPRESSIVE AFTER *LISTERIA* VACCINATION.

[00443] As in the above, the same experiments were carried out using 4T1 tumors and the same observations were made, namely, that MDSCs are less suppressive after Listeria vaccination (Figs. 19 & 21), that *Listeria* has no specific effect on splenic monocytic MDSCs (Figs. 20 & 22), that there is a decrease in the suppressive ability of Tregs from 4T1 tumors after *Listeria* vaccination (Fig. 23), and that *Listeria* has no effect on the suppressive ability of splenic Tregs (Fig. 24).

[00444] Finally, it was observed that *Listeria* has no effect on the suppressive ability of splenic Tregs.

EXAMPLE 13: CHANGE IN THE SUPPRESSIVE ABILITY OF THE GRANULOCITY AND MONOCYTIC MDSC IS DUE TO THE OVEREXPRESSION OF tLLO.

[00445] The LLO plasmid shows similar results as the *Listerias* with either the TAA or an irrelvant antigen (Figure 25). 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.

[00446] Similar to the granulocytic MDSC, the average of the 3 independent experiments shows that the differences observed in the suppressive ability of the monocytic MDSCs purified from the tumors after vaccination with the *Lm*-empty plasmid vaccine are significant when compared to the other vaccine constructs (Figure 26).

[00447] Similar to the above observations, granulocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination (Figure 27). However, after non-specific stimulation, activated T cells (with PMA/ionomycin) are still capable of dividing. None of these results are altered with the use of the LLO only or the empty plasmid vaccines showing that the *Lm*-based vaccines are not affecting the splenic granulocytic MDSC (Figure 27).

[00448] Similarly, monocytic MDSC purified from the spleen retain their ability to suppress the division of the antigen-specific responder T cells after Lm vaccination. However, after non-specific activation (stimulated by PMA/ionomycin), T cells are still capable of dividing. None of these results are altered with the use of the LLO only or the empty plasmid vaccines showing that the Lm vaccines are not affecting the splenic monocytic MDSC (Figure 28).

[00449] Tregs purified from the tumors of any of the *Lm*-treated groups have a slightly diminished ability to suppress the division of the responder T cells, regardless of whether the responder cells are antigen specific or non-specifically activated. Especially for the non-specifically activated responder T cells, it looks as though the vaccine with the empty plasmid shows the same results as all the vaccines that contain LLO on the plasmid. Averaging this experiment with the others shows that the differences are not significant (Figure 29).

[00450] Tregs purified from the spleen are still capable of suppressing the division of both antigen specific and non-specifically activated responder T cells. There is no effect of *Lm* treatment on the suppressive ability of splenic Tregs (Figure 30).

[00451] 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 31-32).

Materials and Methods for Examples 14-20

[00452] Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and DNA sequencing was done by Genewiz Inc, South Plainfield, NJ. Flow cytometry reagents were purchased from Becton Dickinson Biosciences (BD, San Diego, CA). Cell culture media, supplements and all other reagents, unless indicated, were from Sigma (St. Louise, MO). Her2/neu HLA-A2 peptides were synthesized by EZbiolabs (Westfield, IN). Complete RPMI 1640 (C-RPMI) medium contained 2mM glutamine, 0.1 mM non-essential amino acids, and 1mM sodium pyruvate, 10% fetal bovine serum, penicillin/streptomycin, Hepes (25mM). The polyclonal anti-LLO antibody was described previously and anti-Her2/neu antibody was purchased from Sigma.

[00453] ***Mice and Cell Lines***

[00454] All animal experiments were performed according to approved protocols by IACUC at the University of Pennsylvania or Rutgers University. FVB/N mice were purchased from Jackson laboratories (Bar Harbor, ME). The FVB/N Her2/neu transgenic mice, which overexpress the rat Her2/neu onco-protein were housed and bred at the animal core facility at the University of Pennsylvania. The NT-2 tumor cell line expresses high levels of rat Her2/neu protein, was derived from a spontaneous mammary tumor in these mice and grown as described previously. DHFR-G8 (3T3/neu) cells were obtained from ATCC and were grown according to the ATCC recommendations. The EMT6-Luc cell line was a generous gift from Dr. John Ohlfest (University of Minnesota, MN) and was grown in complete C-RPMI medium. Bioluminescent work was conducted under guidance by the Small Animal Imaging Facility (SAIF) at the University of Pennsylvania (Philadelphia, PA).

[00455] ***Listeria* constructs and antigen expression**

[00456] Her2/neu-pGEM7Z was kindly provided by Dr. Mark Greene at the University of Pennsylvania and contained the full-length human Her2/neu (hHer2) gene cloned into the pGEM7Z plasmid (Promega, Madison WI). This plasmid was used as a template to amplify three segments of hHer-2/neu, namely, EC1, EC2, and IC1, by PCR using pfx DNA polymerase (Invitrogen) and the oligos indicated in **Table 3**.

[00457] **Table 3**: Primers for cloning of Human her-2-Chimera

	DNA sequence	Base pair region	Amino acid region or junctions
Her-2-Chimera (F)	TGATCTCGAGACCCACCTGGACATGCTC (SEQ ID NO:74)	120-510	40-170
HerEC1-EC2F (Junction)	CTACCAGGACACGATTTTGTGGAAG-AATATCCAGGAGTTTGCTGGCTGC (SEQ ID NO: 75)	510/1077	170/359
HerEC1-EC2R (Junction)	GCAGCCAGCAAACTCCTGGATATT-CTTCCACAAAATCGTGTCCTGGTAG (SEQ ID NO: 76)		

HerEC2-ICIF (Junction)	CTGCCACCAGCTGTGCGCCCGAGGG-CAGCAGAAGATCCGGAAGTACACGA (SEQ ID NO: 77)	1554/2034	518/679
HerEC2-ICIR (Junction)	TCGTGTACTTCCGGATCTTCTGCTGCCCTC GGGC GCACAGCTGGTGGCAG (SEQ ID NO: 78)		
Her-2-Chimera (R)	GTGGCCCGGGTCTAGATTAGTCTAAGAGG CAGCCATAG11G (SEQ ID NO: 79)	2034-2424	679-808

[00458] The Her-2/neu chimera construct was generated by direct fusion by the SOEing PCR method and each separate hHer-2/neu segment as templates. Primers are shown in **Table 4**.

[00459] **Table 4**

	DNA sequence	Base pair region	Amino acid region
Her-2-EC1(F)	CCGCCTCGAGGCCGCGAGCACCCAAGTG (SEQ ID NO: 80)	58-979	20-326
Her-2-EC1(R)	CGCGACTAGTTTAATCCTCTGCTGTCACCTC (SEQ ID NO: 81)		
Her-2-EC2(F)	CCGCCTCGAGTACCTTTCTACGGACGTG (SEQ ID NO:82)	907-1504	303-501
Her- 2- EC2(R)	CGCGACTAGTTTACTCTGGCCGGTTGGCAG (SEQ ID NO: 83)		
Her-2-Her-2-IC1(F)	CCGCCTCGAGCAGCAGAAGATCCGGAAGTAC (SEQ ID NO: 84)	2034-3243	679-1081
Her-2-IC1(R)	CGCGACTAGTTTAAGCCCCTTCGGAGGGTG (SEQ ID NO: 85)		

[00460] Sequence of primers for amplification of different segments human Her2 regions

[00461] ChHer2 gene was excised from pAdv138 using XhoI and SpeI restriction enzymes, and cloned in frame with a truncated, non-hemolytic fragment of LLO in the *Lmdd* shuttle vector, pAdv134. The sequences of the insert, LLO and *hly* promoter were confirmed by DNA sequencing analysis. This plasmid was electroporated into electro-competent *actA, dal, dat* mutant *Listeria monocytogenes* strain, *LmddA* and positive clones were selected on Brain Heart infusion (BHI) agar plates containing streptomycin (250µg/ml). In some experiments similar *Listeria* strains expressing hHer2/neu (*Lm*-hHer2) fragments were used for comparative purposes. In all studies, an irrelevant *Listeria* construct (*Lm*-control) was included to account for the antigen independent effects of *Listeria* on the immune system. *Lm*-controls were based on the same *Listeria* platform as ADXS31-164 (*LmddA*-ChHer2), but expressed a different antigen such as HPV16-E7 or NY-ESO-1. Expression and secretion of fusion proteins from *Listeria* were tested. Each construct was passaged twice *in vivo*.

[00462] **Cytotoxicity assay**

[00463] Groups of 3-5 FVB/N mice were immunized three times with one week intervals with 1

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

[00464] **Interferon-γ secretion by splenocytes from immunized mice**

[00465] Groups of 3-5 FVB/N or HLA-A2 transgenic mice were immunized three times with one week intervals with 1 x 10^8 CFU of ADXS31-164, a negative *Listeria* control (expressing an irrelevant antigen) or were left naïve. Splenocytes from FVB/N mice were isolated one week after the last immunization and co-cultured in 24 well plates at 5 x 10^6 cells/well in the presence of mitomycin C treated NT-2 cells in C-RPMI medium. Splenocytes from the HLA-A2 transgenic mice were incubated in the presence of 1μM of HLA-A2 specific peptides or 1μg/ml of a recombinant His-tagged ChHer2 protein, produced in *E. coli* and purified by a nickel based affinity chromatography system. Samples from supernatants were obtained 24 or 72 hours later and tested for the presence of interferon-γ (IFN-γ) using mouse IFN-γ Enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's recommendations.

[00466] **Tumor studies in Her2 transgenic animals**

[00467] Six weeks old FVB/N rat Her2/neu transgenic mice (9-14/group) were immunized 6 times with 5 x 10^8 CFU of *Lm*-LLO-ChHer2, ADXS31-164 or *Lm*-control. They were observed twice a week for the emergence of spontaneous mammary tumors, which were measured using an electronic caliper, for up to 52 weeks. Escaped tumors were excised when they reached a size 1cm^2 in average diameter and preserved in RNA later at -20°C. In order to determine the effect of mutations in the Her2/neu protein on the escape of these tumors, genomic DNA was extracted using a genomic DNA isolation kit, and sequenced.

[00468] **Effect of ADXS31-164 on regulatory T cells in spleens and tumors**

[00469] Mice were implanted subcutaneously (s.c.) with 1 x 10^6 NT-2 cells. On days 7, 14 and 21, they were immunized with 1 x 10^8 CFUs of ADXS31-164, *LmddA*-control or left naïve. Tumors and spleens were extracted on day 28 and tested for the presence of $CD3^+/CD4^+/FoxP3^+$ Tregs by FACS analysis. Briefly, splenocytes were isolated by homogenizing the spleens

between two glass slides in C-RPMI medium. Tumors were minced using a sterile razor blade and digested with a buffer containing DNase (12U/ml), and collagenase (2mg/ml) in PBS. After 60 min incubation at RT with agitation, cells were separated by vigorous pipetting. Red blood cells were lysed by RBC lysis buffer followed by several washes with complete RPMI-1640 medium containing 10% FBS. After filtration through a nylon mesh, tumor cells and splenocytes were resuspended in FACS buffer (2% FBS/PBS) and stained with anti-CD3-PerCP-Cy5.5, CD4-FITC, CD25-APC antibodies followed by permeabilization and staining with anti-Foxp3-PE. Flow cytometry analysis was performed using 4-color FACS calibur (BD) and data were analyzed using cell quest software (BD).

[00470] **Statistical analysis**

[00471] The log-rank Chi-Squared test was used for survival data and student's *t*-test for the CTL and ELISA assays, which were done in triplicates. A p-value of less than 0.05 (marked as *) was considered statistically significant in these analyzes. All statistical analysis was done with either Prism software, V.4.0a (2006) or SPSS software, V.15.0 (2006). For all FVB/N rat Her2/neu transgenic studies we used 8-14 mice per group, for all wild-type FVB/N studies we used at least 8 mice per group unless otherwise stated. All studies were repeated at least once except for the long term tumor study in Her2/neu transgenic mouse model.

EXAMPLE 14

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

[00472] Construction of the chimeric *Her2/neu* gene (ChHer2) was as follows. Briefly, *ChHer2* gene was generated by direct fusion of two extracellular (aa 40-170 and aa 359-433) and one intracellular fragment (aa 678-808) of the Her2/neu protein by SOEing PCR method. The chimeric protein harbors most of the known human MHC class I epitopes of the protein. *ChHer2* gene was excised from the plasmid, pAdv138 (which was used to construct *Lm*-LLO-ChHer2) and cloned into *LmddA* shuttle plasmid, resulting in the plasmid pAdv164 (**Figure 33A**). There are two major differences between these two plasmid backbones. 1) Whereas pAdv138 uses the chloramphenicol resistance marker (*cat*) for *in vitro* selection of recombinant bacteria, pAdv164 harbors the D-alanine racemase gene (*dal*) from *bacillus subtilis*, which uses a metabolic complementation pathway for *in vitro* selection and *in vivo* plasmid retention in *LmddA* strain which lacks the *dal-dat* genes. This vaccine platform was designed and developed to address FDA concerns about the antibiotic resistance of the engineered *Listeria* strains. 2) Unlike pAdv138, pAdv164 does not harbor a copy of the *prfA* gene in the plasmid (see sequence below and **Fig. 33A**), as this is not necessary for *in vivo* complementation of the *Lmdd* strain. The *LmddA* vaccine strain also lacks the *actA* gene (responsible for the intracellular

movement and cell-to-cell spread of *Listeria*) so the recombinant vaccine strains derived from this backbone are 100 times less virulent than those derived from the *Lmdd*, its parent strain. *LmddA*-based vaccines are also cleared much faster (in less than 48 hours) than the *Lmdd*-based vaccines from the spleens of the immunized mice. The expression and secretion of the fusion protein tLLO-ChHer2 from this strain was comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (**Figure 33B**) as a band of ~104 KD was detected by an anti-LLO antibody using Western Blot analysis. The *Listeria* backbone strain expressing only tLLO was used as negative control.

[00473] pAdv164 sequence (7075 base pairs) (see **Figure 33**):

[00474] cggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcac
cggtgcgtcagcagaatatgtgatacaggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaa
atggcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgttttc
cataggctccgccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgacaggactataaagataccaggc
gtttcccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcattccacg
cctgacactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaacccccgttcagtccgaccgctgcgccttatccggt
aactatcgtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagttagtcttgaagt
catgcgccggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctcggttcaaagagttggtagctcag
agaaccttcgaaaaaccgccctgcaaggcggtttttcgttttcagagcaagagattacgcgcagaccaaaacgatctcaagaagatcatct
tattaatcagataaaatatttctagccctcctttgattagtatattcctatcttaaagttacttttatgtggaggcattaacatttgttaatgacgtcaaa
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agaggggtggcaaacggtatttggcattattaggttaaaaaatgtagaaggagagtgaaacccatgaaaaaaataatgctagtttttattaca
cttatattagttagtctaccaattgcgcaacaaactgaagcaaaggatgcatctgcattcaataaagaaaattcaatttcatccatggcaccac
cagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcgataagtatatacaaggattggattacaataa
aaacaatgtattagtataccacggagatgcagtgacaaatgtgccgccaagaaaaggttacaaagatggaaatgaatatattgttgtggaga
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aattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcattaacactcagcattgatttgccaggtatgactaatcaaga
caataaaatagttgtaaaaaatgccactaaatcaaacgttaacaacgcagtaaatacattagtggaaagatggaatgaaaaatatgctcaag
cttatccaaatgtaagtgcaaaaattgattatgatgacgaaatggcttacagtgaatcacaattaattgcgaaatttggtacagcatttaaagct
gtaaataatagcttgaatgtaaacttcggcgcaatcagtgaagggaaaatgcaagaagaagtcattagttttaaacaaatttactataacgtga
atgttaatgaacctacaagaccttccagatttttcggcaaagctgttactaaagagcagttgcaagcgcttggagtgaatgcagaaaatcctc
ctgcatatatctcaagtgtggcgtatggccgtcaagtttatttgaaattatcaactaattcccatagtactaaagtaaaagctgcttttgatgctgc
cgtaagcggaaaatctgtctcaggtgatgtagaactaacaaatatcatcaaaaattcttccttcaaagccgtaatttacggaggttccgcaaaa
gatgaagttcaaatcatcgacggcaacctcggagacttacgcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcc
cattgcttatacaacaaacttcctaaaagacaatgaattagctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacag
atggaaaaattaacatcgatcactctggaggatacgttgctcaattcaacatttcttgggatgaagtaaattatgatctcgagacccacctgga

catgctccgccacctctaccagggctgccaggtggtgcagggaaacctggaactcacctacctgcccaccaatgccagcctgtccttcct
gcaggatatccaggaggtgcagggctacgtgctcatcgctcacaaccaagtgaggcaggtcccactgcagaggctgcggattgtgcga
ggcacccagctctttgaggacaactatgccctggccgtgctagacaatggagacccgctgaacaataccacccctgtcacaggggcctc
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accaggacacgattttgtggaagaatatccaggagtttgctggctgcaagaagatctttgggagcctggcatttctgccggagagctttgat
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tcagcatggccggacagcctgcctgacctcagcgtcttccagaacctgcaagtaatccggggacgaattctgcacaatggcgcctactcg
ctgaccctgcaagggctgggcatcagctggctggggctgcgctcactgagggaactgggcagtggactggccctcatccaccataaca
cccacctctgcttcgtgcacacggtgccctgggaccagctctttcggaacccgcaccaagctctgctccacactgccaaccggccagagg
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ctgcaggaaacggagctggtggagccgctgacacctagcggagcgatgcccaaccaggcgcagatgcggatcctgaaagagacgga
gctgaggaaggtgaaggtgcttggatctggcgcttttggcacagtctacaagggcatctggatccctgatggggagaatgtgaaaattcca
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gacccgggccactaactcaacgctagtagtggatttaatcccaaatgagccaacagaaccagaaccagaaacagaacaagtaacattgg
agttagaaatggaagaagaaaaaagcaatgatttcgtgtgaataatgcacgaaatcattgcttatttttttaaaaagcgatatactagatataac
gaaacaacgaactgaataaagaatacaaaaaaagagccacgaccagttaaagcctgagaaactttaactgcgagccttaattgattaccac
caatcaattaaagaagtcgagacccaaaatttggtaaagtatttaattactttattaatcagatacttaaatatctgtaaacccattatatcgggttt
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ggcaaaaaacaagtagatgaaccctggtttaatctcttattgcacgaaacgaaattttcaggagaaaagggtttagtagggcgcaatagcgt
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aaatggtggcattcaacttgctagtgttaaatcattgttgctatcgatcattaaattaaaaaaagaagaacgagaaagctatataaaggcgctg
acagcttcgtttaatttagaacgtacatttattcaagaaactctaaacaaattggcagaacgccccaaaacggacccacaactcgatttgttta

gctacgatacaggctgaaaataaaacccgcactatgccattacatttatatctatgatacgtgtttgtttttctttgctggctagcttaattgcttata
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gttaatggtttcgagccttcctgcaatctcatccatggaaatatattcatccccctgccggcctattaatgtgacttttgtgcccggcggatattc
ctgatccagctccaccataaattggtccatgcaaattcggccggcaattttcaggcgttttcccttcacaaggatgtcggtccctttcaatttttcg
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ctgatcagtttgacatgtgacagtgtcgaatgcagggtaaatgccggacgcagctgaaacggtatctcgtccgacatgtcagcagacgggg
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gggtaaataccccttgcactttaaacgagggttgcggtcaagaattgccatcacgttctgaacttcttcctctgtttttacaccaagtctgttcat
ccccgtatcgaccttcagatgaaaatgaagagaaccttttttcgtgtggcgggctgcctcctgaagccattcaacagaataacctgttaaggt
cacgtcatactcagcagcgattgccacatactccgggggaaccgcgccaagcaccaatataggcgccttcaatccctttttgcgcagtgaa
atcgcttcatccaaaatggccacggccaagcatgaagcacctgcgtcaagagcagcctttgctgtttctgcatcaccatgcccgtaggcgtt
tgctttcacaactgccatcaagtggacatgttcaccgatatgtttttttcatattgctgacattttcctttatcgcggacaagtcaatttccgcccac
gtatctctgtaaaaaggttttgtgctcatggaaaactcctctctttttttcagaaaatcccagtacgtaattaagtatttgagaattaatttttatattgat
taatactaagtttacccagttttcacctaaaaaacaaatgatgagataatagctccaaaggctaaagaggactataccaactatttgttaattaa
(SED ID NO: 86)

EXAMPLE 15: ADXS31-164 IS AS IMMUNOGENIC AS LM-LLO-ChHER2

[00475] Immunogenic properties of ADXS31-164 in generating anti-Her2/neu specific cytotoxic T cells were compared to those of the *Lm*-LLO-ChHer2 vaccine in a standard CTL assay. Both vaccines elicited strong but comparable cytotoxic T cell responses toward Her2/neu antigen expressed by 3T3/neu target cells. Accordingly, mice immunized with a *Listeria* expressing only an intracellular fragment of Her2-fused to LLO showed lower lytic activity than the chimeras which contain more MHC class I epitopes. No CTL activity was detected in naïve animals or mice injected with the irrelevant *Listeria* (**Figure 34A**). ADXS31-164 was also able to stimulate the secretion of IFN-γ by the splenocytes from wild type FVB/N mice (**Figure 34B**). This was detected in the culture supernatants of these cells that were co-cultured with mitomycin C treated NT-2 cells, which express high levels of Her2/neu antigen (**Figure 34C**).

[00476] Proper processing and presentation of the human MHC class I epitopes after immunizations with ADXS31-164 was tested in HLA-A2 mice. Splenocytes from immunized HLA-A2 transgenics were co-incubated for 72 hours with peptides corresponding to mapped HLA-A2 restricted epitopes located at the extracellular (HLYQGCQVV SEQ ID NO: 87 or KIFGSLAFL SEQ ID NO: 88) or intracellular (RLLQETELV SEQ ID NO: 89) domains of the Her2/neu molecule (**Figure 34C**). A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide as negative controls. The data from this experiment show that ADXS31-164 is able to elicit anti-Her2/neu specific immune responses to human epitopes

that are located at different domains of the targeted antigen.

EXAMPLE 16: ADXS31-164 WAS MORE EFFICACIOUS THAN LM-LLO-ChHER2 IN PREVENTING THE ONSET OF SPONTANEOUS MAMMARY TUMORS

[00477] Anti-tumor effects of ADXS31-164 were compared to those of *Lm*-LLO-ChHer2 in Her2/neu transgenic animals which develop slow growing, spontaneous mammary tumors at 20-25 weeks of age. All animals immunized with the irrelevant *Listeria*-control vaccine developed breast tumors within weeks 21-25 and were sacrificed before week 33. In contrast, *Liseria*-Her2/neu recombinant vaccines caused a significant delay in the formation of the mammary tumors. On week 45, more than 50% of ADXS31-164 vaccinated mice (5 out of 9) were still tumor free, as compared to 25% of mice immunized with *Lm*-LLO-ChHer2. At week 52, 2 out of 8 mice immunized with ADXS31-164 still remained tumor free, whereas all mice from other experimental groups had already succumbed to their disease (**Figure 35**). These results indicate that despite being more attenuated, ADXS31-164 is more efficacious than *Lm*-LLO-ChHer2 in preventing the onset of spontaneous mammary tumors in Her2/neu transgenic animals.

EXAMPLE 17: MUTATIONS IN HER2/NEU GENE UPON IMMUNIZATION WITH ADXS31-164

[00478] Mutations in the MHC class I epitopes of Her2/neu have been considered responsible for tumor escape upon immunization with small fragment vaccines or trastuzumab (Herceptin), a monoclonal antibody that targets an epitope in the extracellular domain of Her2/neu. To assess this, genomic material was extracted from the escaped tumors in the transgenic animals and sequenced the corresponding fragments of the *neu* gene in tumors immunized with the chimeric or control vaccines. Mutations were not observed within the Her-2/neu gene of any vaccinated tumor samples suggesting alternative escape mechanisms (data not shown).

EXAMPLE 18: ADXS31-164 CAUSES A SIGNIFICANT DECREASE IN INTRA-TUMORAL T REGULATORY CELLS

[00479] To elucidate the effect of ADXS31-164 on the frequency of regulatory T cells in spleens and tumors, mice were implanted with NT-2 tumor cells. Splenocytes and intra-tumoral lymphocytes were isolated after three immunizations and stained for Tregs, which were defined as $CD3^+/CD4^+/CD25^+/FoxP3^+$ cells, although comparable results were obtained with either FoxP3 or CD25 markers when analyzed separately. The results indicated that immunization with ADXS31-164 had no effect on the frequency of Tregs in the spleens, as compared to an irrelevant *Listeria* or the naïve animals (**Figure 36**). In contrast, immunization with the *Listerias* caused a considerable impact on the presence of Tregs in the tumors (**Figure 37**). Whereas in average 19.0% of all $CD3^+$ T cells in untreated tumors were Tregs, this frequency was reduced to 4.2% for the irrelevant vaccine and 3.4% for ADXS31-164, a 5-fold reduction in the

frequency of intra-tumoral Tregs (**Figure 37B**). The decrease in the frequency of intra-tumoral Tregs in mice treated with either of the *LmddA* vaccines could not be attributed to differences in the sizes of the tumors. In a representative experiment, the tumors from mice immunized with ADXS31-164 were significantly smaller [mean diameter (mm) ±SD, 6.71±0.43, n=5] than the tumors from untreated mice (8.69±0.98, n=5, $p<0.01$) or treated with the irrelevant vaccine (8.41±1.47, n=5, p=0.04), whereas comparison of these last two groups showed no statistically significant difference in tumor size (p=0.73). The lower frequency of Tregs in tumors treated with *LmddA* vaccines resulted in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* vaccines. However, only the vaccine expressing the target antigen HER2/neu (ADXS31-164) was able to reduce tumor growth, indicating that the decrease in Tregs has an effect only in the presence on antigen-specific responses in the tumor.

EXAMPLE 19: NO ESCAPE MUTATIONS WERE INTRODUCED BY LISTERIA EXPRESSING HER-2 CHIMERA

[00480] Tumor samples of the mice immunized with different vaccines such as Lm-LLO-138, LmddA164 and irrelevant vaccine Lm-LLO-NY were harvested. The DNA was purified from these samples and the DNA fragments corresponding to Her-2/neu regions IC1, EC1 and EC2 were amplified and were sequenced to determine if there were any immune escape mutations. The alignment of sequence from each DNA was performed using CLUSTALW. The results of the analysis indicated that there were no mutations in the DNA sequences harvested from tumors. The detailed analysis of these sequences is shown below.

[00481] **Alignment of EC2 (975 -1029 bp of Her-2-neu)**

[00482] Reference

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT (SEQ ID NO:90)

[00483] Lm-LLO-138-2

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00484] Lm-LLO-138-3

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00485] Lm-ddA-164-1

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00486] LmddA164-2

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00487] Lm-ddA-164-3

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00488] LmddA164-4

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00489] Lm-ddA-164-5

GGTCACAGCTGAGGACGGAACACAGCGTTGTGAGAAATGCAGCAAGCCCTGTGCT

[00490] LmddA-164-6

GGTCACAGCTGAGGACGGAACACAGCGTTCTGAGAAATGCAGCAAGCCCTGTGCT

[00491] Reference

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

(SEQ ID NO:91)

[00492] Lm-LLO-138-2

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00493] Lm-LLO-138-3

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00494] Lm-ddA-164-1

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00495] LmddA164-2

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00496] Lm-ddA-164-3

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00497] LmddA164-4

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00498] Lm-ddA-164-5

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00499] LmddA-164-6

CGAGTGTGCTATGGTCTGGGCATGGAGCACCTTCGAGGGGCGAGGGCCATCACCAGTGAC

[00500] Reference

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

(SEQ ID No:92)

[00501] Lm-LLO-138-2

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00502] Lm-LLO-138-3

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00503] Lm-ddA-164-1

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00504] LmddA164-2

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00505] Lm-ddA-164-3

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00506] LmddA164-4

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00507] Lm-ddA-164-5

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00508] LmddA-164-6

AATGTCCAGGAGTTTGATGGCTGCAAGAAGATCTTTGGGAGCCTGGCATTTTTGCCGGAG

[00509] Reference

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

(SEQ ID No:93)

[00510] Lm-LLO-138-2

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00511] Lm-LLO-138-3

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00512] Lm-ddA-164-1

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00513] LmddA164-2

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00514] Lm-ddA-164-3

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00515] LmddA164-4

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00516] Lm-ddA-164-5

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00517] LmddA-164-6

AGCTTTGATGGGGACCCCTCCTCCGGCATTGCTCCGCTGAGGCCTGAGCAGCTCCAAGTG

[00518] Reference

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

(SEQ ID NO: 94)

[00519] Lm-LLO-138-2

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

[00520] Lm-LLO-138-3

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

[00521] Lm-ddA-164-1

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

[00522] LmddA164-2

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

[00523] Lm-ddA-164-3

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

[00524] LmddA164-4

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCTC

[00525] Lm-ddA-164-5

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCANACAGTCTC

[00526] LmddA-164-6

TTCGAAACCCTGGAGGAGATCACAGGTTACCTGTACATCTCAGCATGGCCAGACAGTCT

[00527] Reference

CGTGACCTCAGTGTCTTCCAGAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

(SEQ ID NO: 95)

[00528] Lm-LLO-138-2

CGTGACCTCAGTGTCTTCCAGAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00529] Lm-LLO-138-3

CGTGACCTCAGTGTCTTCCAGAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00530] Lm-ddA-164-1

CGTGACCTCAGTGTCTTCCAGAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00531] LmddA164-2

CGTGACCTCAGTGTCTTCCAGAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00532] Lm-ddA-164-3

CGTGACCTCAGTGTCTTCCAGAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00533] LmddA164-4

CGTGACCTCAGTGTCTTCCAAAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00534] Lm-ddA-164-5

CGTGACCTCAGTGTCTTCCAAAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00535] LmddA-164-6

CGTGACCTCAGTGTCTTCCAAAACCTTCGAATCATTCGGGGACGGATTCTCCACGATGGC

[00536] Reference

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

(SEQ ID NO: 96)

[00537] Lm-LLO-138-2

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

[00538] Lm-LLO-138-3

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

[00539] Lm-ddA-164-1

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

[00540] LmddA164-3

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

[00541] Lm-ddA-164-5

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

[00542] Lm-ddA-164-6

GCGTACTCATTGACACTGCAAGGCCTGGGGATCCACTCGCTGGGGCTGCGCTCACTGCGG

[00543] Reference

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

(SEQ ID NO: 97)

[00544] Lm-LLO-138-2

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

[00545] Lm-LLO-138-3

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

[00546] Lm-ddA-164-1

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

[00547] LmddA164-3

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

[00548] Lm-ddA-164-5

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

[00549] Lm-ddA-164-6

GAGCTGGGCAGTGGATTGGCTCTGATTCACCGCAACGCCCATCTCTGCTTTGTACACACT

[00550] Reference

GTACCTTGGGACCAGCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

(SEQ ID NO: 98)

[00551] Lm-LLO-138-2

GTACCTTGGGACCAGCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

[00552] Lm-LLO-138-3

GTACCTTGGGACCAGCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

[00553] Lm-ddA-164-1

GTACCTTGGGACCAGCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

[00554] LmddA164-3

GTACCTTGGGACCAGCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

[00555] Lm-ddA-164-5

GTACCTTGGGACCANCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

[00556] Lm-ddA-164-6

GTACCTTGGGACCAGCTCTTCCGGAACCCACATCAGGCCCTGCTCCACAGTGGGAACCGG

[00557] Reference

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCCACGGGCAC

(SEQ ID NO: 99)

[00558] Lm-LLO-138-2

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCCACGGGCAC

[00559] Lm-LLO-138-3

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCCACGGGCAC

[00560] Lm-ddA-164-1

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCCACGGGCAC

[00561] LmddA164-3

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCCACGGGCAC

[00562] Lm-ddA-164-6

CCGGAAGAGGATTGTGGTCTCGAGGGCTTGGTCTGTAACTCACTGTGTGCCCACGGGCAC

[00563] Reference

TGCTGGGGGCCAGGGCCCACCCAGTGTGTCAACTGCAGTCATTTCCTTCGGGGCCAGGAG

(SEQ ID NO: 100)

[00564] Lm-LLO-138-2

TGCTGGGGGCCAGGGCCCACCCAGTGTGTCAACTGCAGTCATTTCCTTCGGGGCCAGGAG

[00565] Lm-LLO-138-3

TGCTGGGGGCCAGGGCCCACCCAGTGTGTCAACTGCAGTCATTTCCTTCGGGGCCAGGAG

[00566] Lm-ddA-164-1

TGCTGGGGGCCAGGGCCCACCCAGTGTGTCAACTGCAGTCATTTCCTTCGGGGCCAGGAG

[00567] LmddA164-3

TGCTGGGGGCCAGGGCCCACCCAGTGTGTCAACTGCAGTCATTTCCTTCGGGGCCAGGAG

[00568] Lm-ddA-164-6 TGCTGGGGGCCAGGGCCCACCCA----------

[00569] **Alignment of IC1 (2114-3042 bp of Her-2-neu)**

[00570] Reference

CGCCCAGCGGAGCAATGCCCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

(SEQ ID NO: 101)

[00571] Lm-LLO-NY-2

CGCCCAGCGGAGCAATGCCCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

[00572] Lm-LLO-138-4

CGCCCAGCGGAGCAATGCCCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

[00573] Lm-ddA-164-2

CGCCCAGCGGAGCAATGCCCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

[00574] Lm-ddA-164-3

CGCCCAGCGGAGCAATGCCCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

[00575] Lm-ddA164-6

CGCCCAGCGGAGCAATGCCCAACCAGGCTCAGATGCGGATCCTAAAAGAGACGGAGC

[00576] Reference

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

(SEQ ID NO: 102)

[00577] Lm-LLO-NY-1

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00578] Lm-LLO-NY-2

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00579] Lm-LLO-138-1

TAAGGAAGGTGAACGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00580] Lm-LLO-138-2

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00581] Lm-LLO-138-3

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00582] Lm-LLO-138-4

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00583] Lm-ddA-164-1

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00584] Lm-ddA-164-2

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00585] Lm-ddA-164-3

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00586] Lm-ddA-164-4

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00587] Lm-ddA-164-5

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00588] Lm-ddA164-6

TAAGGAAGGTGAAGGTGCTTGGATCAGGAGCTTTTGGCACTGTCTACAAGGGCATCTGGA

[00589] Reference

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

(SEQ ID NO: 103)

[00590] Lm-LLO-NY-1

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00591] Lm-LLO-NY-2

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00592] Lm-LLO-138-1

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00593] Lm-LLO-138-2

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00594] Lm-LLO-138-3

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00595] Lm-LLO-138-4

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00596] Lm-ddA-164-1

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00597] Lm-ddA-164-2

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00598] Lm-ddA-164-3

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00599] Lm-ddA-164-4

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00600] Lm-ddA-164-5

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00601] Lm-ddA164-6

TCCCAGATGGGGAGAATGTGAAAATCCCCGTGGCTATCAAGGTGTTGAGAGAAAACACAT

[00602] Reference

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

(SEQ ID NO: 104)

[00603] Lm-LLO-NY-1

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00604] Lm-LLO-NY-2

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00605] Lm-LLO-138-1

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00606] Lm-LLO-138-2

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00607] Lm-LLO-138-3

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00608] lm-LLO-138-4

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00609] Lm-ddA-164-1

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00610] Lm-ddA-164-2

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00611] Lm-ddA-164-3

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00612] Lm-ddA-164-4

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00613] Lm-ddA-164-5

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00614] Lm-ddA164-6

CTCCTAAAGCCAACAAAGAAATTCTAGATGAAGCGTATGTGATGGCTGGTGTGGGTTCTC

[00615] Reference

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

(SEQ ID NO: 105)

[00616] Lm-LLO-NY-1

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00617] Lm-LLO-NY-2

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00618] Lm-LLO-138-1

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00619] Lm-LLO-138-2

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00620] Lm-LLO-138-3

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00621] Lm-LLO-138-4

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00622] Lm-ddA-164-1

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00623] Lm-ddA-164-2

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00624] Lm-ddA-164-3

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00625] Lm-ddA-164-4

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00626] Lm-ddA-164-5

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00627] Lm-ddA164-6

CGTATGTGTCCCGCCTCCTGGGCATCTGCCTGACATCCACAGTACAGCTGGTGACACAGC

[00628] Reference

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

(SEQ ID NO: 106)

[00629] Lm-LLO-NY-1

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00630] Lm-LLO-NY-2

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00631] Lm-LLO-138-1

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00632] Lm-LLO-138-2

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00633] Lm-LLO-138-3

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00634] Lm-LLO-138-4

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00635] Lm-ddA-164-1

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00636] Lm-ddA-164-2

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00637] Lm-ddA-164-3

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00638] Lm-ddA-164-4

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00639] Lm-ddA-164-5

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00640] Lm-ddA164-6

TTATGCCCTACGGCTGCCTTCTGGACCATGTCCGAGAACACCGAGGTCGCCTAGGCTCCC

[00641] Reference

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

(SEQ ID NO: 107)

[00642] Lm-LLO-NY-1

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00643] Lm-LLO-NY-2

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00644] Lm-LLO-138-1

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00645] Lm-LLO-138-2

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00646] Lm-LLO-138-3

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00647] Lm-LLO-138-4

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00648] Lm-ddA-164-1

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00649] Lm-ddA-164-2

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00650] Lm-ddA-164-3

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00651] Lm-ddA-164-4

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00652] Lm-ddA-164-5

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00653] Lm-ddA164-6

AGGACCTGCTCAACTGGTGTGTTCAGATTGCCAAGGGGATGAGCTACCTGGAGGACGTGC

[00654] Reference

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

(SEQ ID NO: 108)

[00655] Lm-LLO-NY-1

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00656] Lm-LLO-NY-2

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00657] Lm-LLO-138-1

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00658] Lm-LLO-138-2

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00659] Lm-LLO-138-3

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00660] Lm-LLO-138-4

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00661] Lm-ddA-164-1

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00662] Lm-ddA-164-2

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00663] Lm-ddA-164-4

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00664] Lm-ddA-164-3

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00665] Lm-ddA-164-5

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00666] Lm-ddA164-6

GGCTTGTACACAGGGACCTGGCTGCCCGGAATGTGCTAGTCAAGAGTCCCAACCACGTCA

[00667] Reference

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

(SEQ ID NO: 109)

[00668] Lm-LLO-NY-1

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00669] Lm-LLO-NY-2

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00670] Lm-LLO-138-1

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00671] Lm-LLO-138-2

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00672] Lm-LLO-138-3

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00673] Lm-LLO-138-4

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00674] Lm-ddA-164-1

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00675] Lm-ddA-164-2

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00676] Lm-ddA-164-3

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00677] Lm-ddA-164-4

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00678] Lm-ddA-164-5

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00679] Lm-ddA164-6

AGATTACAGATTTCGGGCTGGCTCGGCTGCTGGACATTGATGAGACAGAGTACCATGCAG

[00680] Reference

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA(

SEQ ID NO: 110)

[00681] Lm-LLO-NY-1

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00682] Lm-LLO-NY-2

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00683] Lm-LLO-138-1

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00684] Lm-LLO-138-2

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00685] Lm-LLO-138-3

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00686] Lm-LLO-138-4

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00687] Lm-ddA-164-1

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00688] Lm-ddA-164-2

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00689] Lm-ddA-164-3

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00690] Lm-ddA-164-4

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00691] Lm-ddA-164-5

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00692] Lm-ddA-164-6

ATGGGGGCAAGGTGCCCATCAAATGGATGGCATTGGAATCTATTCTCAGACGCCGGTTCA

[00693] Reference

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

(SEQ ID NO: 111)

[00694] Lm-LLO-NY-1

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00695] Lm-LLO-NY-2

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00696] Lm-LLO-138-1

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00697] Lm-LLO-138-2

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00698] Lm-LLO-138-3

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00699] Lm-LLO-138-4

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00700] Lm-ddA-164-1

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00701] Lm-ddA-164-2

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00702] Lm-ddA-164-3

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00703] Lm-ddA-164-4

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00704] Lm-ddA-164-5

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00705] Lm-ddA164-6

CCCATCAGAGTGATGTGTGGAGCTATGGAGTGACTGTGTGGGAGCTGATGACTTTTGGGG

[00706] Reference

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA (SEQ ID NO: 112)

[00707] Lm-LLO-NY-1

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00708] Lm-LLO-NY-2

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00709] Lm-LLO-138-1

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00710] Lm-LLO-138-3

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00711] Lm-LLO-138-4

CCAAACCTTACGATGNAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00712] Lm-ddA164-6

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00713] Lm-ddA-164-2

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00714] Lm-LLO-138-2

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00715] Lm-ddA-164-3

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00716] Lm-ddA-164-5

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00717] Lm-ddA-164-1

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00718] Lm-ddA-164-4

CCAAACCTTACGATGGAATCCCAGCCCGGGAGATCCCTGATTTGCTGGAGAAGGGAGAA

[00719] Reference

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT (SEQ ID NO: 113)

[00720] Lm-LLO-NY-1

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00721] Lm-LLO-NY-2

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00722] Lm-LLO-138-1

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00723] Lm-LLO-138-2

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00724] Lm-LLO-138-3

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00725] Lm-LLO-138-4

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00726] Lm-ddA-164-1

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00727] Lm-ddA-164-2

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00728] Lm-ddA-164-3

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00729] Lm-ddA-164-4

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00730] Lm-ddA-164-5

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00731] Lm-ddA164-6

CGCCTACCTCAGCCTCCAATCTGCACCATTGATGTCTACATGATTATGGTCAAATGTT

[00732] Reference

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT (SEQ ID NO: 114)

[00733] Lm-LLO-NY-1

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00734] Lm-LLO-NY-2

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00735] Lm-LLO-138-2

GGATGATTGACTCTGAATGTCCCCCGAGATTCCGGGAGTTGGTGTCAAAATTTT

[00736] Lm-LLO-138-3

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00737] Lm-LLO-138-4

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00738] Lm-ddA-164-1

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00739] Lm-ddA-164-2

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00740] Lm-ddA-164-3

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00741] Lm-ddA-164-5

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00742] Lm-ddA-164-4

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00743] Lm-ddA164-6

GGATGATTGACTCTGAATGTCGCCCGAGATTCCGGGAGTTGGTGTCAGAATTTT

[00744] Reference

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT (SEQ ID NO: 115)

[00745] Lm-LLO-NY-1

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00746] Lm-LLO-NY-2

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00747] Lm-LLO-138-2

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00748] Lm-LLO-138-3

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00749] Lm-LLO-138-4

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00750] Lm-ddA-164-1

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00751] Lm-ddA-164-2

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00752] Lm-ddA-164-3

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00753] Lm-ddA-164-5

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00754] Lm-ddA-164-6

CACGTATGGCGAGGGACCCCCAGCGTTTTGTGGTCATCCAGAACGAGGACTT

[00755] **Alignment of EC1 (399-758 bp of Her-2-neu)**

[00756] Reference

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT (SEQ ID NO: 116)

[00757] Lm-LLO-138-1

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT

[00758] Lm-LLO-138-2

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT

[00759] Lm-ddA-164-1

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT

[00760] LmddA-164-2

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT

[00761] LmddA-164-3

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT

[00762] LmddA164-4

CCCAGGCAGAACCCCAGAGGGGCTGCGGGAGCTGCAGCTTCGAAGTCTCACAGAGATCCT

[00763] Reference

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

(SEQ ID NO: 117)

[00764] Lm-LLO-138-1

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

[00765] Lm-LLO-138-2

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

[00766] Lm-ddA-164-1

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

[00767] LmddA-164-2

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

[00768] LmddA-164-3

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

[00769] LmddA164-4

GAAGGGAGGAGTTTTGATCCGTGGGAACCCTCAGCTCTGCTACCAGGACATGGTTTTGTG

[00770] Reference

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

(SEQ ID NO: 118)

[00771] Lm-LLO-138-1

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

[00772] Lm-LLO-138-2

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

[00773] Lm-ddA-164-1

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

[00774] LmddA-164-2

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

[00775] LmddA-164-3

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

[00776] LmddA164-4

CCGGGCCTGTCCACCTTGTGCCCCCGCCTGCAAAGACAATCACTGTTGGGGTGAGAGTCC

[00777] Reference

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

(SEQ ID NO: 119)

[00778] Lm-LLO-138-1

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

[00779] Lm-LLO-138-2

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

[00780] Lm-ddA-164-1

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

[00781] LmddA-164-2

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

[00782] LmddA-164-3

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

[00783] LmddA164-4

GGAAGACTGTCAGATCTTGACTGGCACCATCTGTACCAGTGGTTGTGCCCGGTGCAAGGG

[00784] Reference

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

(SEQ ID NO: 120)

[00785] Lm-LLO-138-1

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

[00786] Lm-LLO-138-2

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

[00787] Lm-ddA-164-1

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGCA

[00788] LmddA-164-2

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGTA

[00789] LmddA-164-3

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGTAL

mddA164-4

CCGGCTGCCCACTGACTGCTGCCATGAGCAGTGTGCCGCAGGCTGCACGGGCCCCAAGTA

EXAMPLE 20: PERIPHERAL IMMUNIZATION WITH ADXS31-164 CAN DELAY THE GROWTH OF A METASTATIC BREAST CANCER CELL LINE IN THE BRAIN

[00790] Mice were immunized IP with ADXS31-164 or irrelevant *Lm*-control vaccines and then implanted intra-cranially with 5,000 EMT6-Luc tumor cells, expressing luciferase and low levels of Her2/neu (**Figure 38C**). Tumors were monitored at different times post-inoculation by *ex vivo* imaging of anesthetized mice. On day 8 post-tumor inoculation tumors were detected in all control animals, but none of the mice in ADXS31-164 group showed any detectable tumors (**Figure 38A and 38B**). ADXS31-164 could clearly delay the onset of these tumors, as on day 11 post-tumor inoculation all mice in negative control group had already succumbed to their tumors, but all mice in ADXS31-164 group were still alive and only showed small signs of tumor growth. These results strongly suggest that the immune responses obtained with the peripheral

administration of ADXS31-164 could possibly reach the central nervous system and that *LmddA*-based vaccines might have a potential use for treatment of CNS tumors.

[00791] While certain features of 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.

What is claimed is:

1. An immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, said composition further comprising an additional active agent.

2. The composition of claim 1, wherein said additional active agent comprises an attenuated oncolytic virus, a chimeric antigen receptor engineered T cell (CAR T cells), a therapeutic or immunomodulating antibody, a targeting thymidine kinase inhibitor (TKI), or T cell receptor engineered T cells (Receptor engineered T cells), or any combination thereof.

3. The composition of claim 2, wherein said attenuated oncolytic virus is selected from the group comprising a vesicular stomatitis virus (VSV), a newcastle disease virus (NDV), a retrovirus, a reovirus, a measles virus, a sinbis virus, an influenza virus, a herpes simplex virus, a vaccinia virus, and an adenovirus.

4. The composition of any one of claims 2-3, wherein said oncolytic virus expresses a programmed cell death receptor (PD-1) binding agonist or antagonist.

5. The composition of any one of claims 2-3, wherein said immunomodulating antibody is a PD-1 antagonist selected from the group comprising an antibody or a fragment thereof, a PD-1 antagonist, or a PD-1 partial antagonist, or any combination thereof.

6. The composition of claim 2, wherein said CAR T cells comprise a nucleic acid that encodes an antigen binding domain.

7. The composition of claim 6, wherein said antigen binding domain is an antibody or an antigen-binding fragment thereof.

8. The composition of claim 7, wherein said antigen binding fragment thereof is a Fab or scFv.

9. The composition of any one of claims 6-8, wherein said antigen binding domain binds to a prostate specific antigen (PSA) domain, a human papilloma virus (HPV) antigen domain or a chimeric Her2/neu antigen domain.

10. The composition of claim 2, wherein said antibody recognizes a prostate specific antigen (PSA) epitope, a human papilloma virus (HPV) antigen epitope or a chimeric

Her2/neu antigen epitope.

11. The composition of claim 2, wherein said Receptor engineered T cells comprise selective binding specificity to a cell-surface tumor ligand.

12. The composition of claim 11, wherein said ligand comprises a prostate specific antigen (PSA) cell-surface tumor ligand, a human papilloma virus (HPV) cell-surface tumor ligand or a chimeric Her2/neu cell-surface tumor ligand.

13. The composition of claim 2, wherein said thymidine kinase inhibitor (TKI) comprises imatinib mesylate (IM), dasatinib (D), nilotinib (N), bosutinib (B), INNO 406, zelborafinib, gefitinib, erlotinib or sunitinib.

14. The composition of claims 1-13, wherein said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, is integrated into the *Listeria* genome.

15. The composition of claims 1-13, wherein said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, is in a plasmid in said recombinant *Listeria* strain.

16. *Listeria*The composition of claim 15, wherein said plasmid is stably maintained in said recombinant *Listeria* strain in the absence of antibiotic selection.

17. The composition of claim 15, wherein said plasmid does not confer antibiotic resistance upon said recombinant *Listeria*.

18. Truncated LLO, a truncated ActA or a PEST-sequence peptide The composition according to any of the claims 1-17, wherein said heterologous antigen is a tumor-associated antigen.

19. The composition according to claim 18, wherein said tumor-associated antigen is a prostate specific antigen (PSA), a human papilloma virus (HPV) antigen or a chimeric Her2/neu antigen.

20. The composition of claim 19, wherein said PSA antigen comprises an amino acid sequence set forth in SEQ ID NO: 26.

21. The composition of claim 19, wherein said HPV antigen comprises an amino acid sequence set forth in SEQ ID NO: 54.

22. The composition of claim 19, wherein said cHER2 antigen comprises an amino acid sequence set forth in SEQ ID NO: 57.

23. The composition of claim 18, wherein said tumor-associated antigen is an angiogenic antigen.

24. The composition of claim 19, wherein when said tumor-associated antigen is a prostate specific antigen (PSA), and if present, said antigen binding domain binds to a PSA domain and/or said monoclonal antibody recognizes a PSA epitope.

25. The composition of claim 19, wherein when said tumor associated antigen is a human papilloma virus (HPV) antigen, and if present, said antigen binding domain binds to an HPV antigen and/or said monoclonal antibody recognizes an HPV epitope.

26. The composition of claim 19, wherein when said tumor-associated antigen is a chimeric Her2/neu antigen, and if present, said antigen binding domain binds to a chimeric Her2/neu antigen domain and/or said monoclonal antibody recognizes a Her2/neu antigen.

27. The composition according to any of the claims 1-26, wherein said recombinant *Listeria* strain is attenuated.

28. The method of claim 27, wherein said attenuated *Listeria* comprises a mutation, in at least one endogenous gene.

29. The method of claim 28, wherein said mutation comprises inactivation, truncation, deletion, replacement or disruption.

30. The method of any one of claims 28-29, wherein said endogenous gene is an *actA* virulence gene, a p*rfA* virulence gene, a *dal* gene, an *inlB* gene, a *dat* gene or a combination thereof.

31. The composition of any one of claims 28-30, wherein said endogenous genes comprise the *dal/dat* and *actA* genes.

32. The composition of any one of claims 1-31, wherein said nucleic acid comprising a first open reading frame, further comprises a second open reading frame.

33. The composition of claim 32, wherein said second open reading frame encodes a PrfA protein comprising a D133V mutation, and wherein said PrfA protein complements said mutation, deletion, disruption, inactivation, replacement, or truncation in said *prfA* gene.

34. The composition of claim 32, wherein said second open reading frame encodes a metabolic enzyme and wherein said metabolic enzyme complements said mutation, deletion, disruption, inactivation, replacement, or truncation in said *dal* and *dat* genes.

35. The composition according to claim 34, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

36. The composition according to any of claims 1-35, further comprising an adjuvant.

37. The composition of claim 36, wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

38. The composition of any one of claims 1-37, wherein said *Listeria* is *Listeria monocytogenes*.

39. A method of eliciting an enhanced anti-tumor T cell response in a subject, said method comprising the step of administering to said subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a Truncated LLO, a truncated ActA or a PEST-sequence peptide or a PEST-sequence peptide fused to a heterologous antigen or fragment thereof, wherein:

(a) said composition further comprises an additional active agent;

(b) said method further comprises a step of administering an effective amount of a composition comprising an additional active agent to said subject; or

(c) said method further comprises a step of administering a targeted radiation therapy to said subject; or any combination thereof of (a)-(c).

40. The method of claim 39, wherein said additional active agent comprises an attenuated oncolytic virus, a chimeric antigen receptor engineered T cell (CAR T cells), a therapeutic or immunomodulating monoclonal antibody, a targeting thymidine kinase inhibitor (TKI), or T cell receptor engineered T cells (Receptor engineered T cells), or any combination thereof.

41. The method of claim 40, wherein said attenuated oncolytic virus is selected from the group comprising a vesicular stomatitis virus (VSV), a newcastle disease virus (NDV), a

retrovirus, a reovirus, a measles virus, a sinbis virus, an influenza virus, a herpes simplex virus, a vaccinia virus, and an adenovirus.

42. The method of any one of claims 40-41, wherein said oncolytic virus expresses a programmed cell death receptor (PD-1) binding agonist or antagonist.

43. The composition of any one of claims 40-41, wherein said immunomodulating antibody is a PD-1 antagonist selected from the group comprising an antibody or a fragment thereof, a PD-1 antagonist, or a PD-1 partial antagonist, or any combination thereof

44. The method of claim 40, wherein said CAR T cells comprise a nucleic acid that encodes an antigen binding domain.

45. The method of claim 44, wherein said antigen binding domain is an antibody or an antigen-binding fragment thereof.

46. The method of claim 45, wherein said antigen binding fragment thereof is a Fab or scFv.

47. The method of any one of claims 44-46, wherein said antigen binding domain binds to a prostate specific antigen (PSA) domain, a human papilloma virus (HPV) antigen domain or a chimeric Her2/neu antigen domain.

48. The method of claim 40, wherein said monoclonal antibody recognizes a prostate specific antigen (PSA) epitope, a human papilloma virus (HPV) antigen epitope or a chimeric Her2/neu antigen epitope.

49. The method of claim 40, wherein said Receptor engineered T cells comprise selective binding specificity to a cell-surface tumor ligand.

50. The method of claim 49, wherein said ligand comprises a prostate specific antigen (PSA) cell-surface tumor ligand, a human papilloma virus (HPV) cell-surface tumor ligand or a chimeric Her2/neu cell-surface tumor ligand.

51. The method of claim 40, wherein said thymidine kinase inhibitor (TKI) comprises imatinib mesylate (IM), dasatinib (D), nilotinib (N), bosutinib (B), INNO 406, zelborafinib, gefitinib, erlotinib or sunitinib.

52. The method of claims 39-51, wherein said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, is integrated into the *Listeria* genome.

53. The method of claims 39-52, wherein said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, is in a plasmid in said recombinant *Listeria* strain.

54. The method of claim 53, wherein said plasmid is stably maintained in said recombinant *Listeria* strain in the absence of antibiotic selection.

55. The method of claim 53, wherein said plasmid does not confer antibiotic resistance upon said recombinant *Listeria*.

56. The method according to any of the claims 39-56, wherein said heterologous antigen is a tumor-associated antigen.

57. The method according to claim 56, wherein said tumor-associated antigen is a prostate specific antigen (PSA), a human papilloma virus (HPV) antigen or a chimeric Her2/neu antigen.

58. The method of claim 57, wherein said PSA antigen comprises an amino acid sequence set forth in SEQ ID NO: 26.

59. The method of claim 57, wherein said HPV antigen comprises an amino acid sequence set forth in SEQ ID NO: 54.

60. The method of claim 57, wherein said cHER2 antigen comprises an amino acid sequence set forth in SEQ ID NO: 57.

61. The method of claim 56, wherein said tumor-associated antigen is an angiogenic antigen.

62. The method of claim 56, wherein when said tumor-associated antigen is a prostate specific antigen (PSA), and if present, said antigen binding domain binds to a PSA domain and/or said monoclonal antibody recognizes a PSA epitope.

63. The method of claim 56, wherein when said tumor associated antigen is a human papilloma virus (HPV) antigen, and if present, said antigen binding domain binds to an HPV antigen and/or said monoclonal antibody recognizes an HPV epitope.

64. The method of claim 56, wherein when said tumor-associated antigen is a chimeric Her2/neu antigen, and if present, said antigen binding domain binds to a chimeric Her2/neu antigen domain and/or said monoclonal antibody recognizes a Her2/neu antigen.

65. The method according to any of the claims 39-64, wherein said recombinant *Listeria* strain is attenuated.

66. The method of claim 65, wherein said attenuated *Listeria* comprises a mutation, in at least one endogenous gene.

67. The method of claim 66, wherein said mutation comprises inactivation, truncation, deletion, replacement or disruption.

68. The method of any one of claims 66-67, wherein said endogenous gene is an *actA* virulence gene, a p*rfA* virulence gene, a *dal* gene, an *inlB* gene, a *dat* gene or a combination thereof.

69. The method of any one of claims 66-68, wherein said endogenous genes comprise the *dal/dat* and *actA* genes.

70. The method of any one of claims 66-69, wherein said nucleic acid comprising a first open reading frame, further comprises a second open reading frame.

71. The method of claim 70, wherein said second open reading frame encodes a PrfA protein comprising a D133V mutation, and wherein said PrfA protein complements said mutation in said *prfA* gene.

72. The method of claim 70, wherein said second open reading frame encodes a metabolic enzyme and wherein said metabolic enzyme complements said mutation, deletion, disruption, inactivation, replacement, or truncation in said *dal* and *dat* genes.

73. The method according to claim 72, wherein said metabolic enzyme encoded by said second open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

74. The method according to any of claims 39-73, further comprising an adjuvant.

75. The method of claim 74, wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

76. ListeriaThe method of any one of claims 40-75, wherein said composition comprising an additional active agent is administered prior to, concurrent with or following the

administration of said composition comprising said recombinant attenuated *Listeria* strain

77. The method of any one of claims 40-76, wherein said targeted radiation therapy comprises the use of a targeted delivery system comprising nanoparticles, microspheres, viruses or antibodies, or any combination thereof.

78. The method of any one of claims 40-77, wherein said targeted radiation therapy is administered prior to, concurrent with or following the administration of said composition.

79. The method of any one of claims 40-78, wherein said CAR T cells are autologous or single-source HLA masked CAR T cells.

80. The method of any one of claims 40-79, wherein said immune response comprises increasing a level of Interferon-gamma producing cells.

81. The method of any one of claims 40-80, wherein said immune response comprises an increase of tumor infiltration by T effector cells.

82. The method of claim 81, wherein said T effector cells are CD45+CD8+Tcells or CD4+Fox3P- T cells.

83. The method of any one of claims 40-82, wherein said immune response comprises a decrease in the frequency of T regulatory cells (Tregs) in the spleen and the tumor microenvironment.

84. The method of any one of claims 40-83, wherein said immune response comprises a decrease in the frequency of myeloid derived suppressor cells (MDSCs) in the spleen and the tumor microenvironment.

85. The method of any one of claims 40-84, wherein said method comprises inhibiting tumor-mediated immunosuppression in a subjectTruncated LLO, a truncated ActA or a PEST-sequence peptide Truncated LLO, a truncated ActA or a PEST-sequence peptide

86. The method of any one of claims 40-84, wherein said method comprises treating a tumor or cancer in a subjectTruncated LLO, a truncated ActA or a PEST-sequence peptide Truncated LLO, a truncated ActA or a PEST-sequence peptide The method of any one of claims 85-86, wherein said tumor is a breast tumor, a head and neck tumor, a cervical tumor, a prostate tumor.

87. The method of any one of claims 85-86, wherein said cancer is a breast cancer, a head

and neck cancer, a cervical cancer, a prostate cancer, an anal cancer, an esophageal cancer, a lung cancer, a melanoma, or an ovarian cancer.

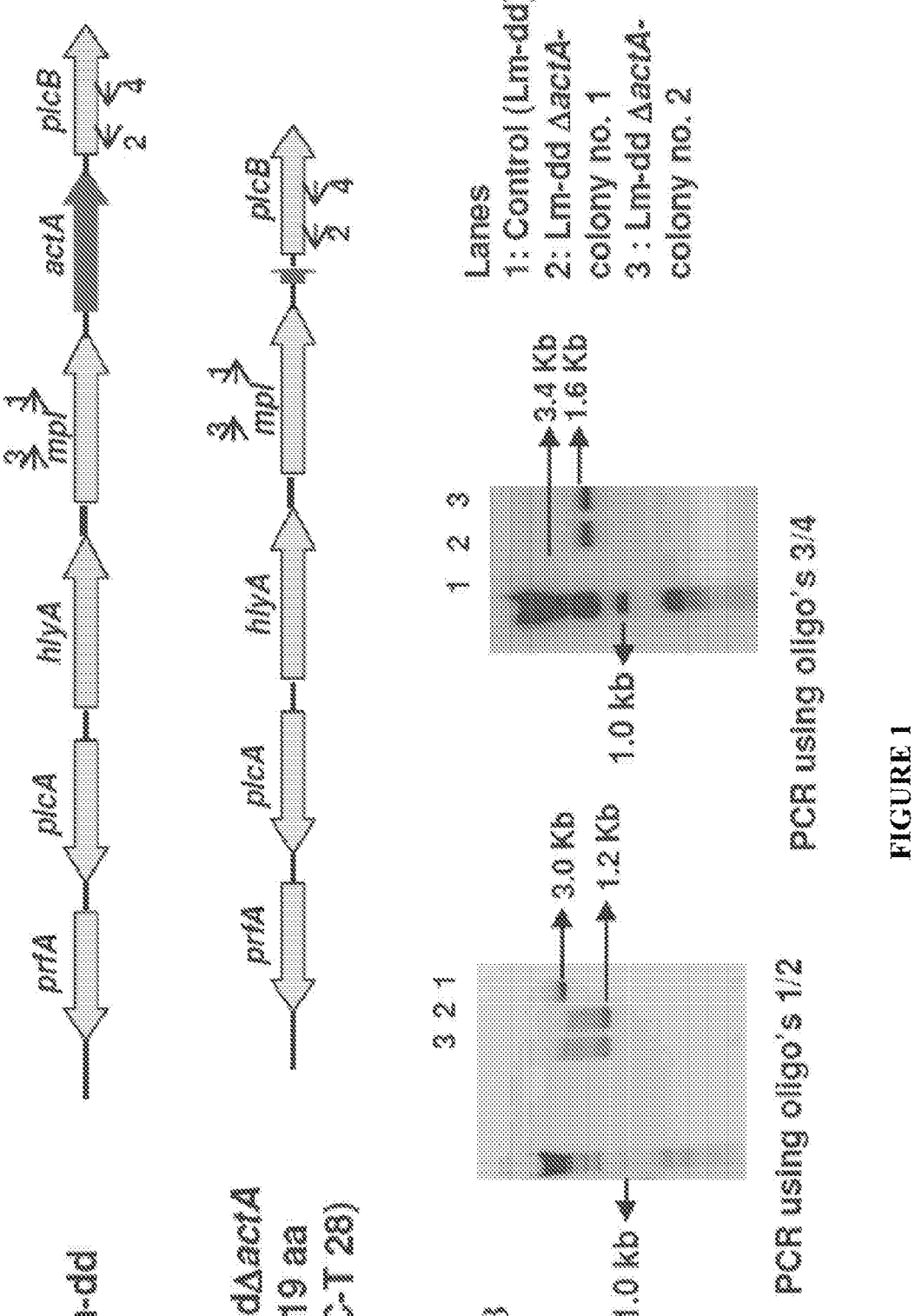


Figure 1A

Figure 1B

FIGURE 1

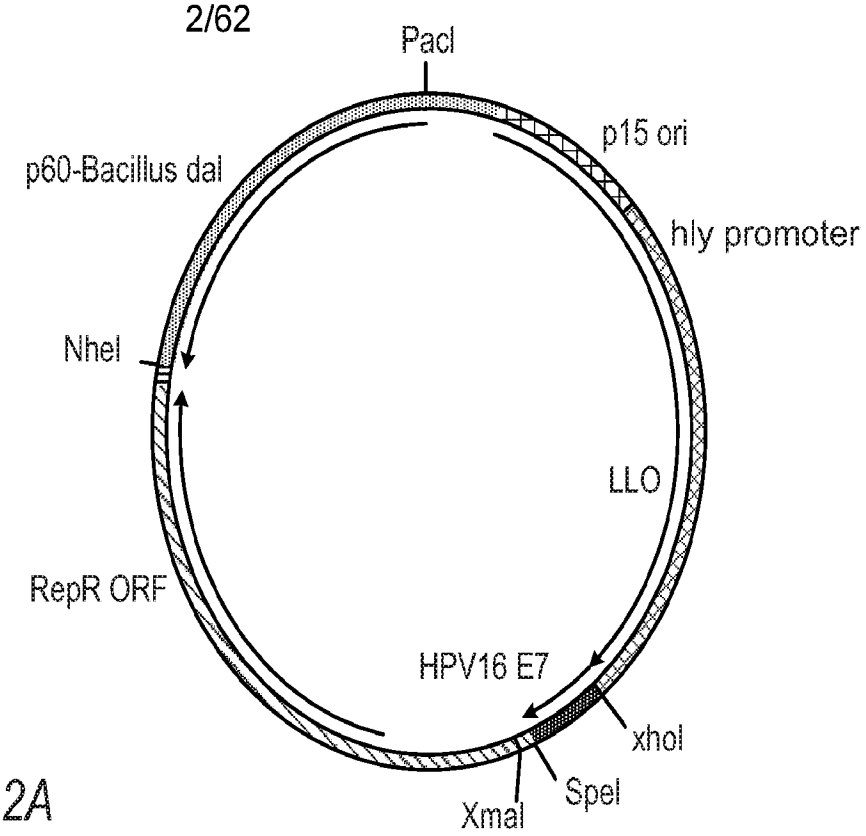


Figure 2A

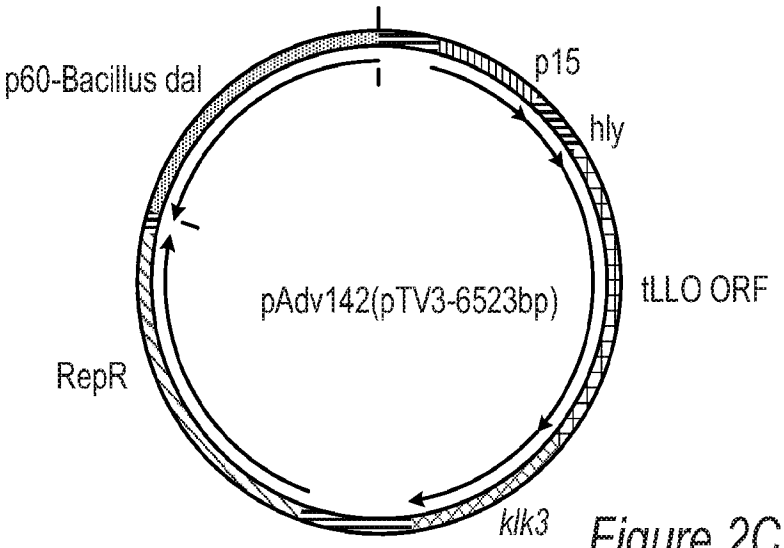


Figure 2C

Figure 2B

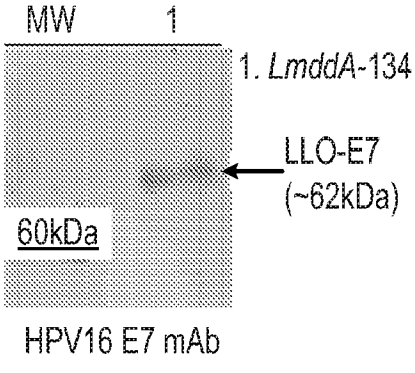
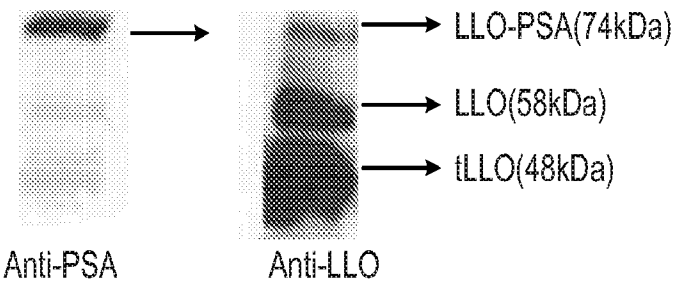


Figure 2D



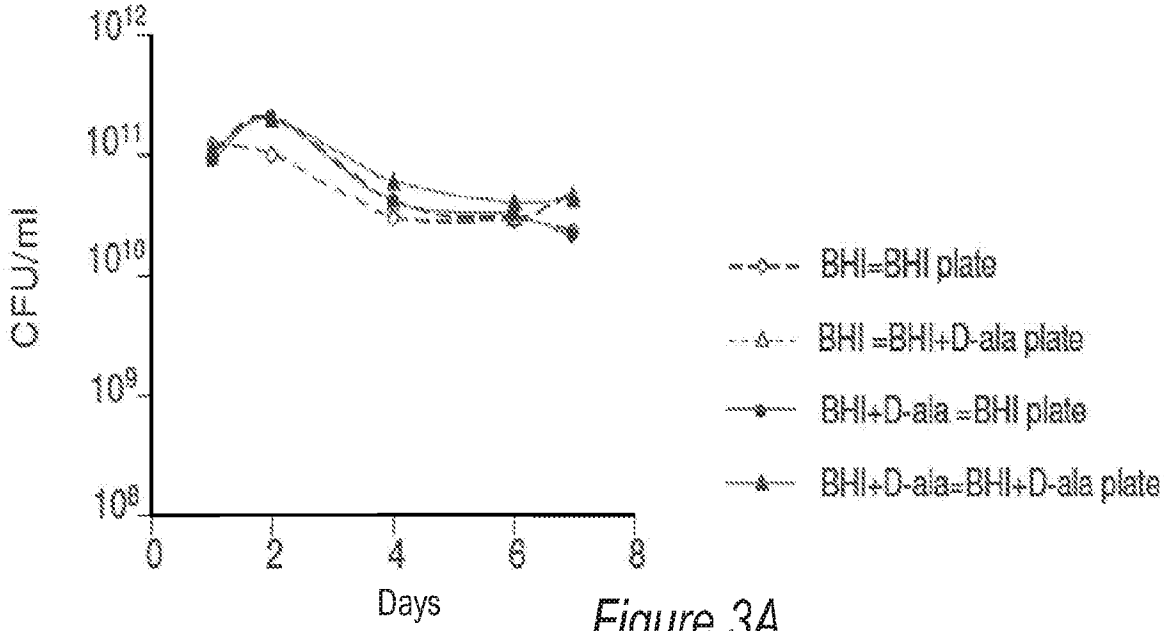


Figure 3A

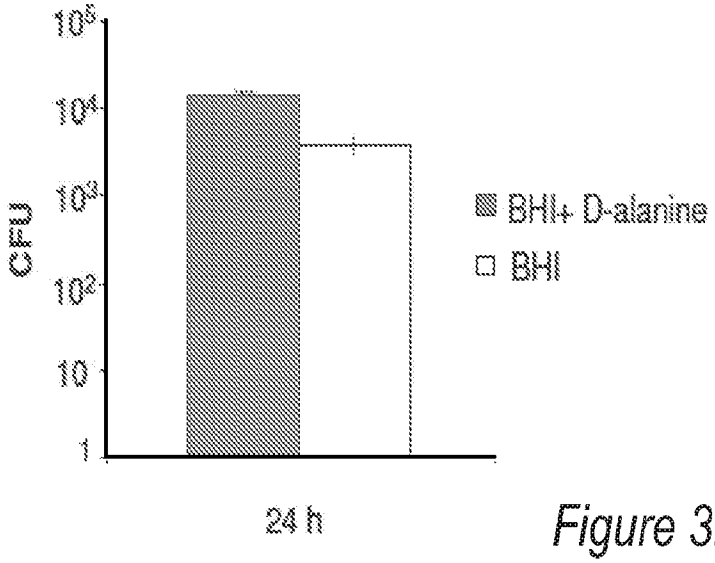


Figure 3B

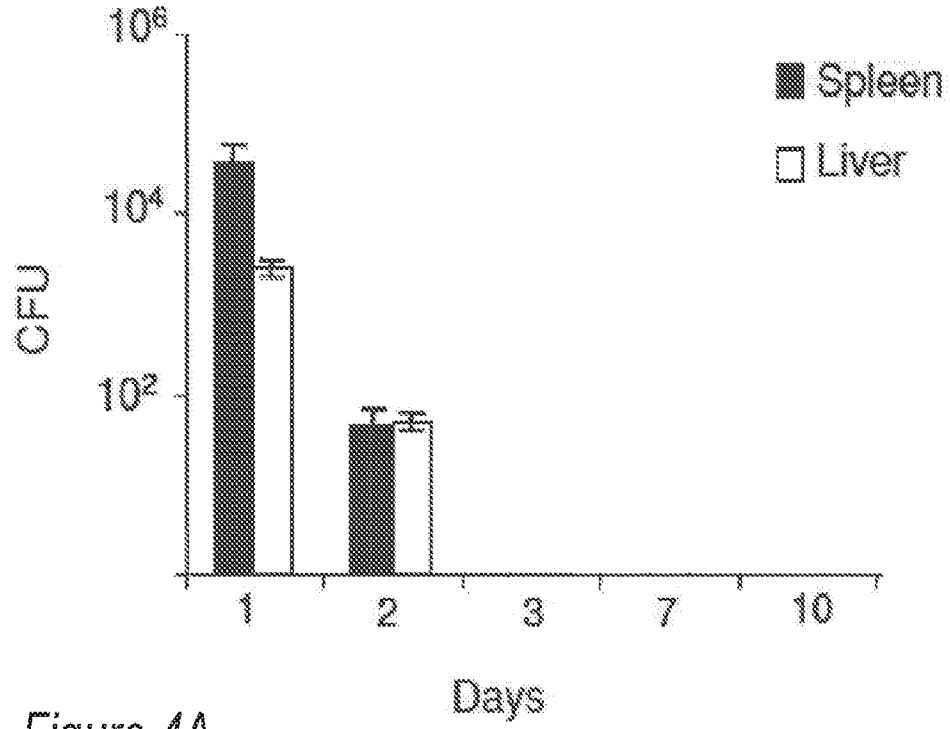


Figure 4A

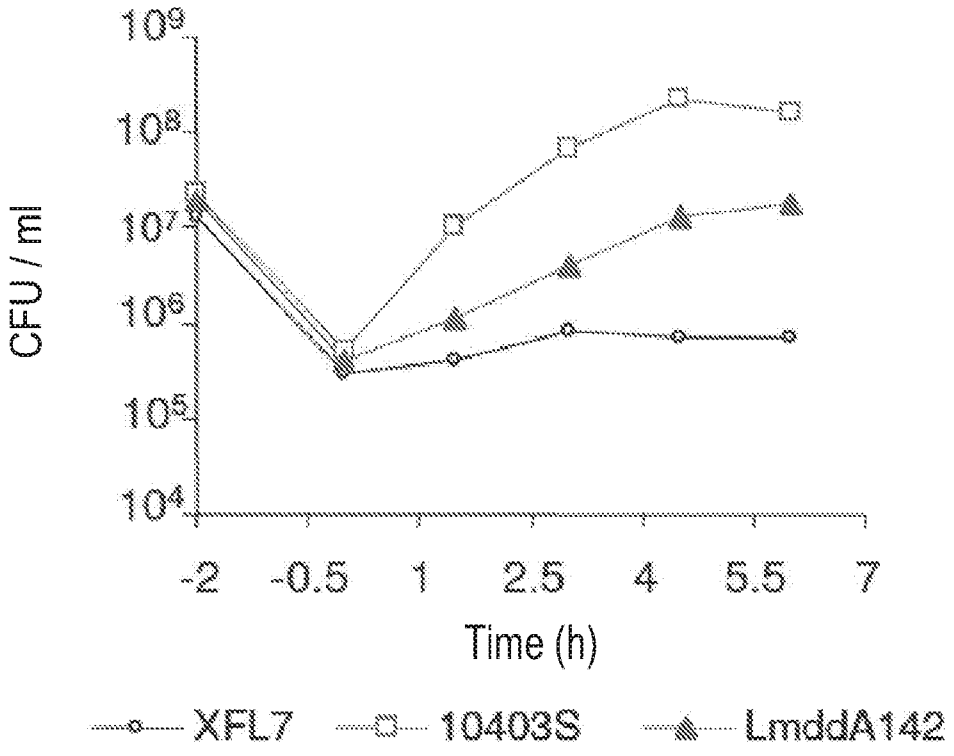


Figure 4B

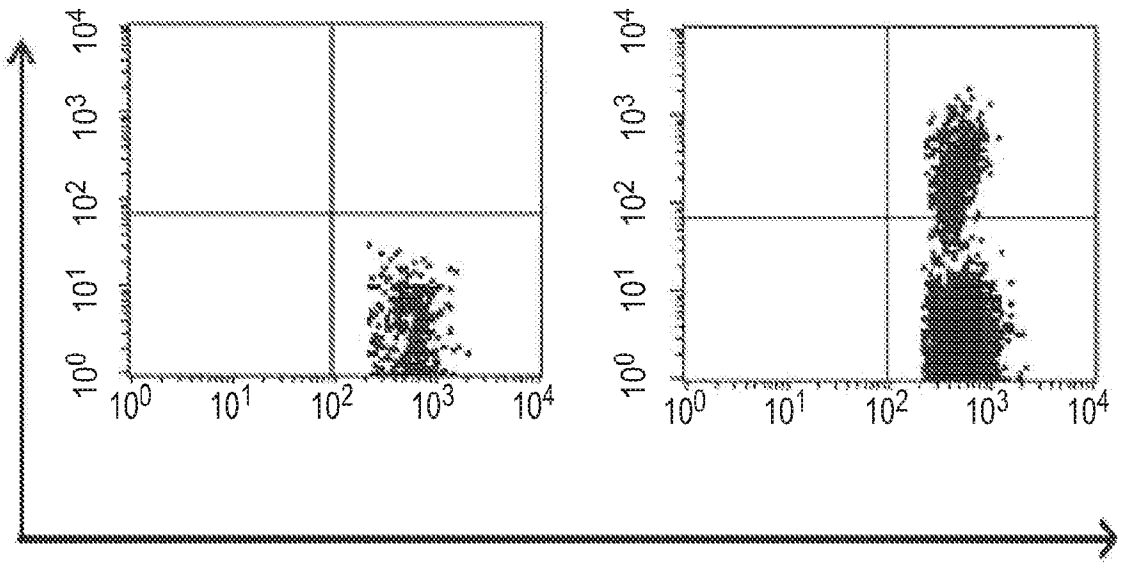


Figure 5A

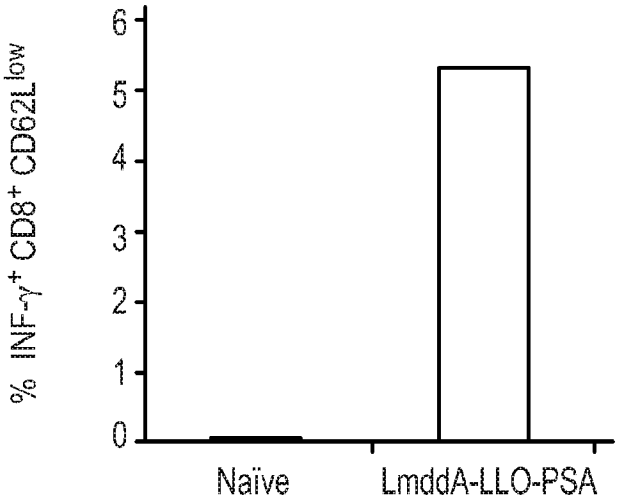


Figure 5B

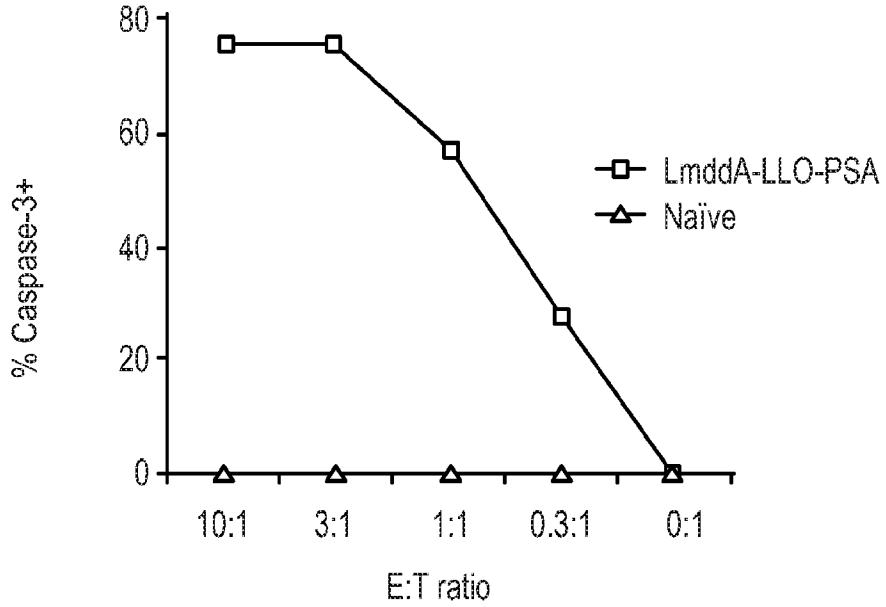


Figure 5C

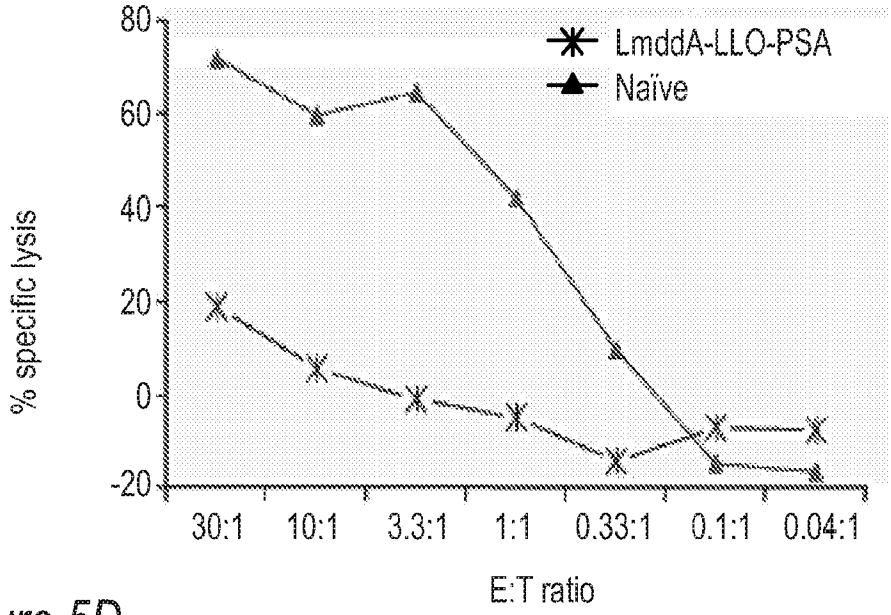


Figure 5D

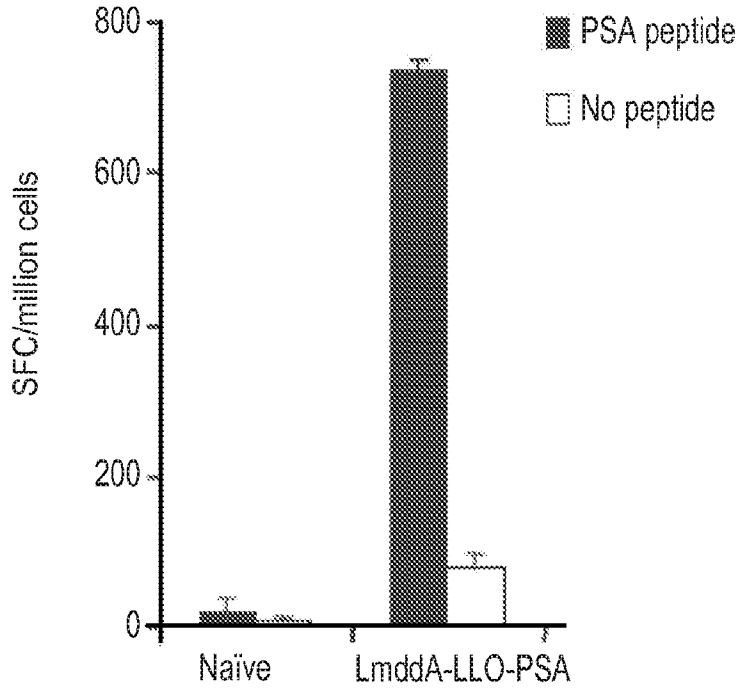


Figure 5E

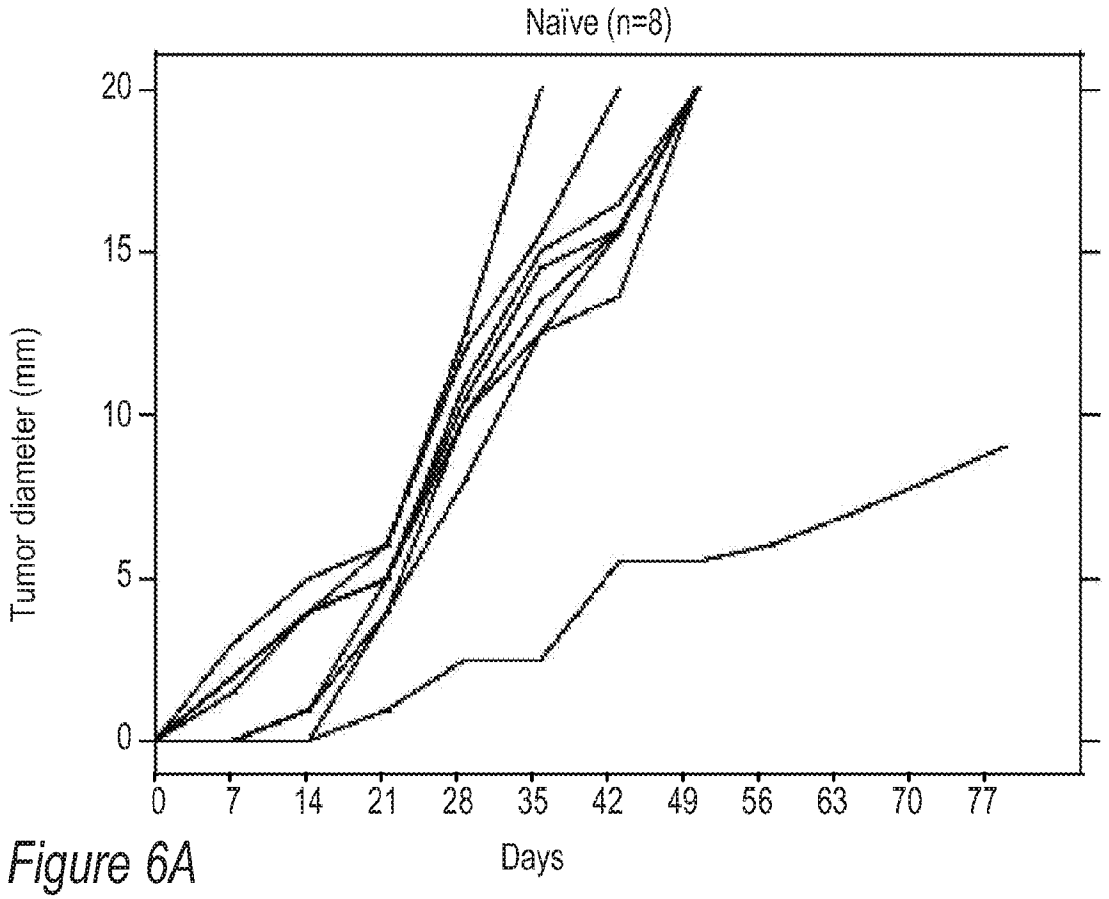


Figure 6A

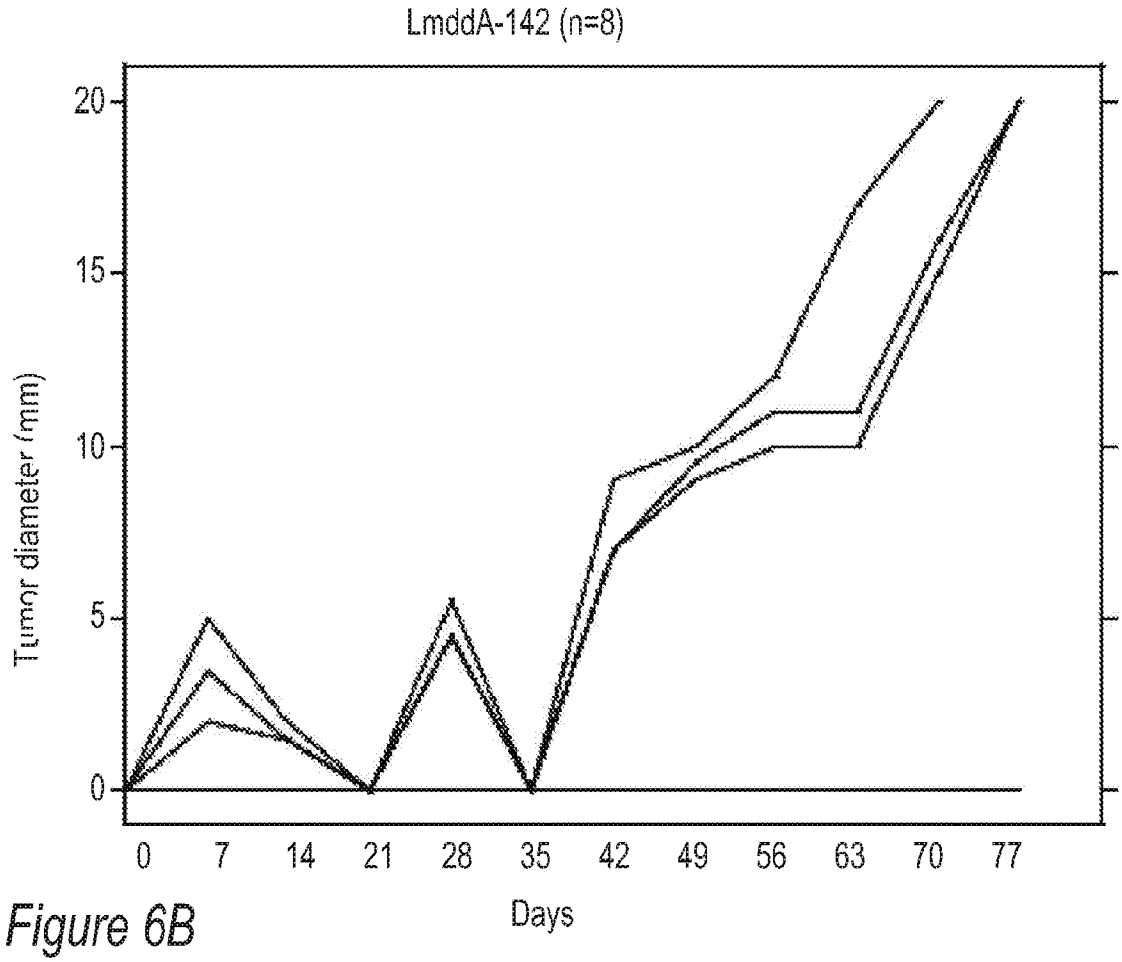


Figure 6B

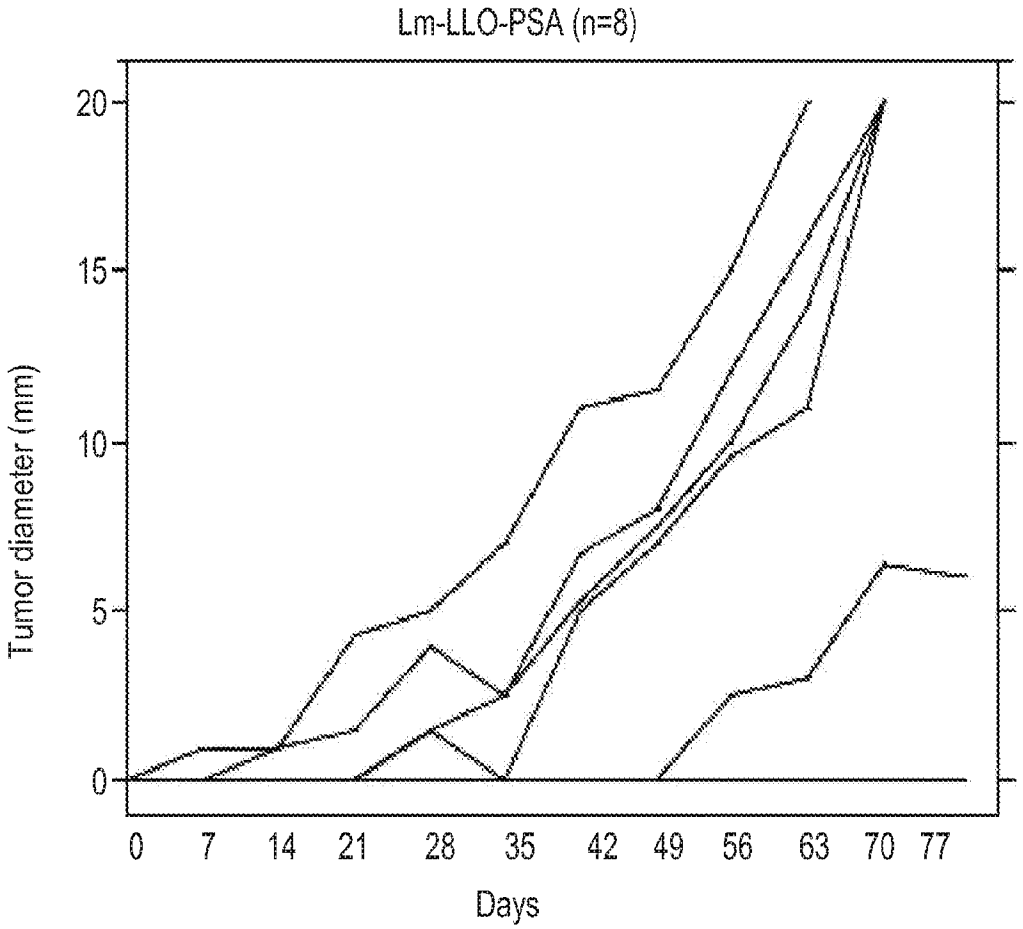


Figure 6C

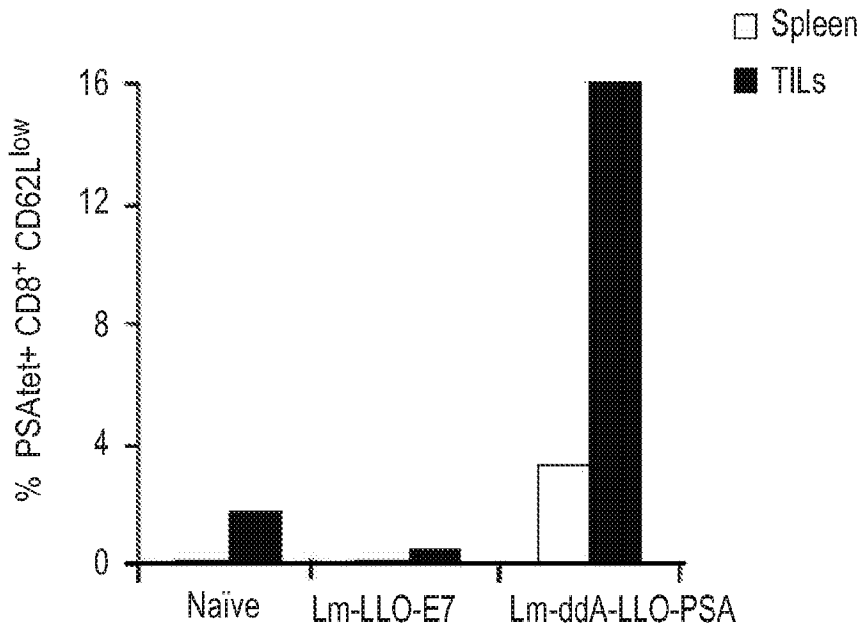


Figure 7A

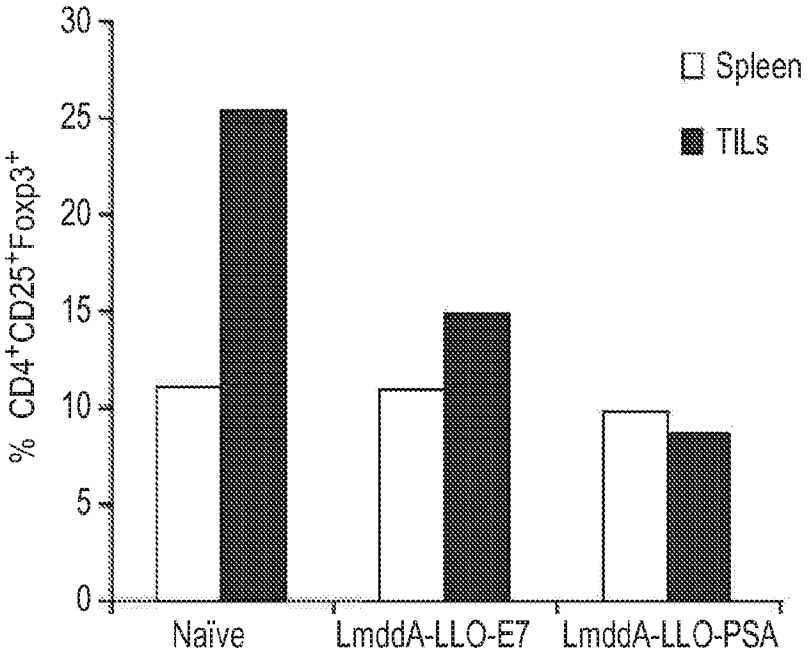


Figure 7B

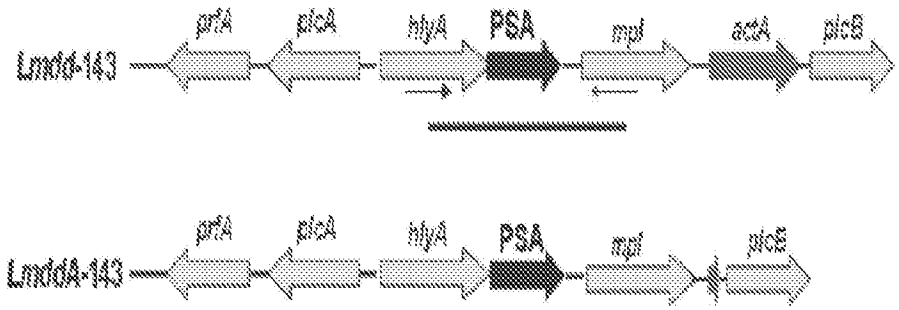


Figure 8A

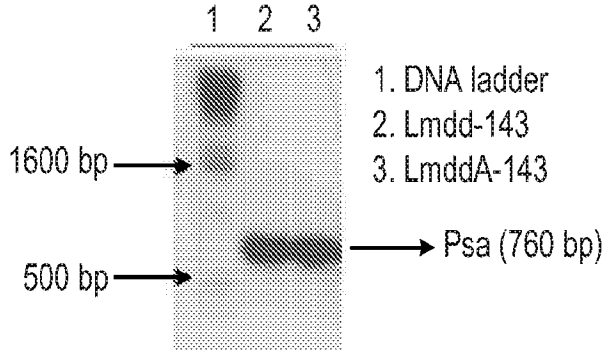


Figure 8B

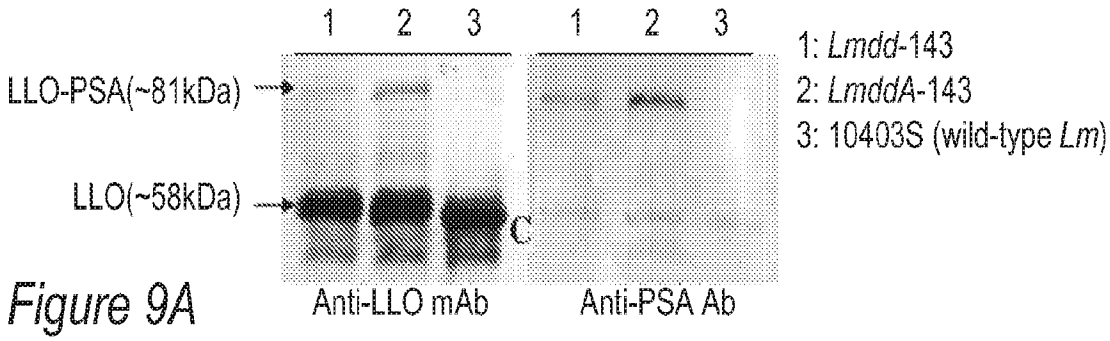


Figure 9A

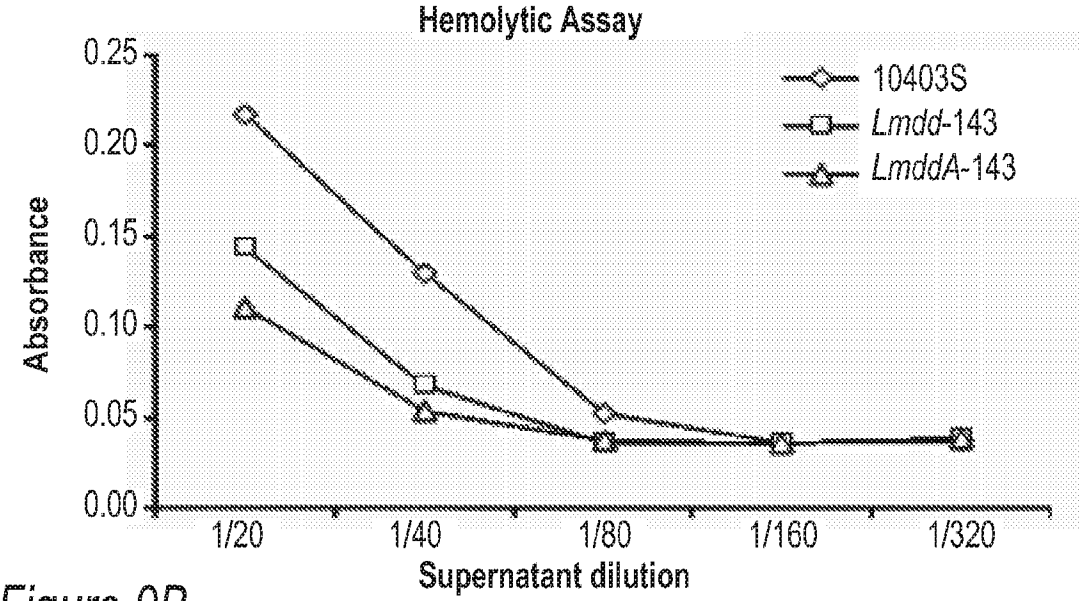
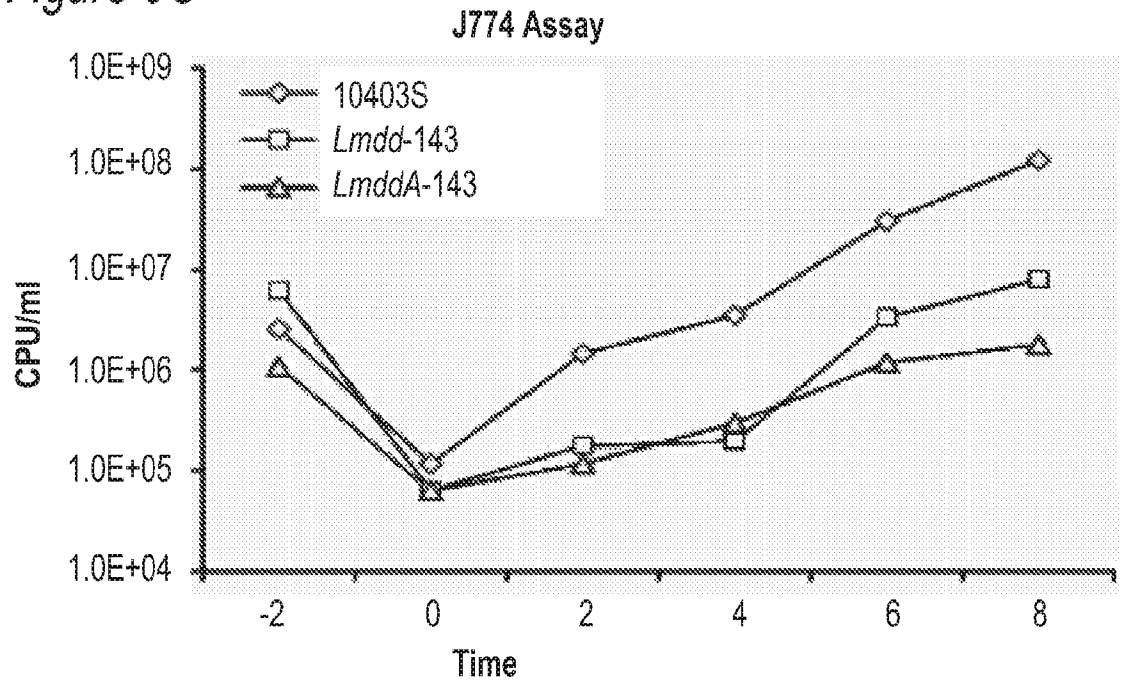


Figure 9B

Figure 9C



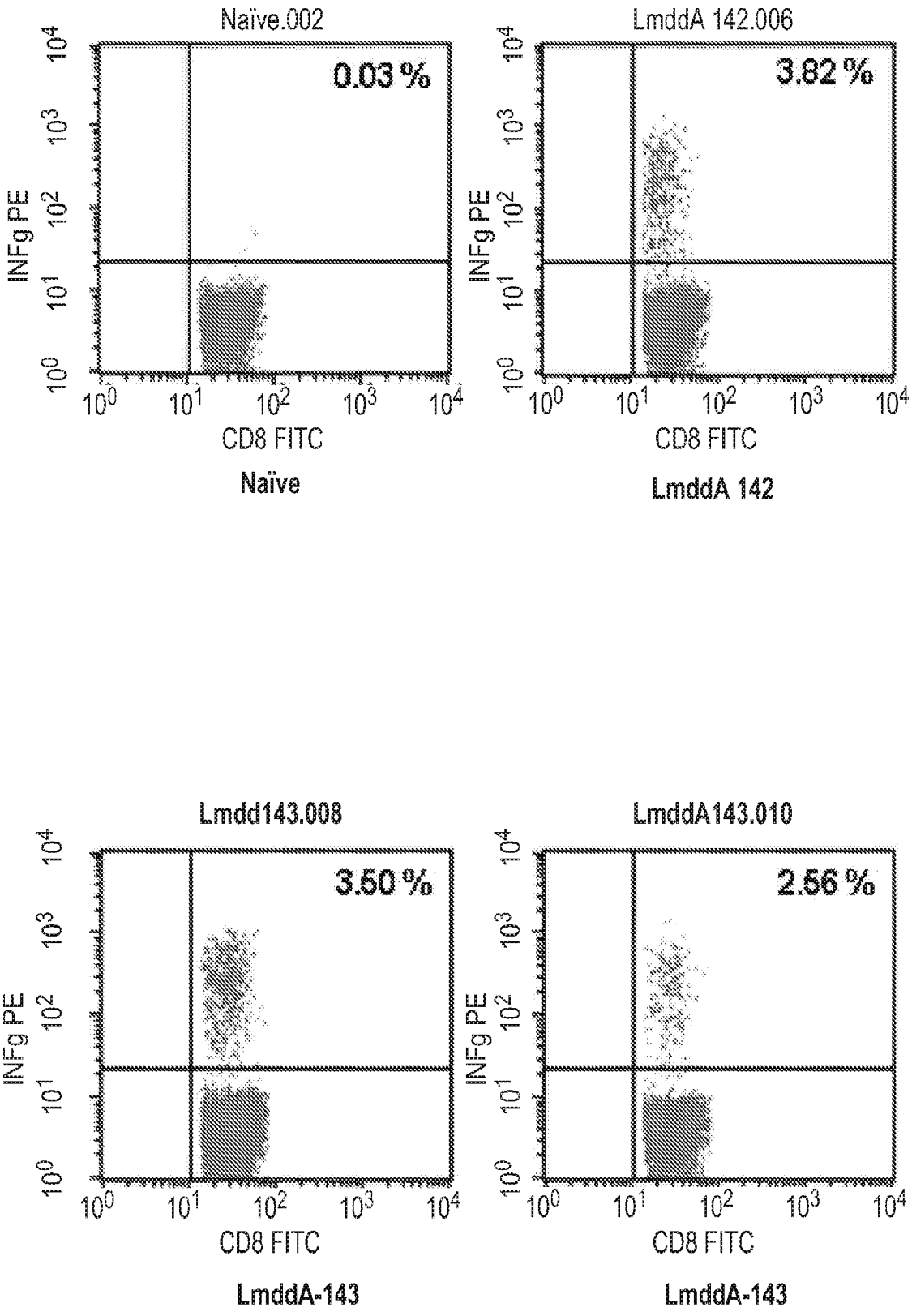

Naïve.002
0.03 %
INFg PE
CD8 FITC
Naïve
LmddA 142.006
3.82 %
INFg PE
CD8 FITC
LmddA 142
Lmdd143.008
3.50 %
INFg PE
CD8 FITC
LmddA-143
LmddA143.010
2.56 %
INFg PE
CD8 FITC
LmddA-143

Figure 10

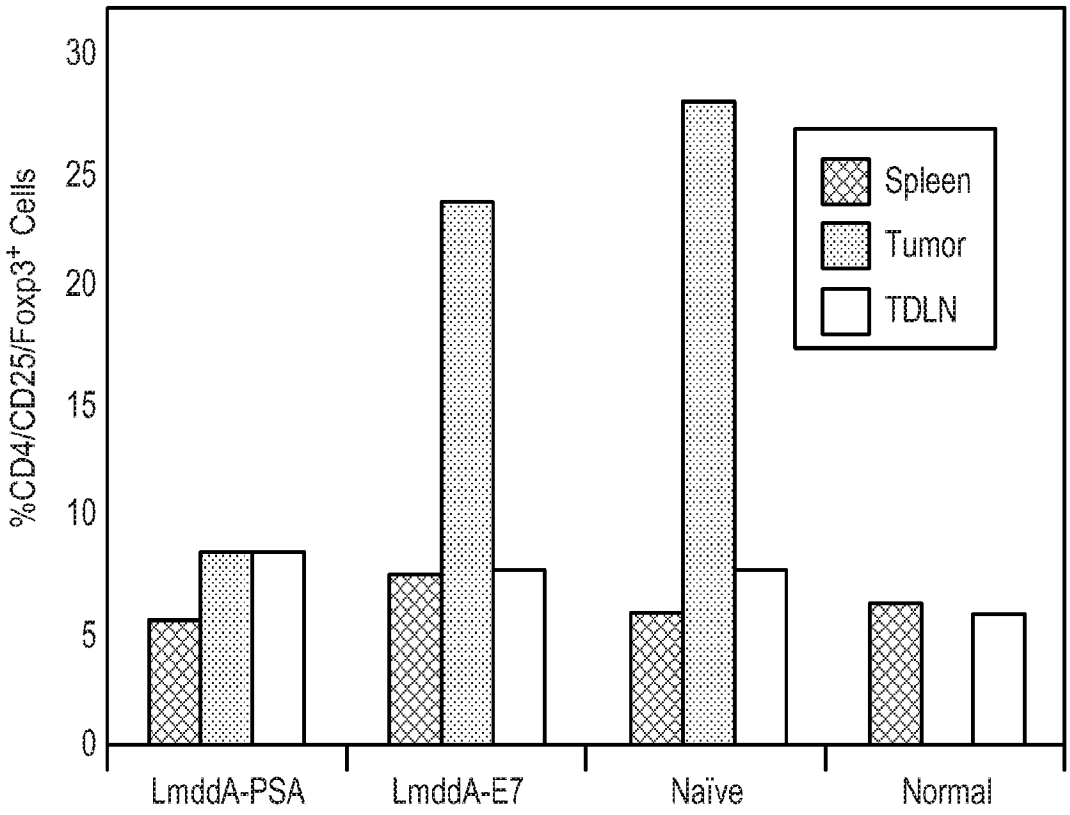


Figure 11A

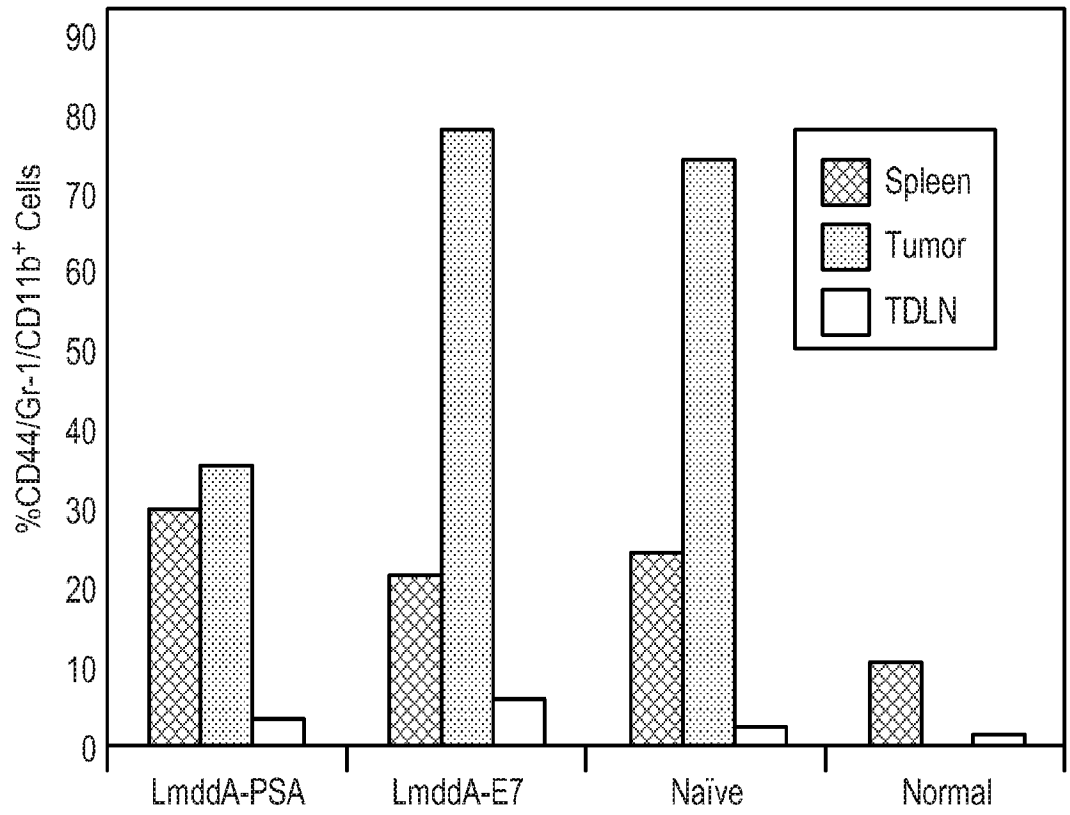


Figure 11B

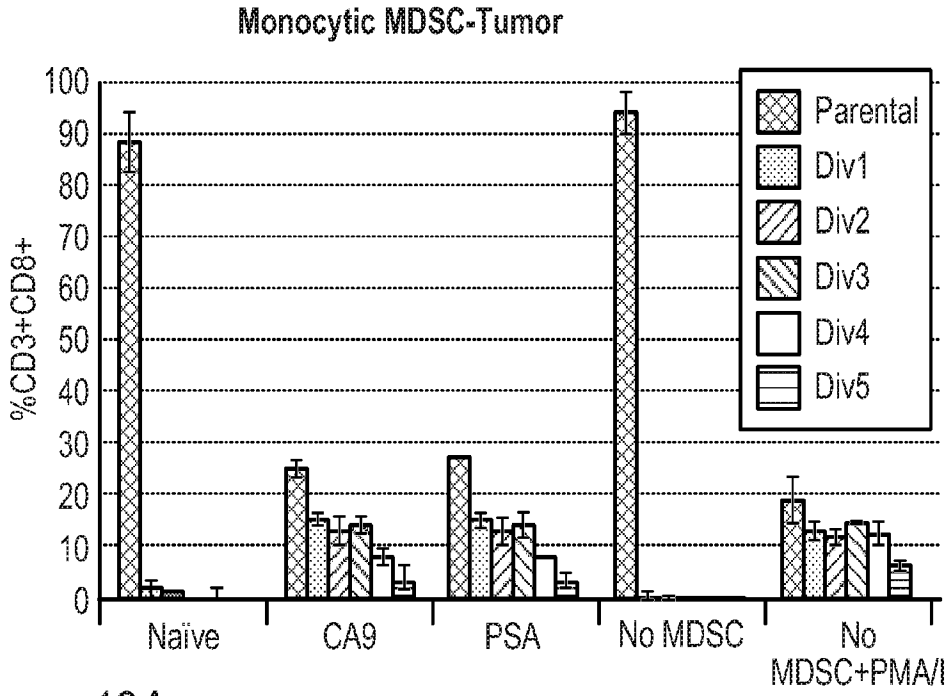


Figure 12A

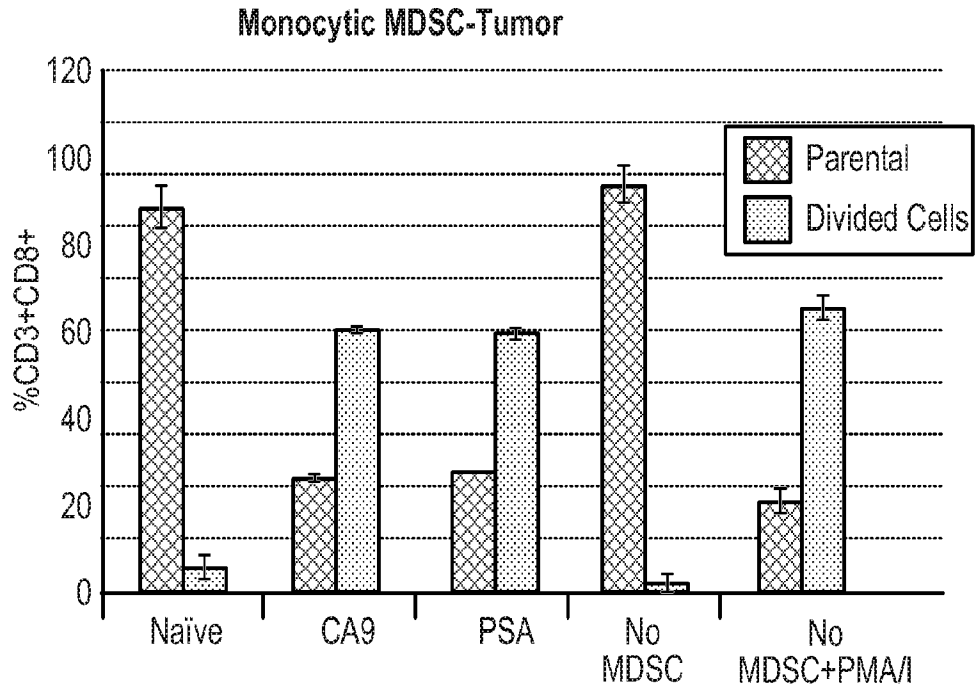


Figure 12B

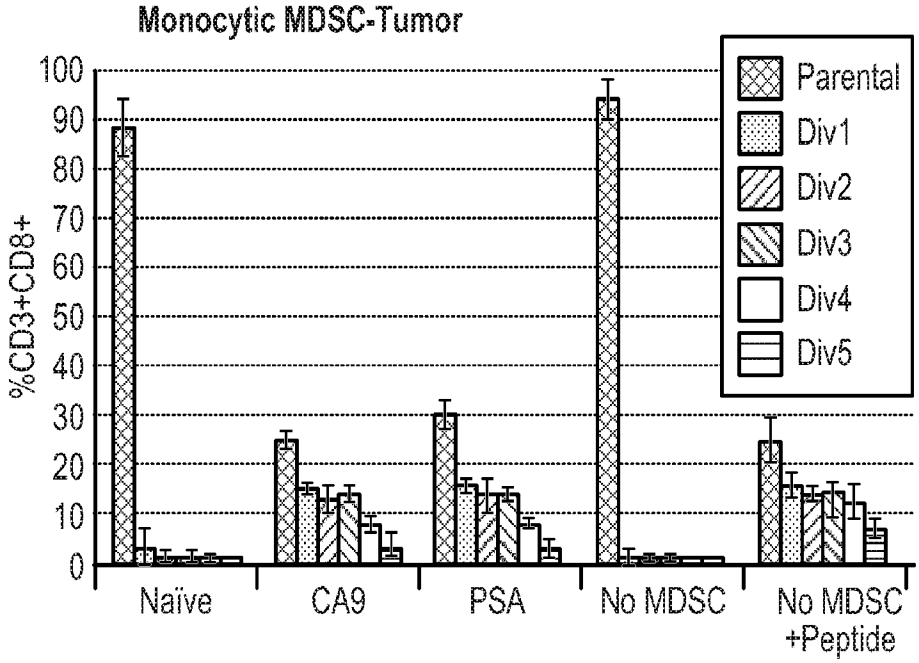


Figure 12C

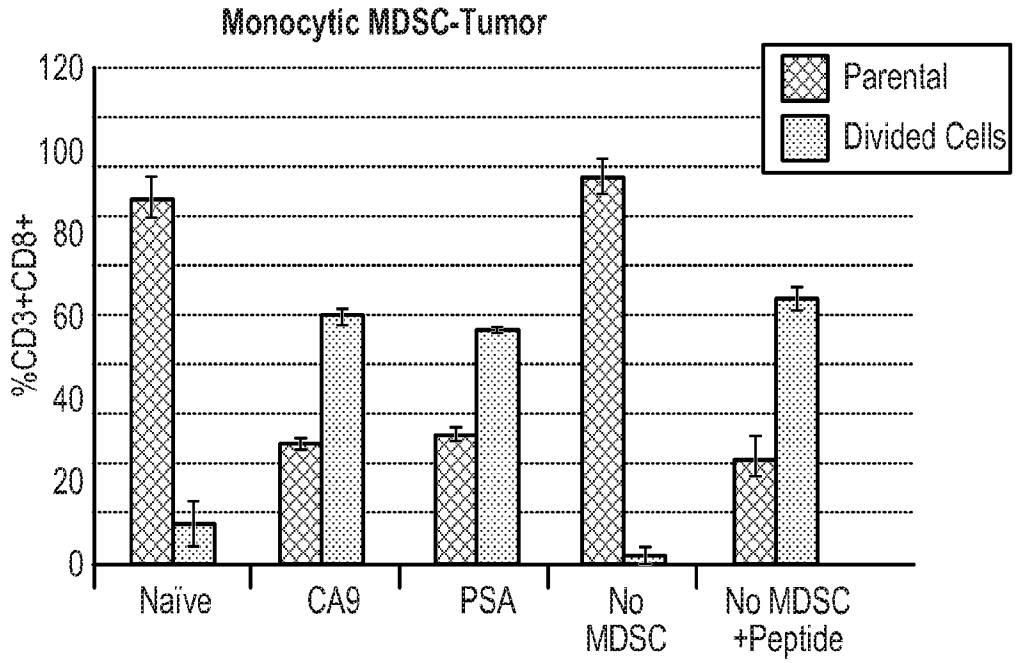


Figure 12D

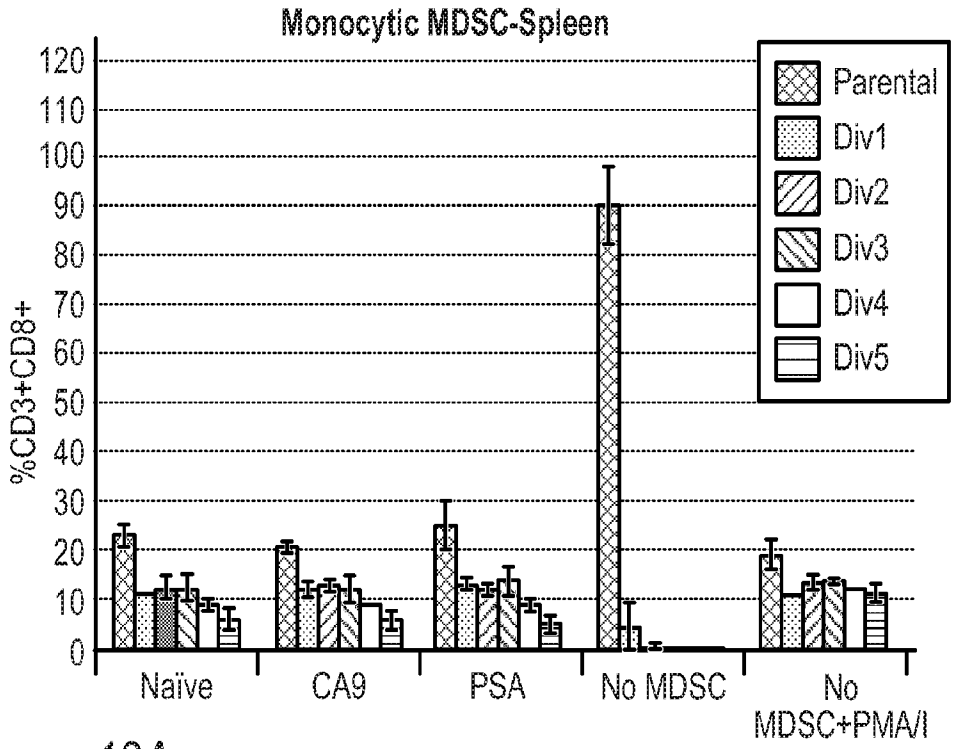


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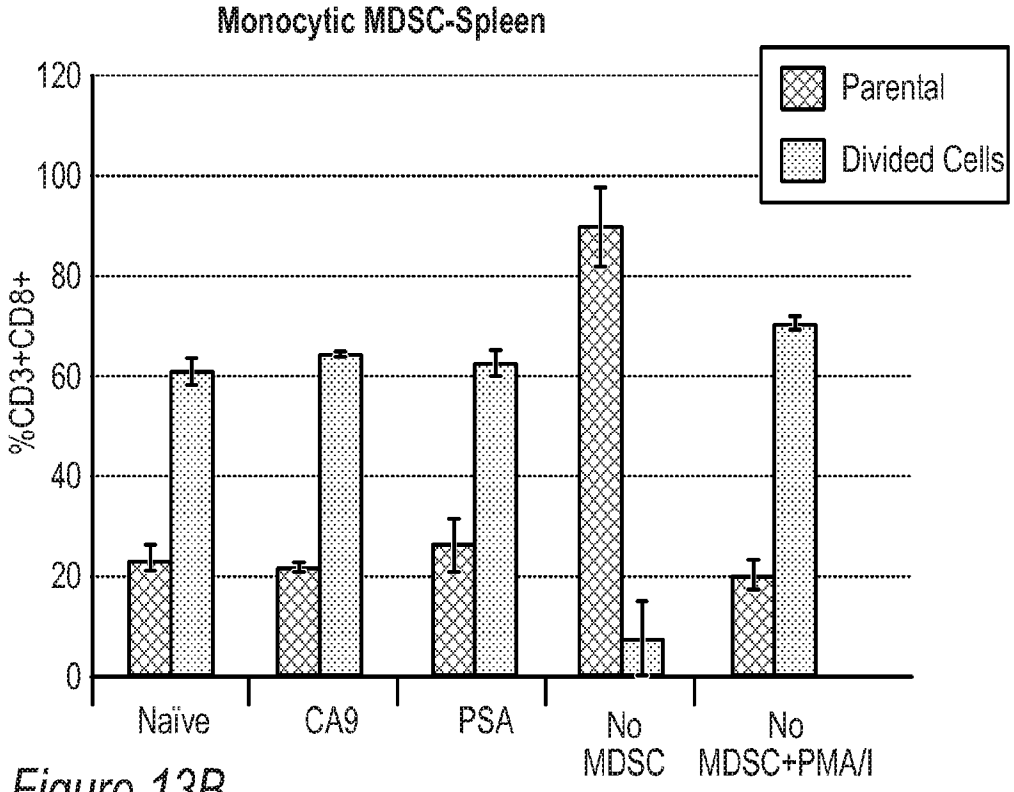


Figure 13B

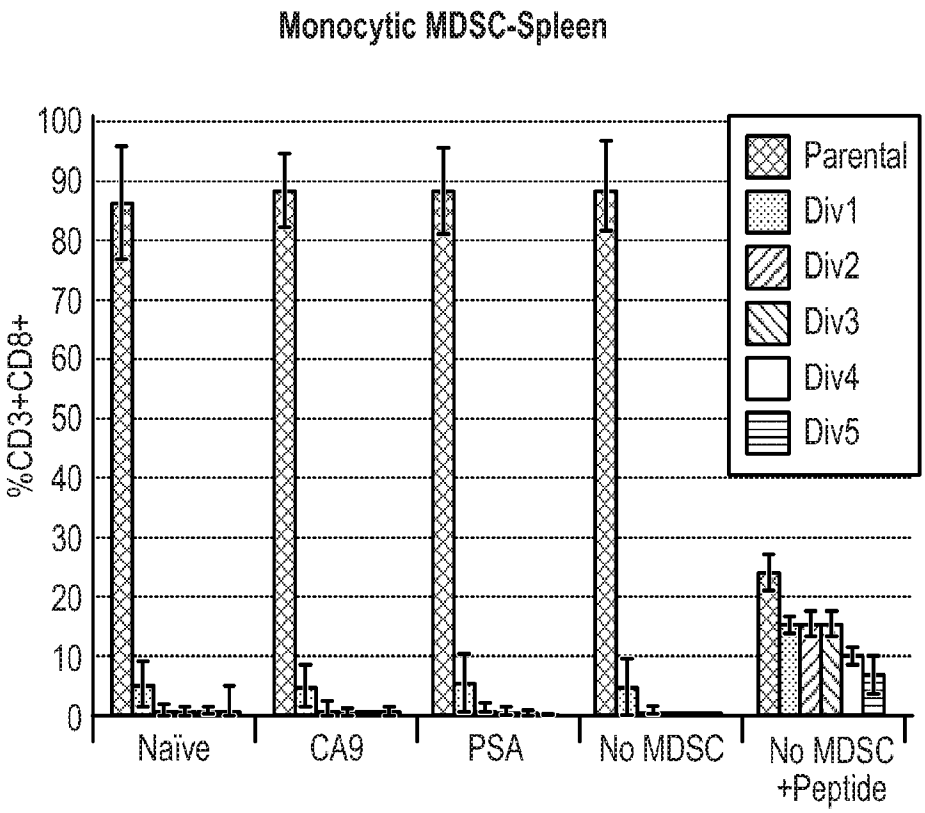


Figure 13C

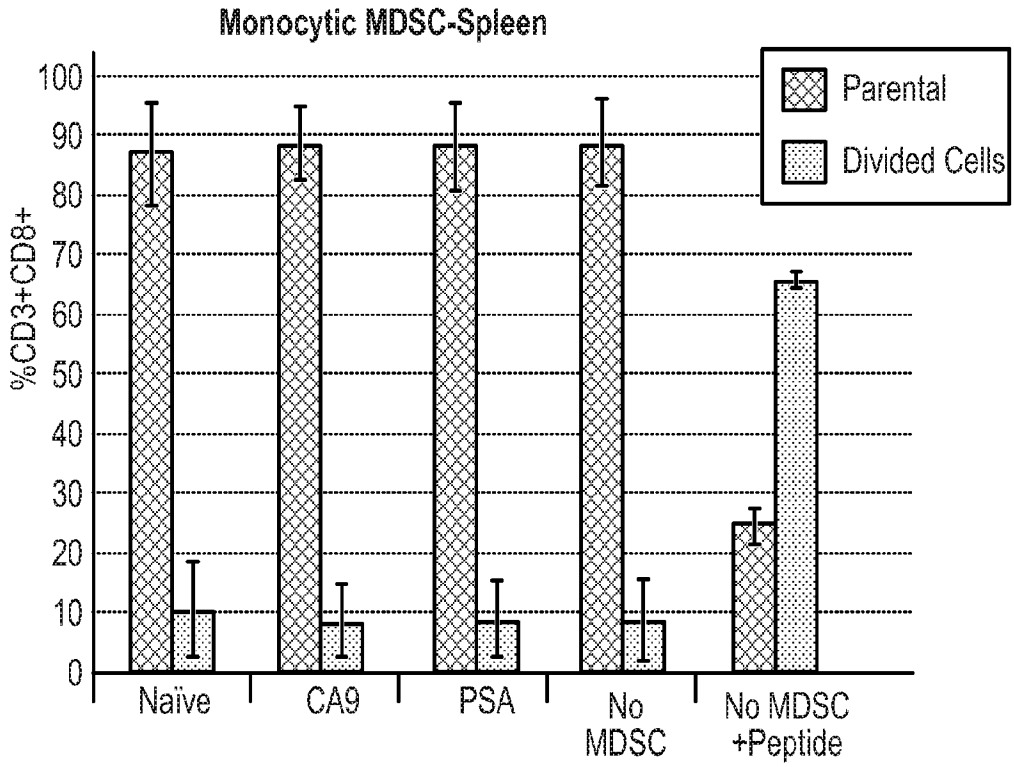


Figure 13D

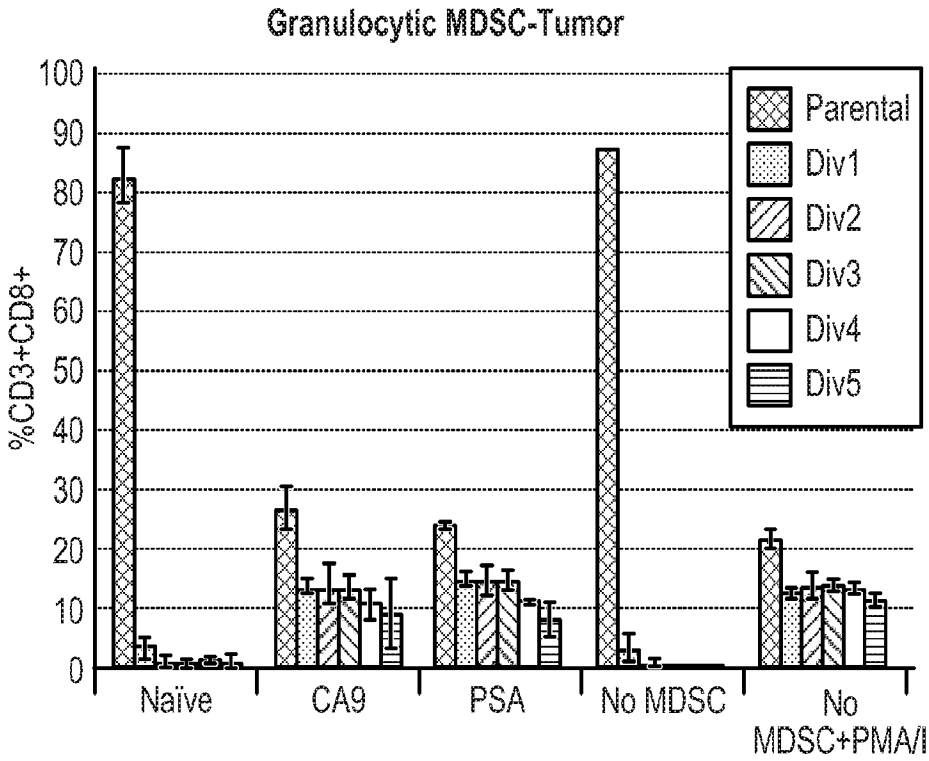


Figure 14A

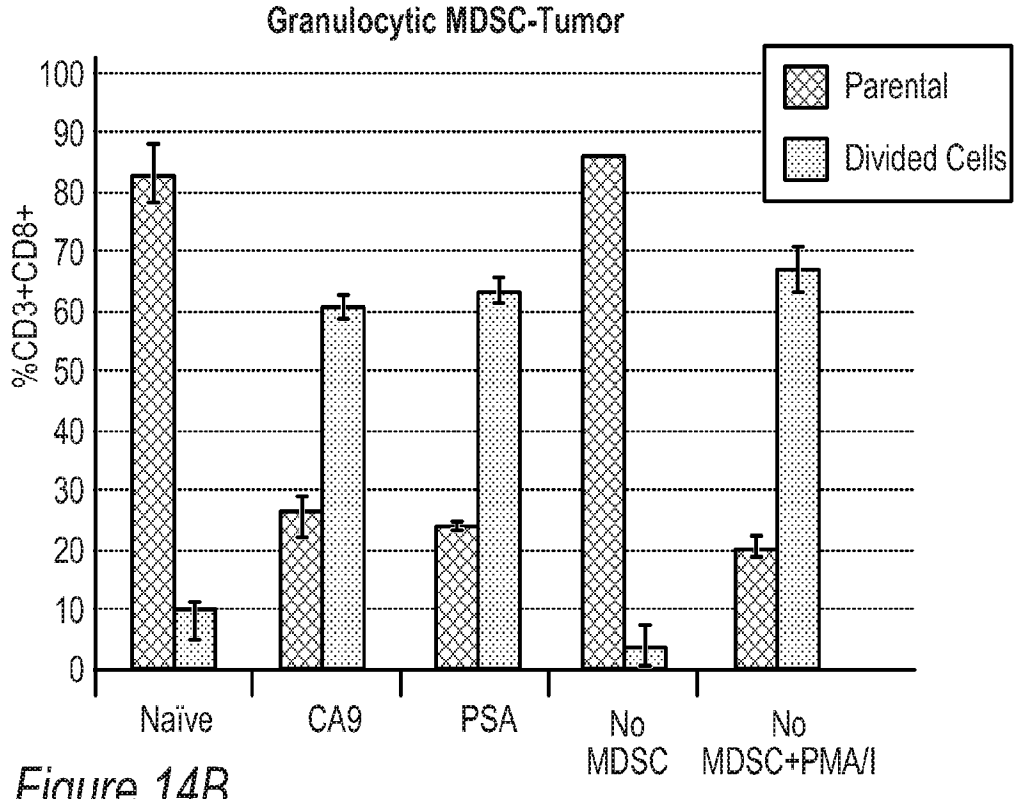


Figure 14B

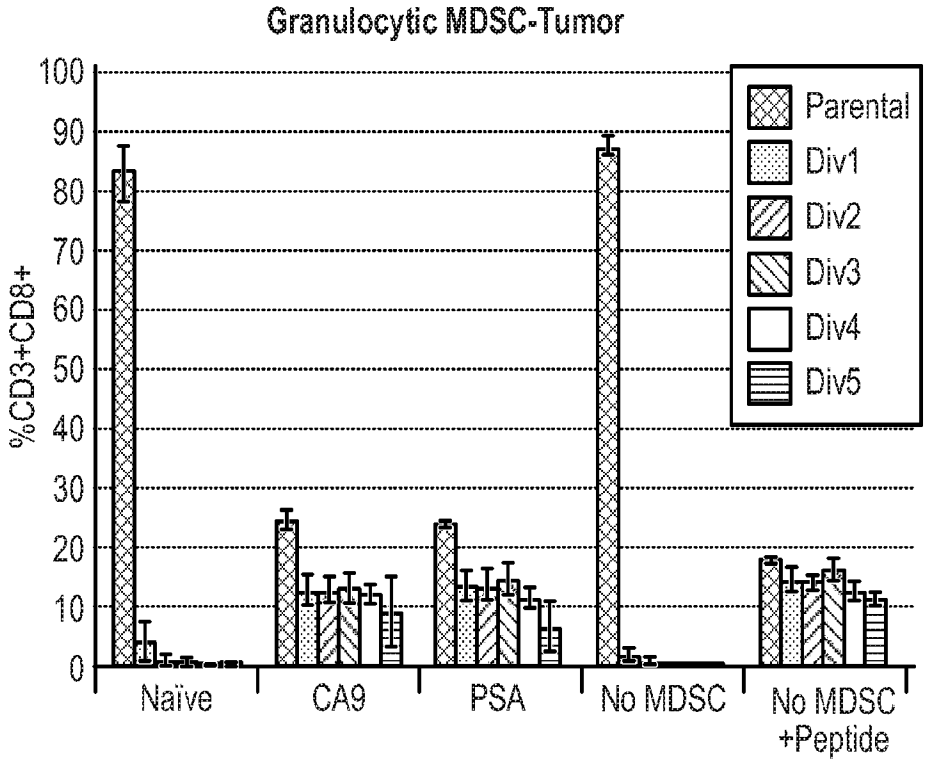


Figure 14C

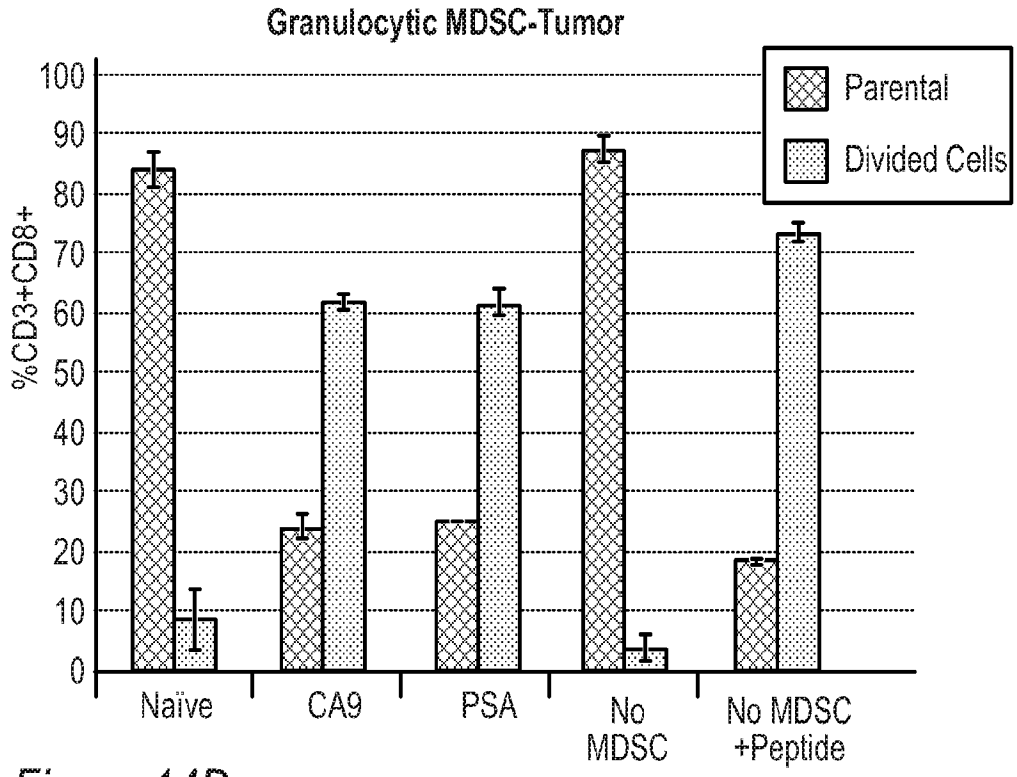


Figure 14D

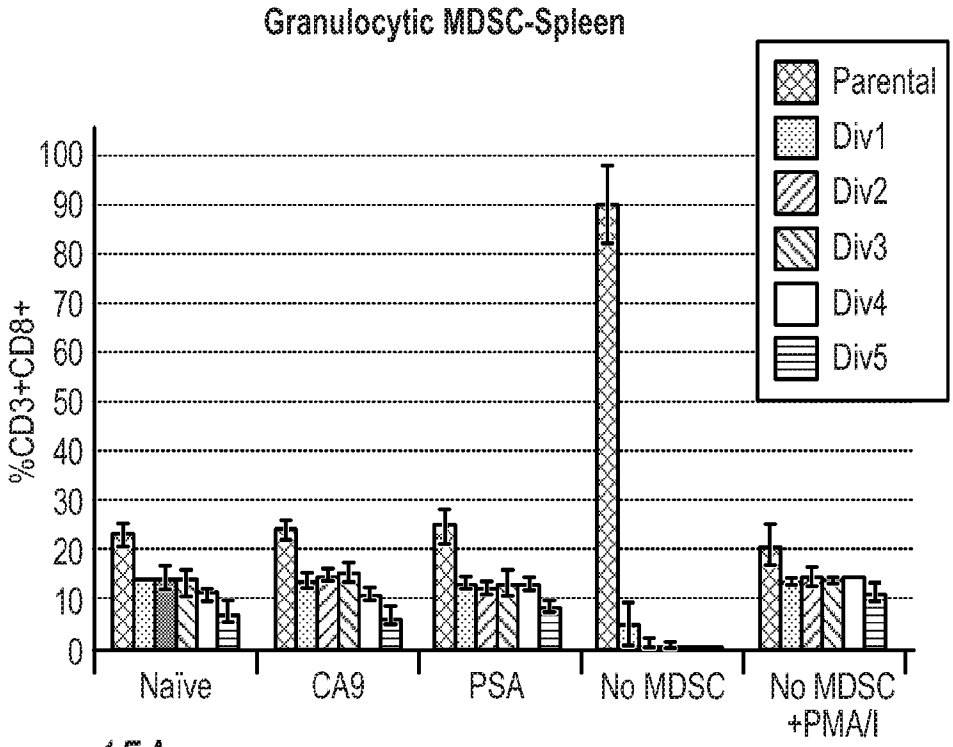


Figure 15A

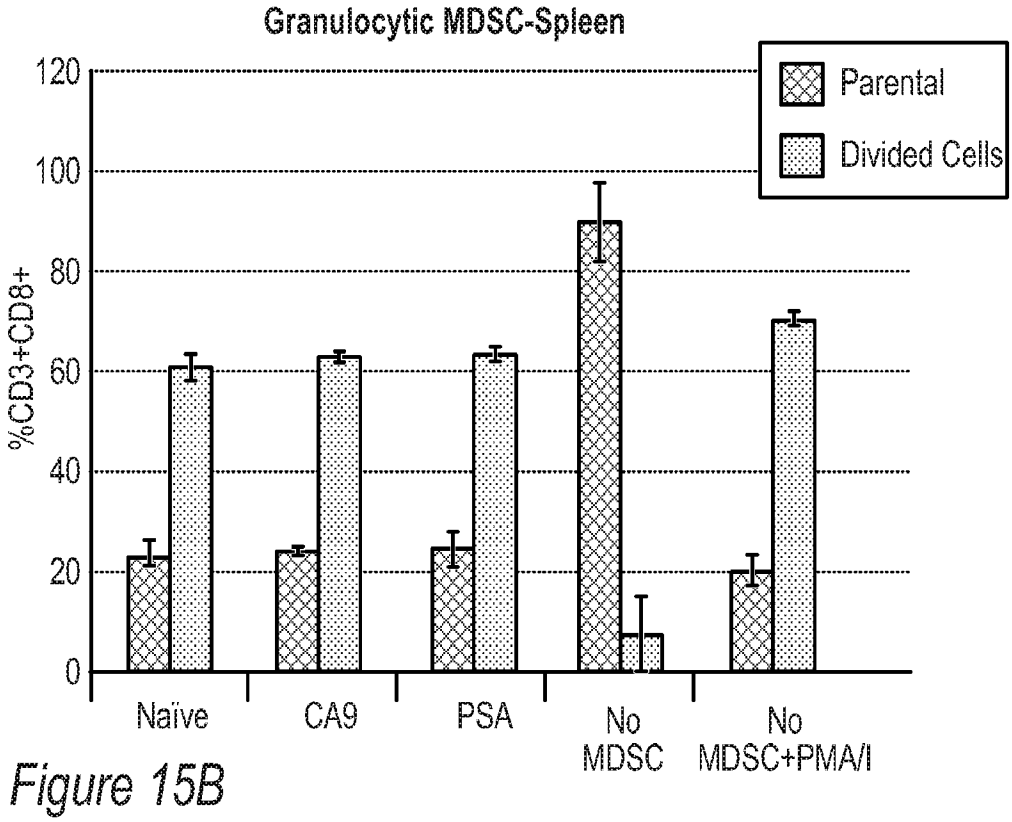


Figure 15B

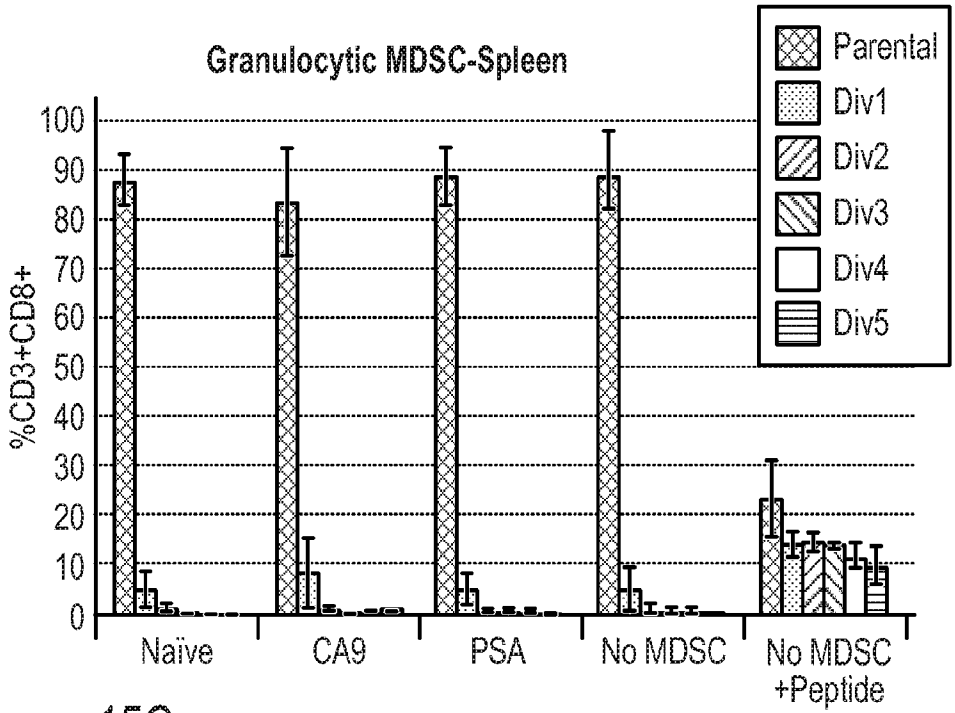


Figure 15C

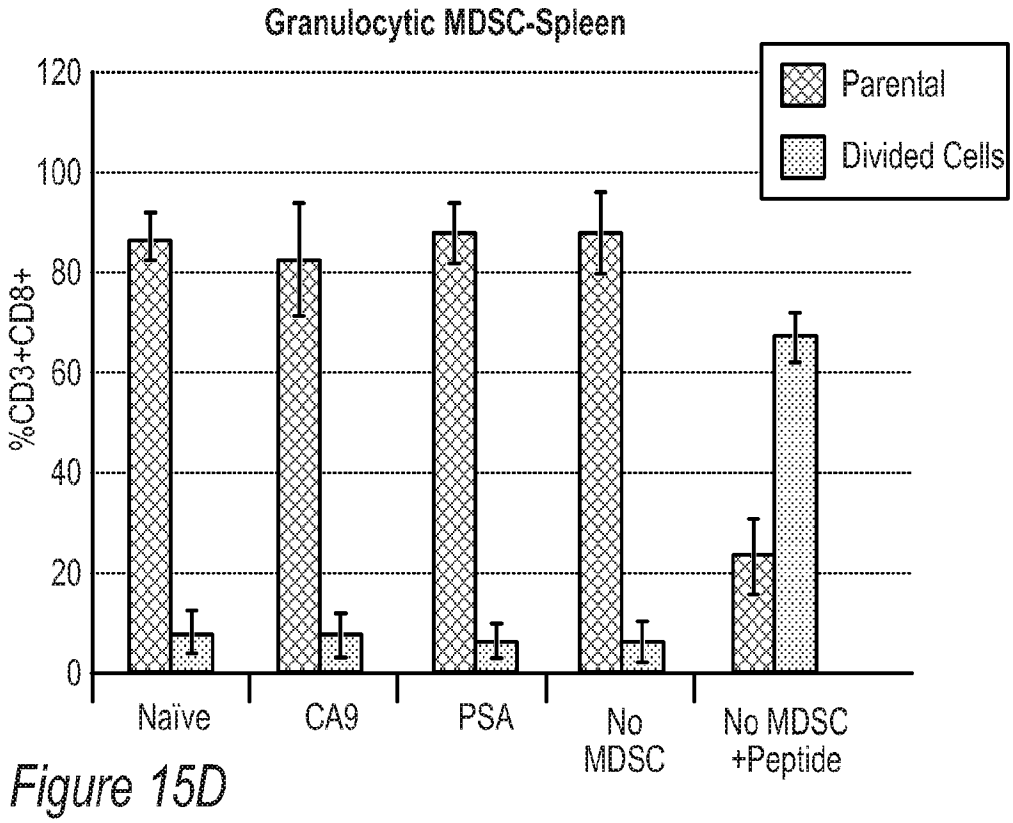


Figure 15D

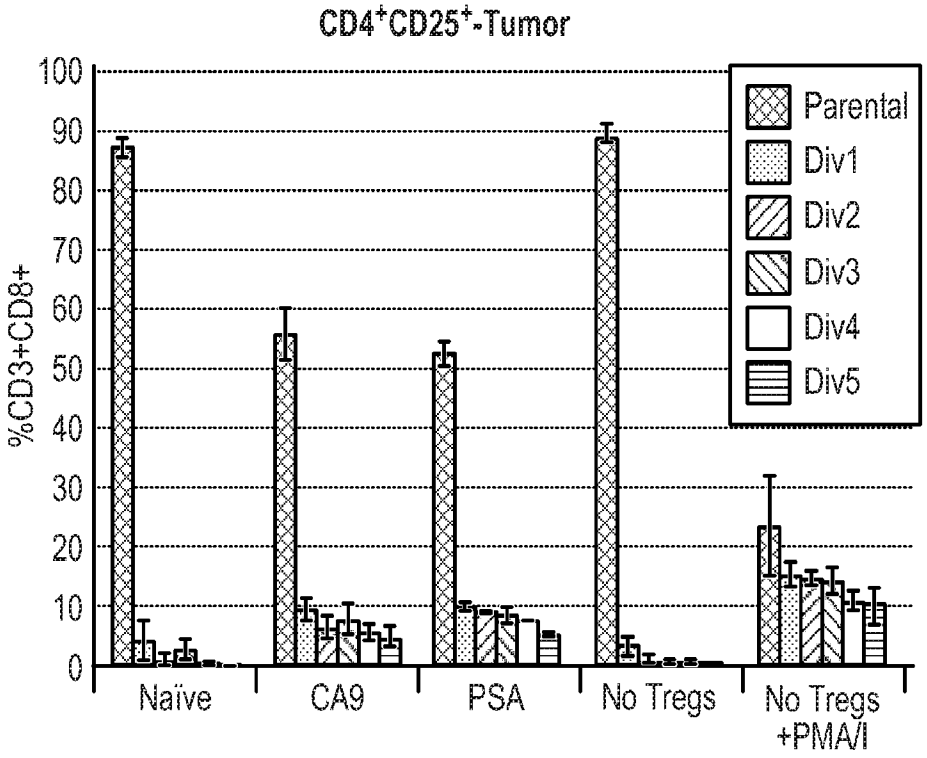


Figure 16A

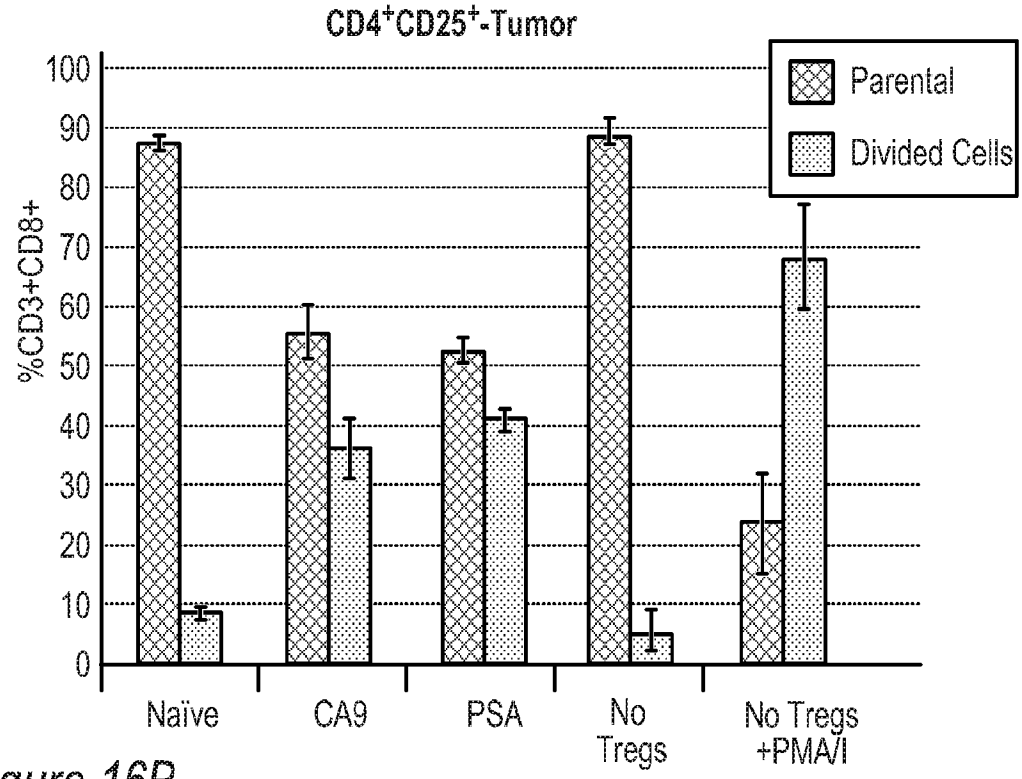


Figure 16B

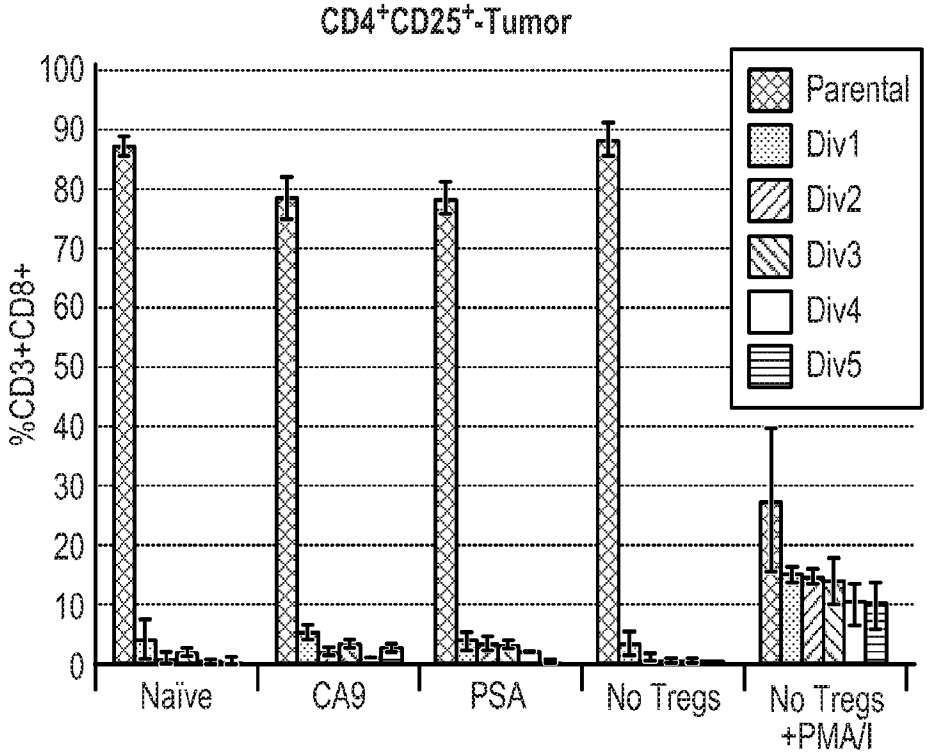


Figure 16C

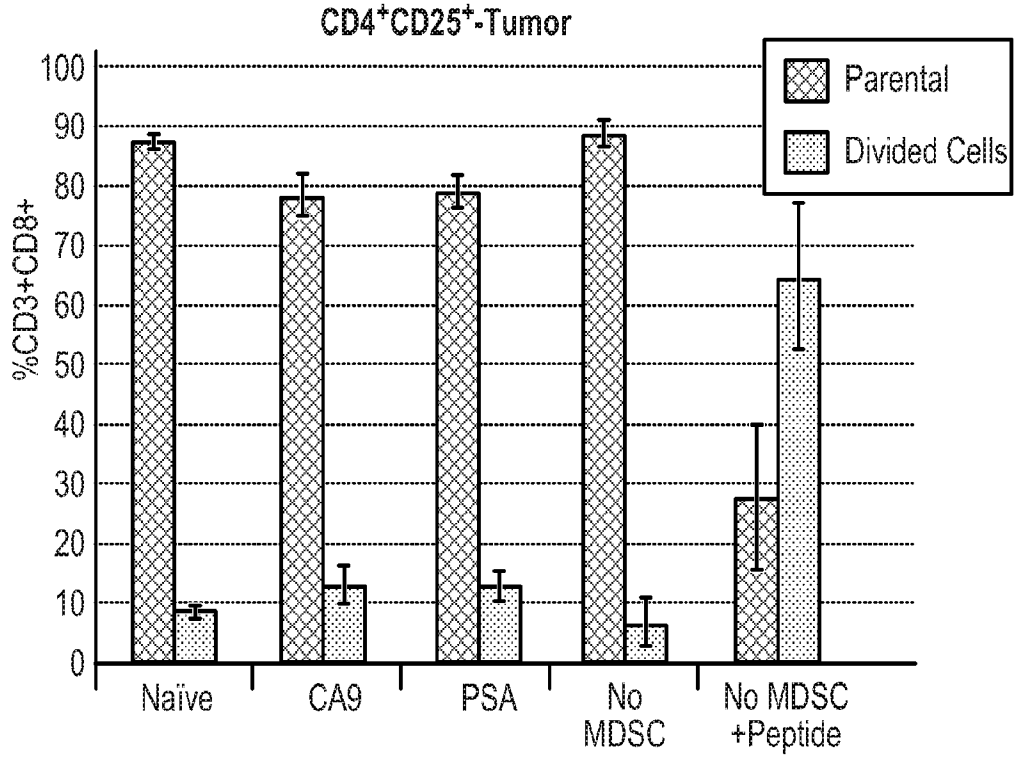


Figure 16D

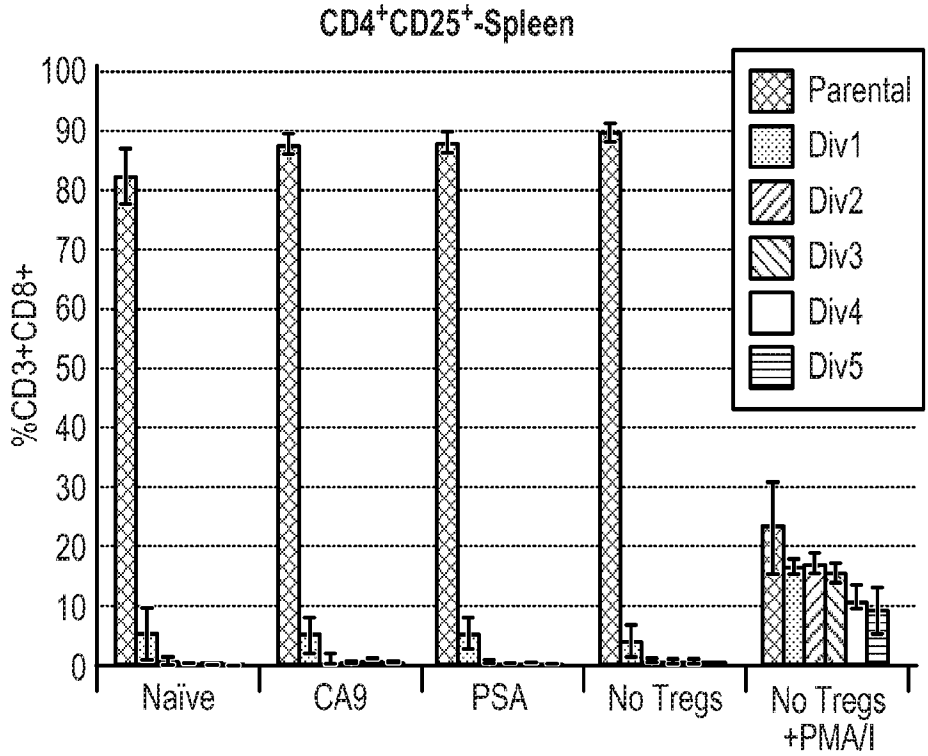


Figure 17A

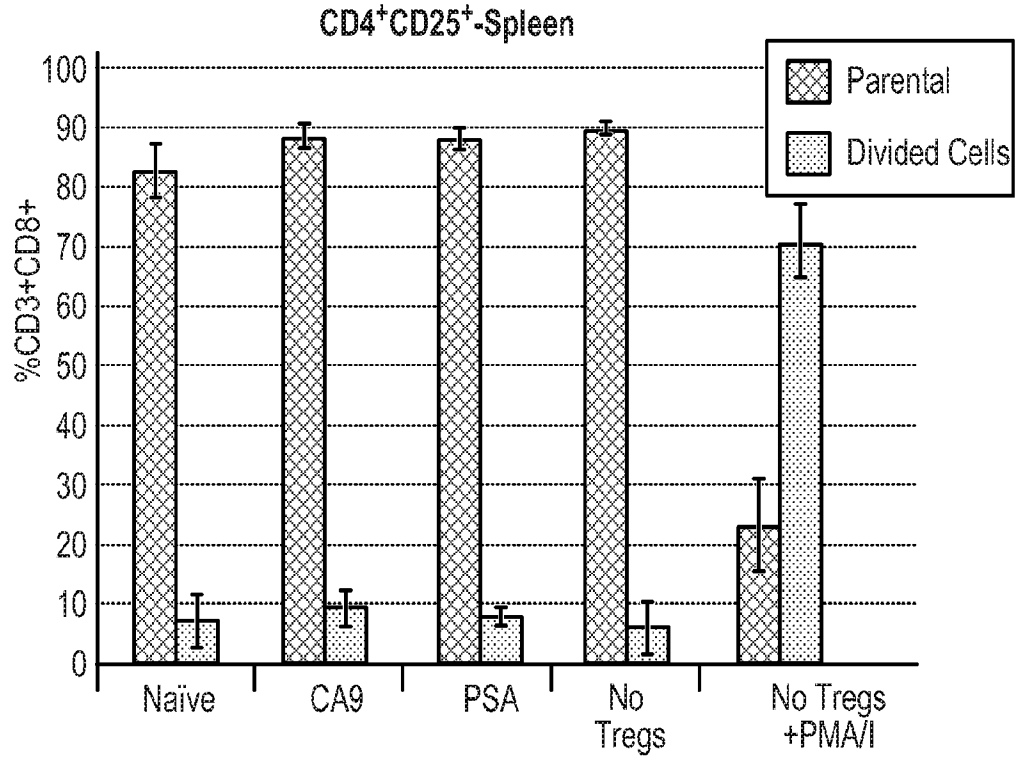


Figure 17B

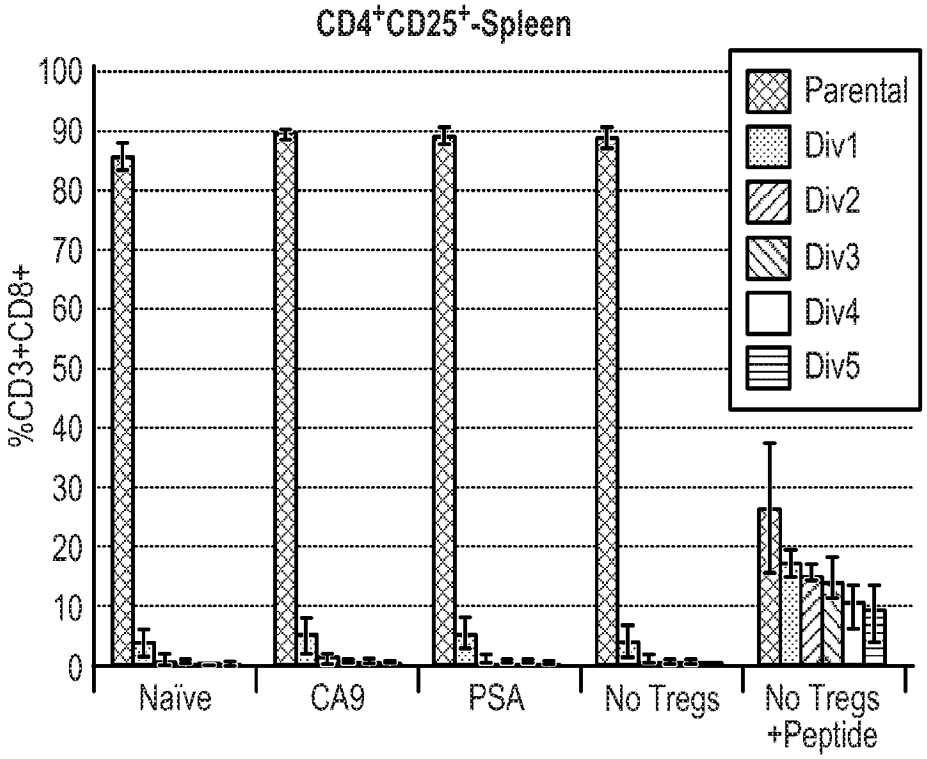


Figure 17C

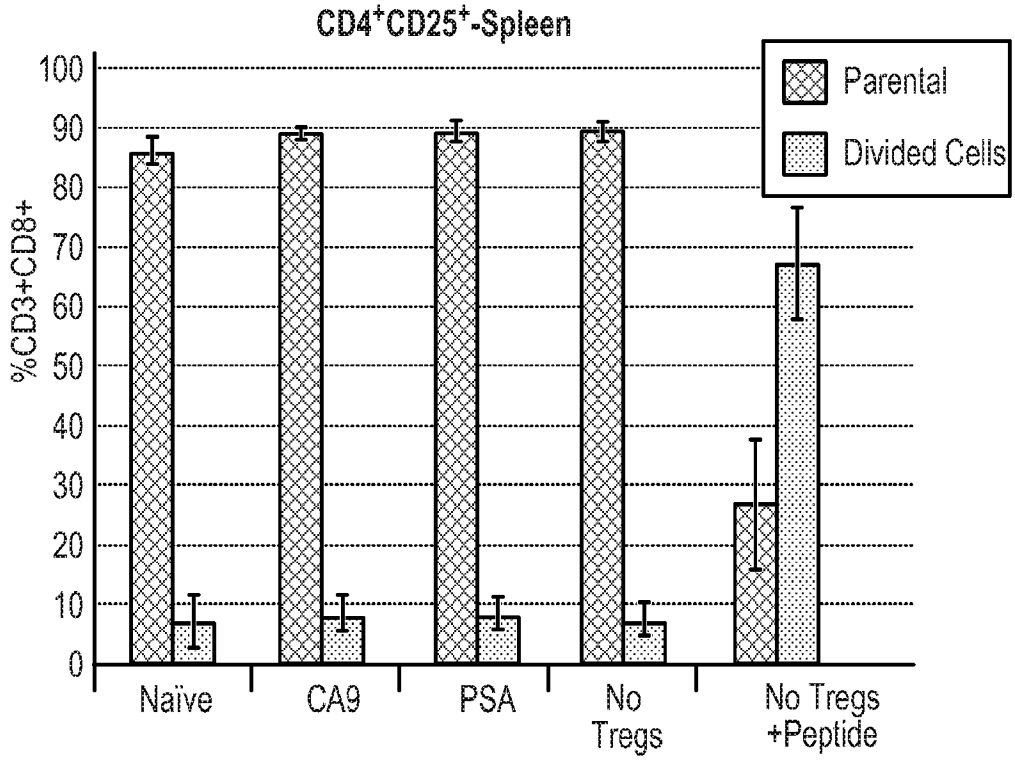


Figure 17D

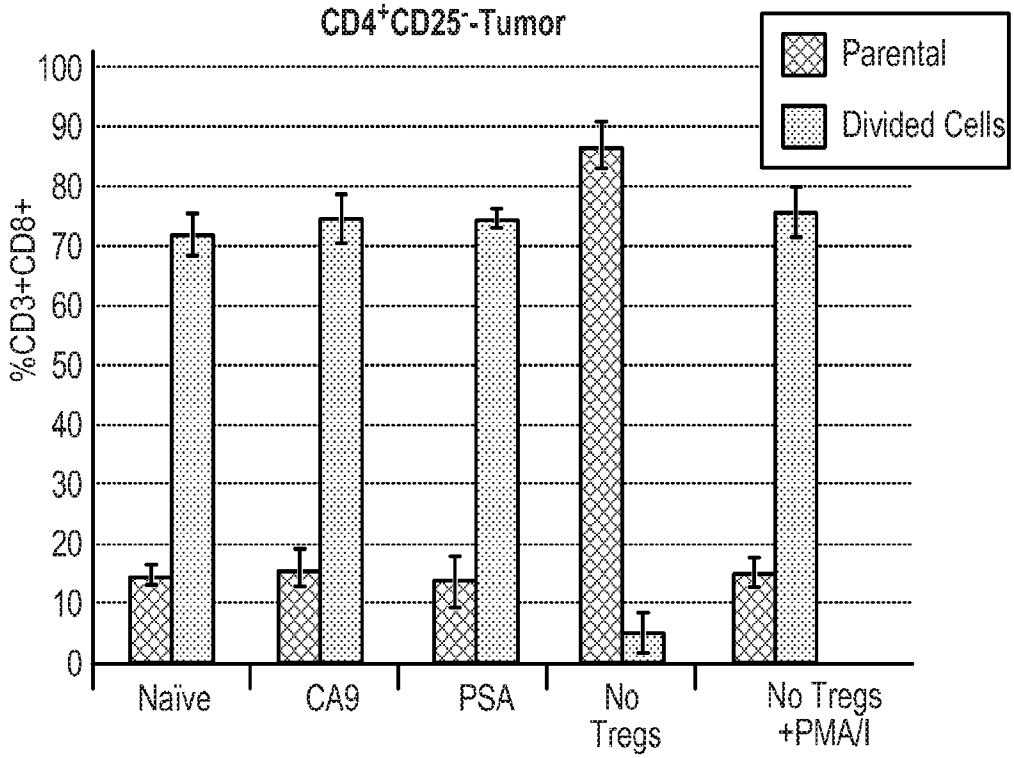


Figure 18A

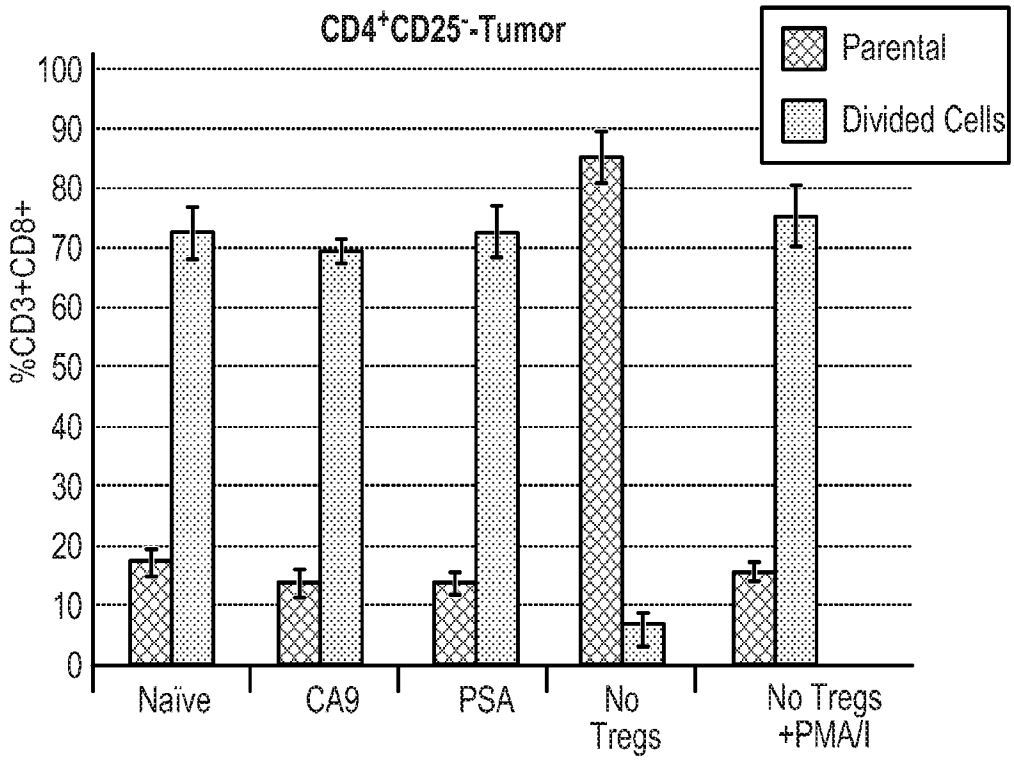


Figure 18B

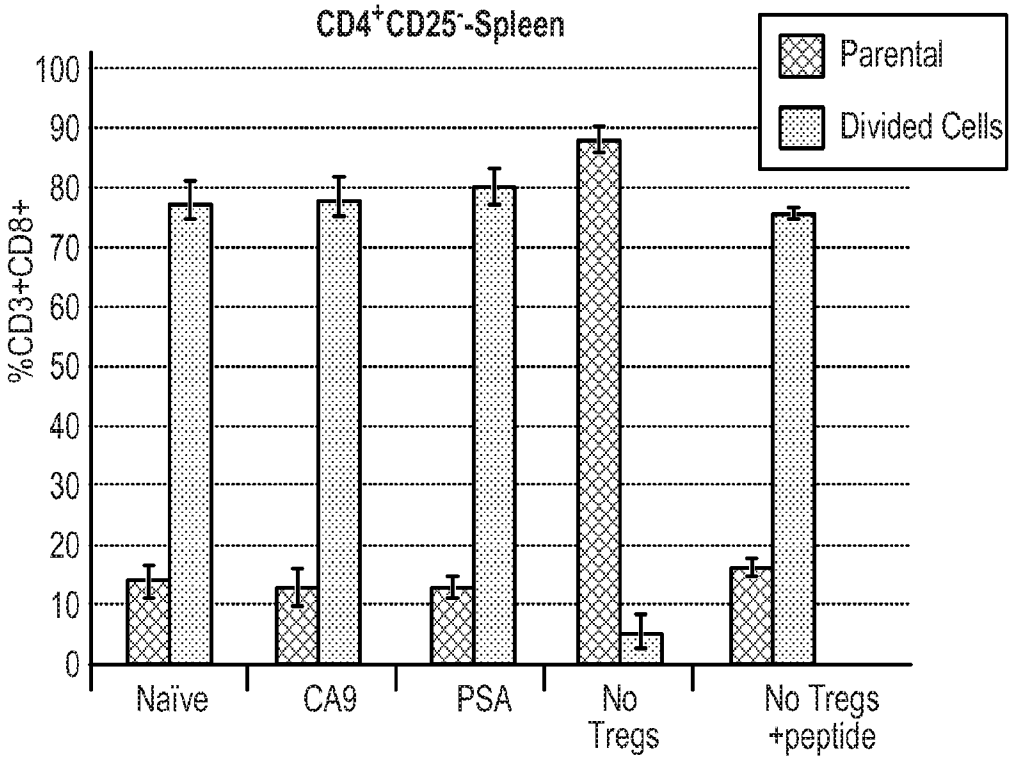


Figure 18C

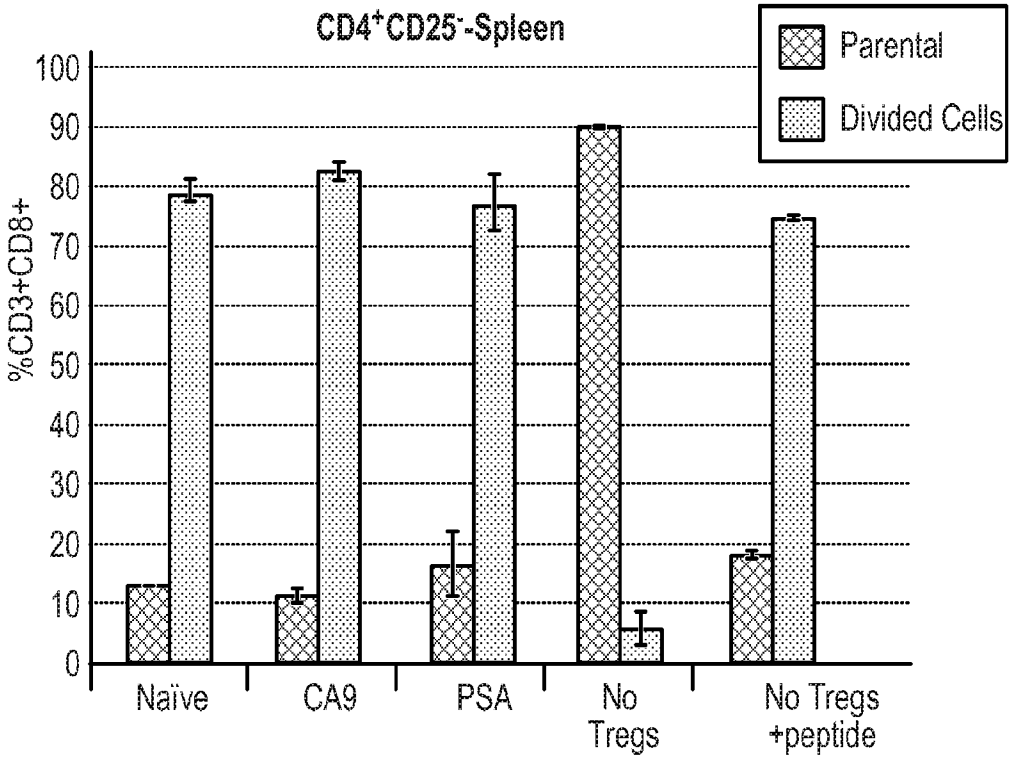


Figure 18D

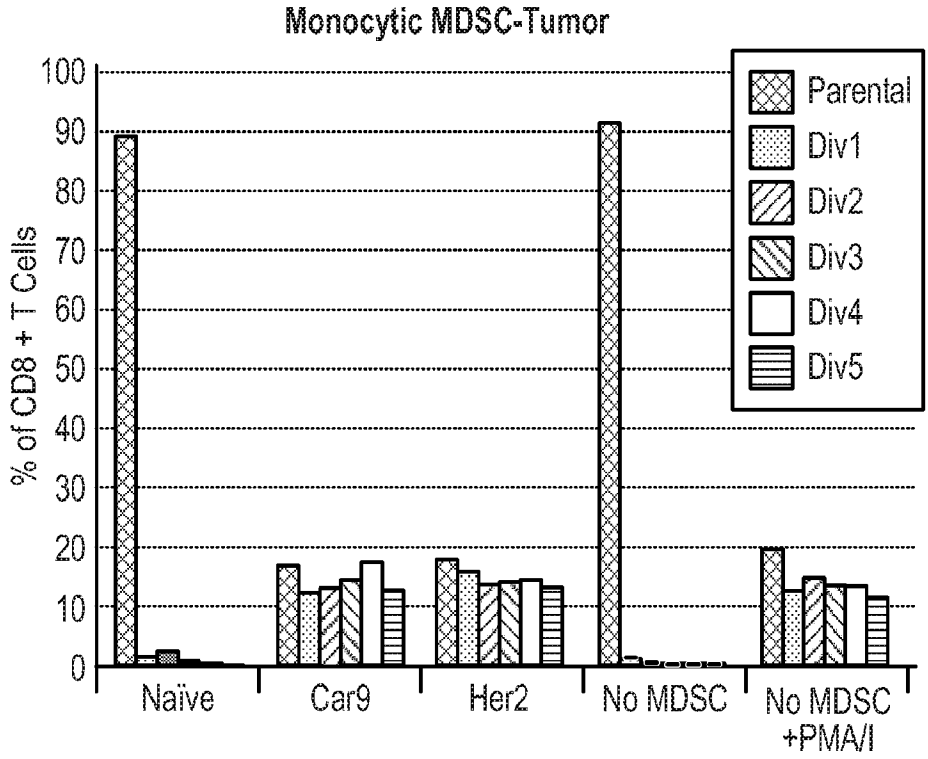


Figure 19A

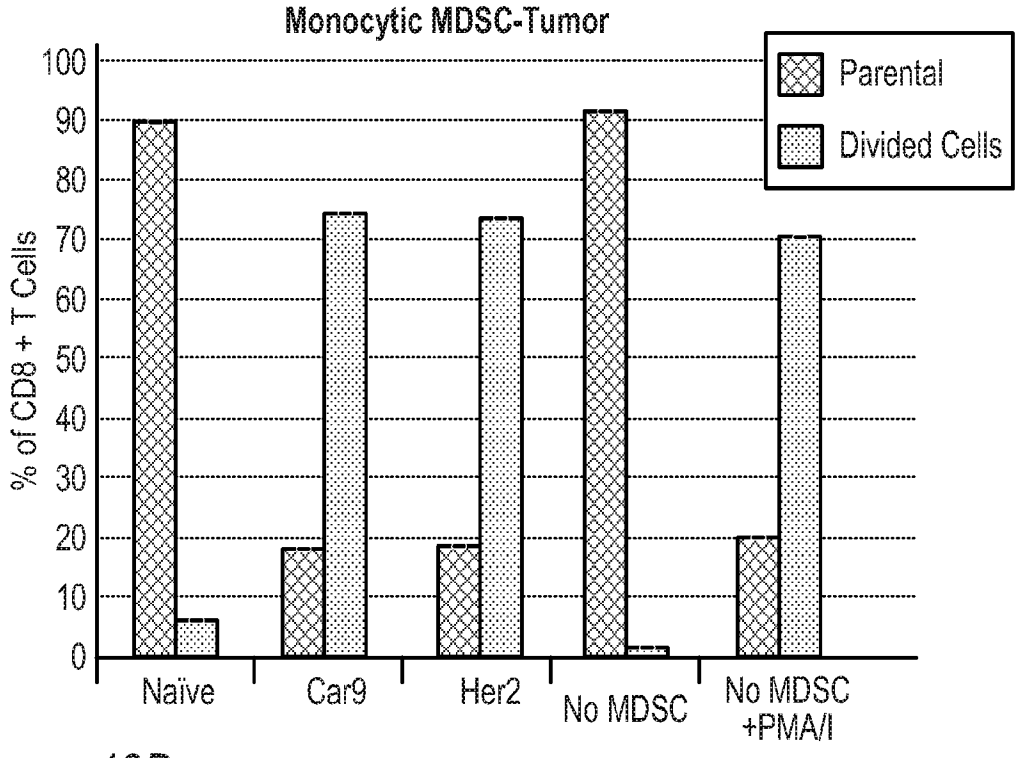


Figure 19B

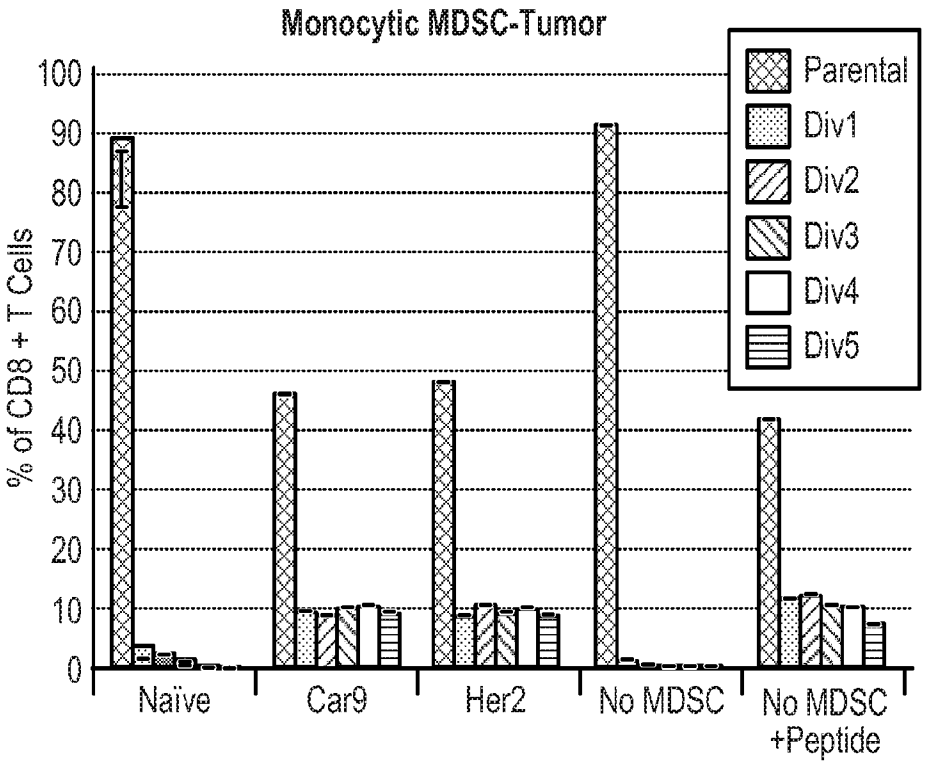


Figure 19C

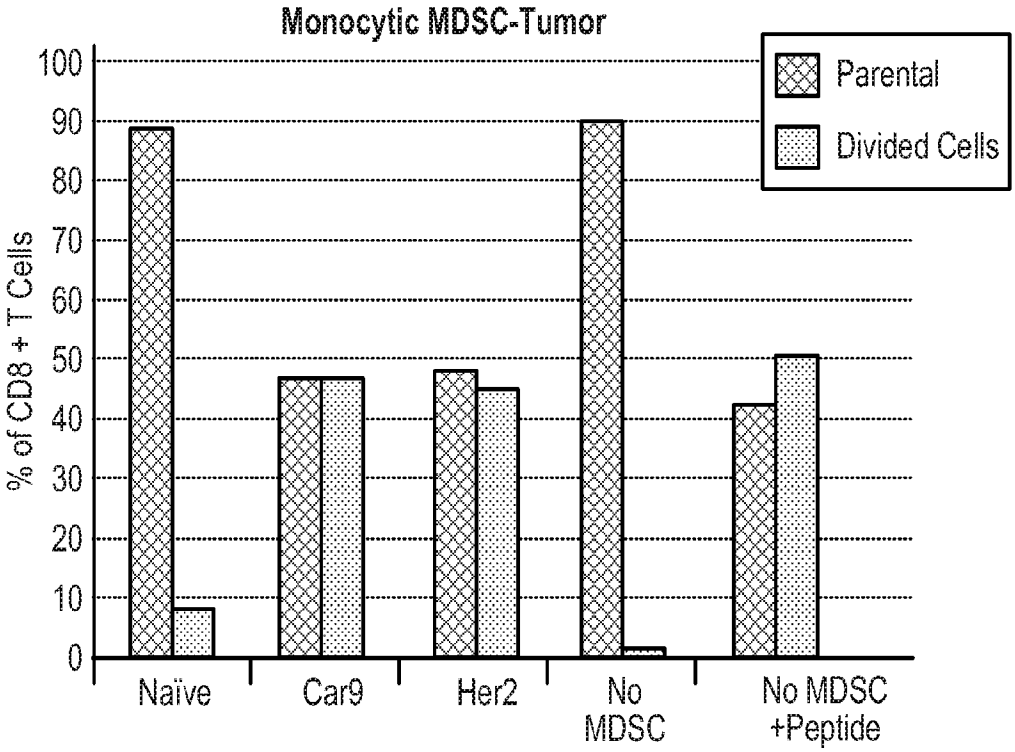


Figure 19D

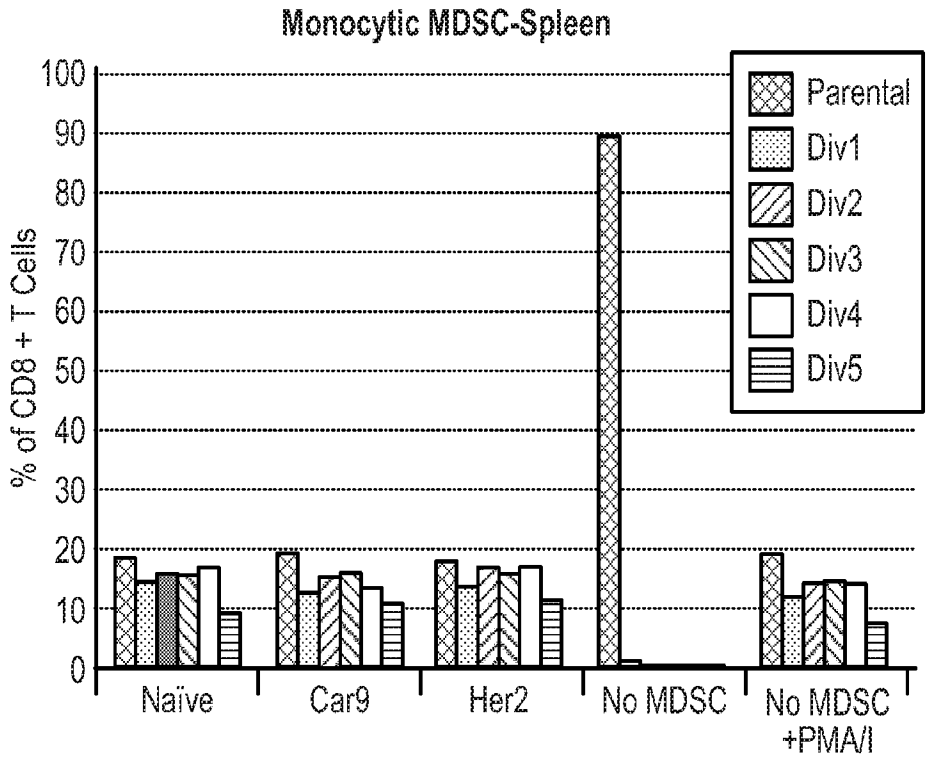


Figure 20A

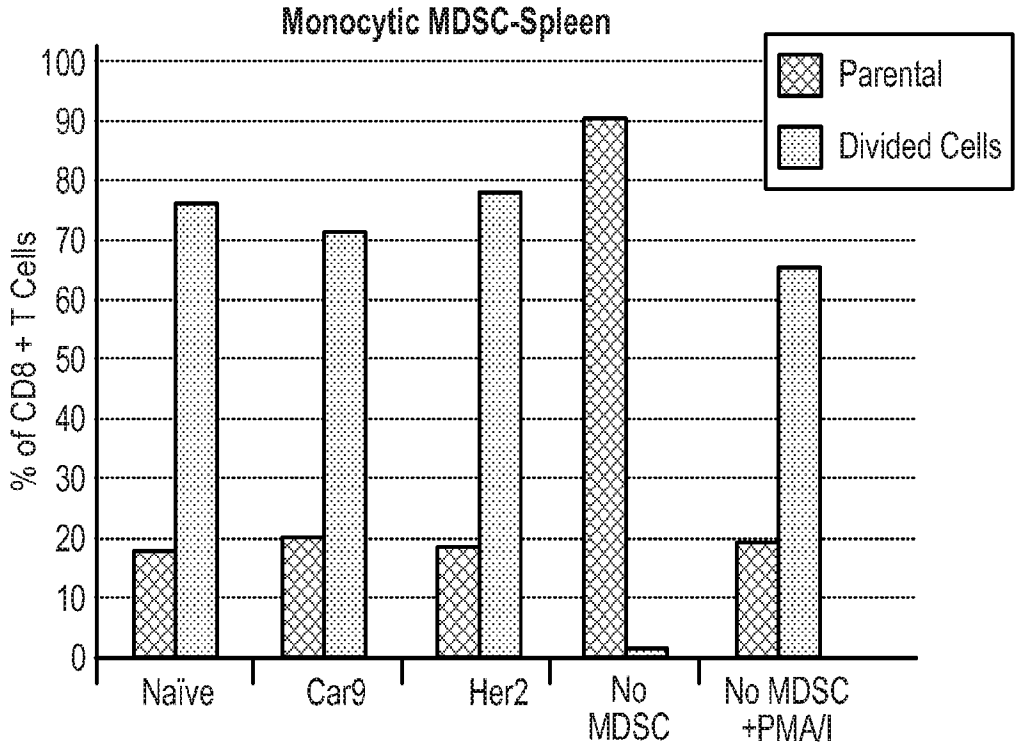


Figure 20B

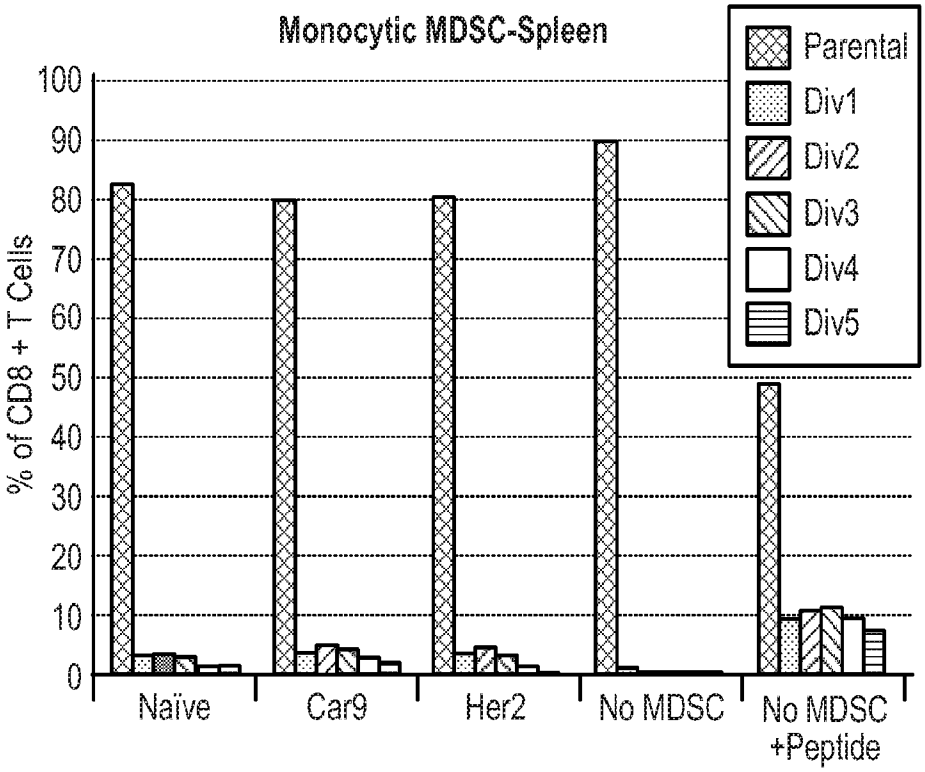


Figure 20C

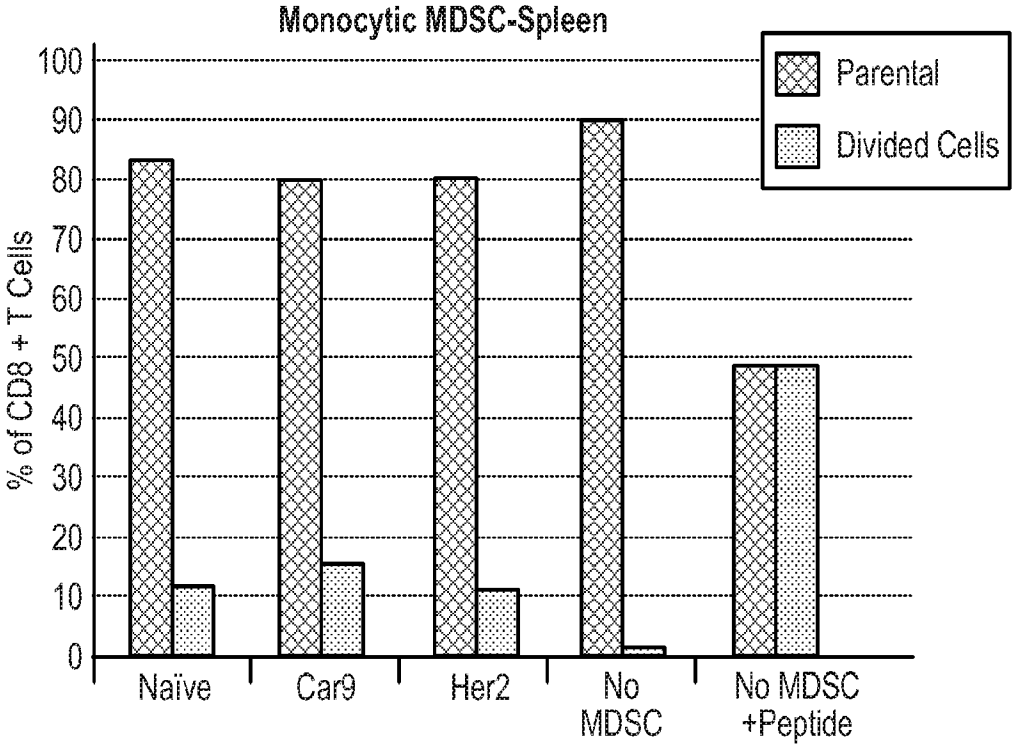


Figure 20D

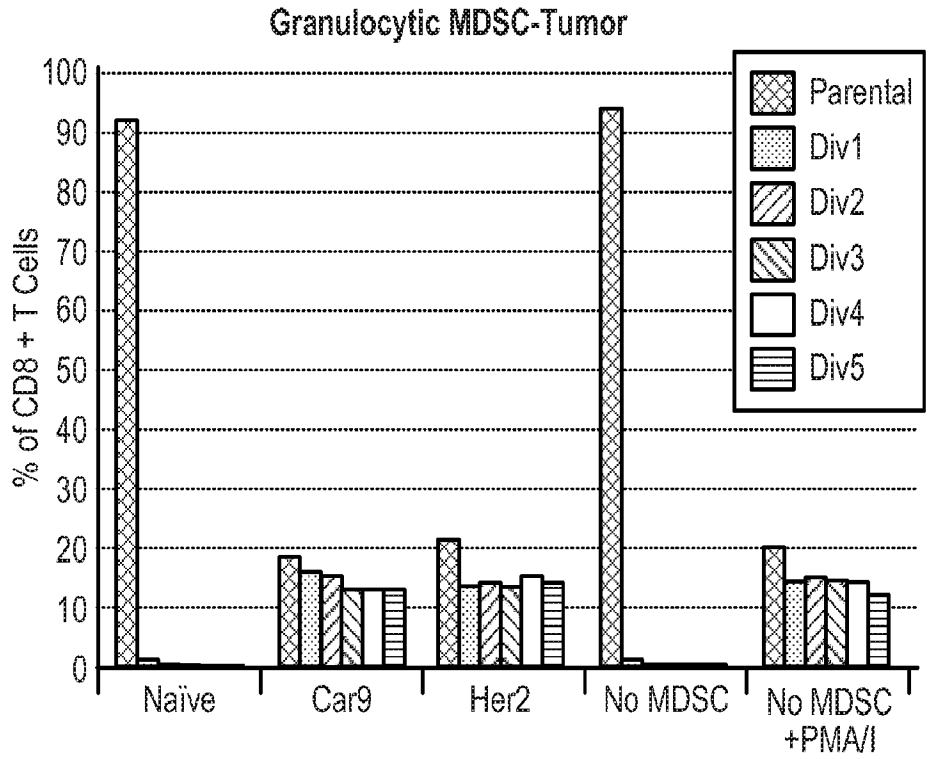


Figure 21A

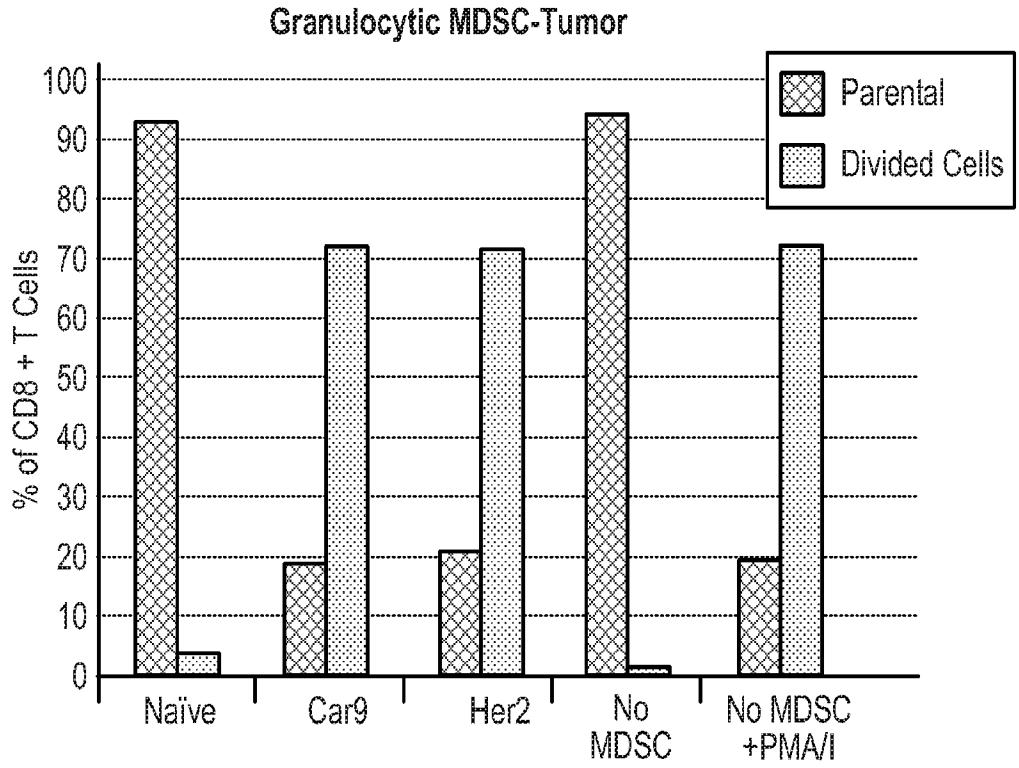


Figure 21B

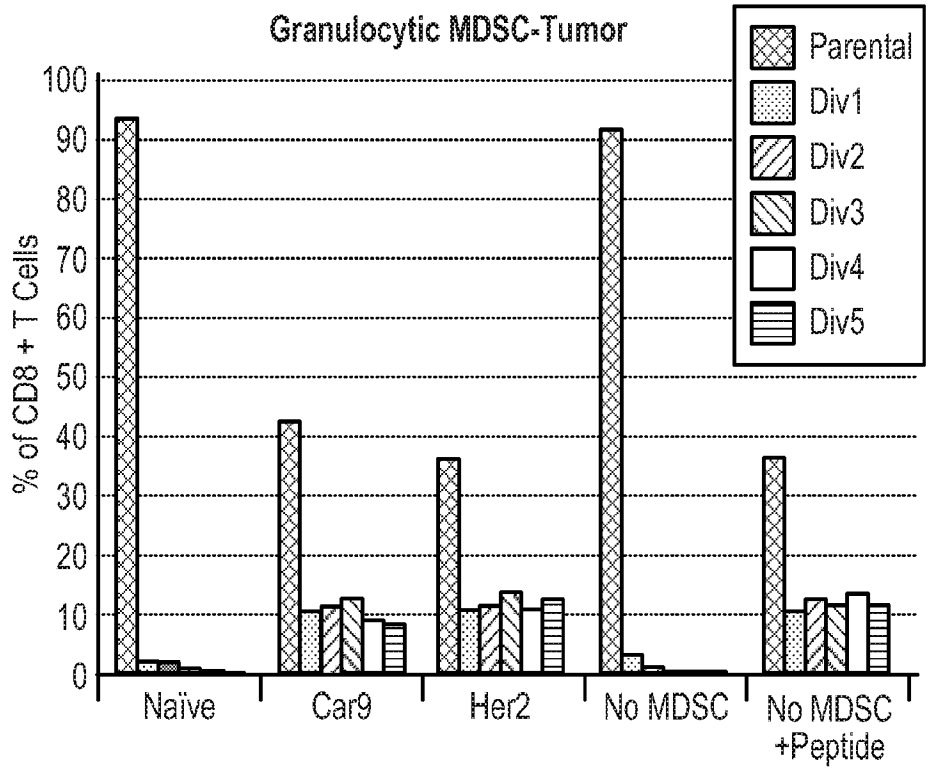


Figure 21C

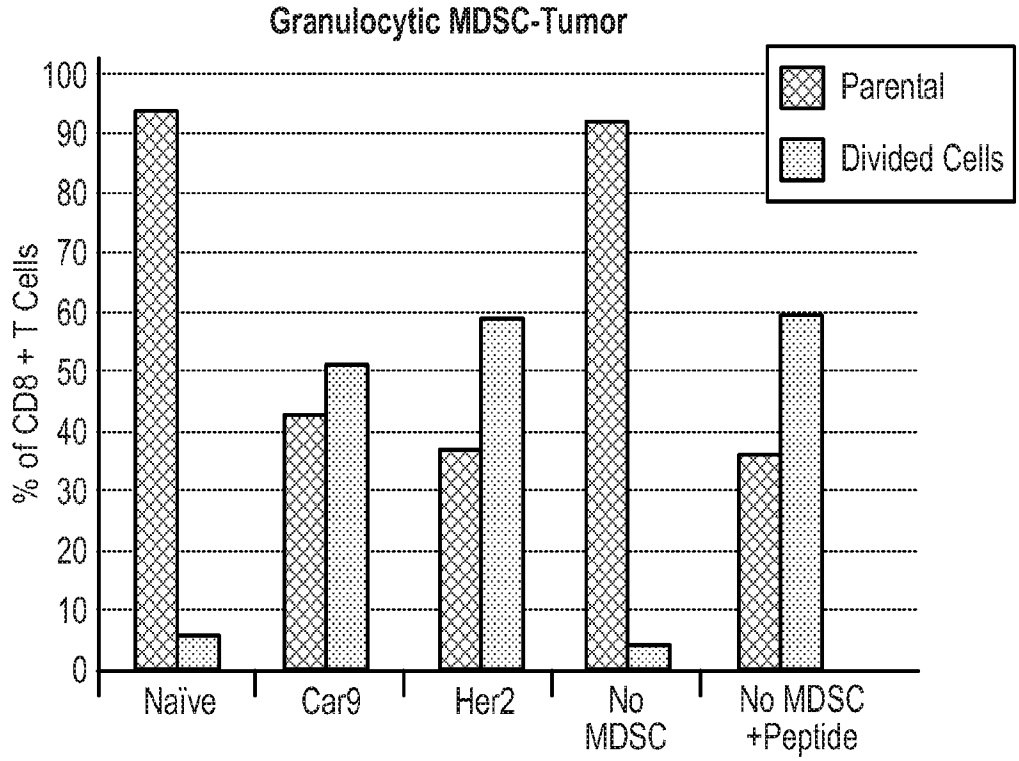


Figure 21D

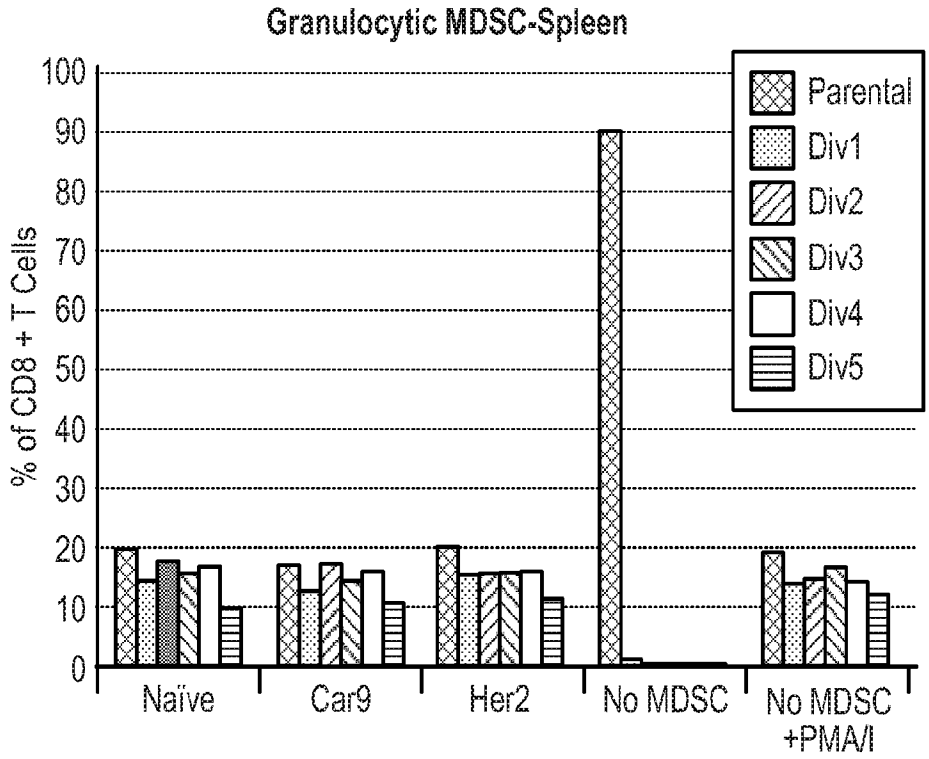


Figure 22A

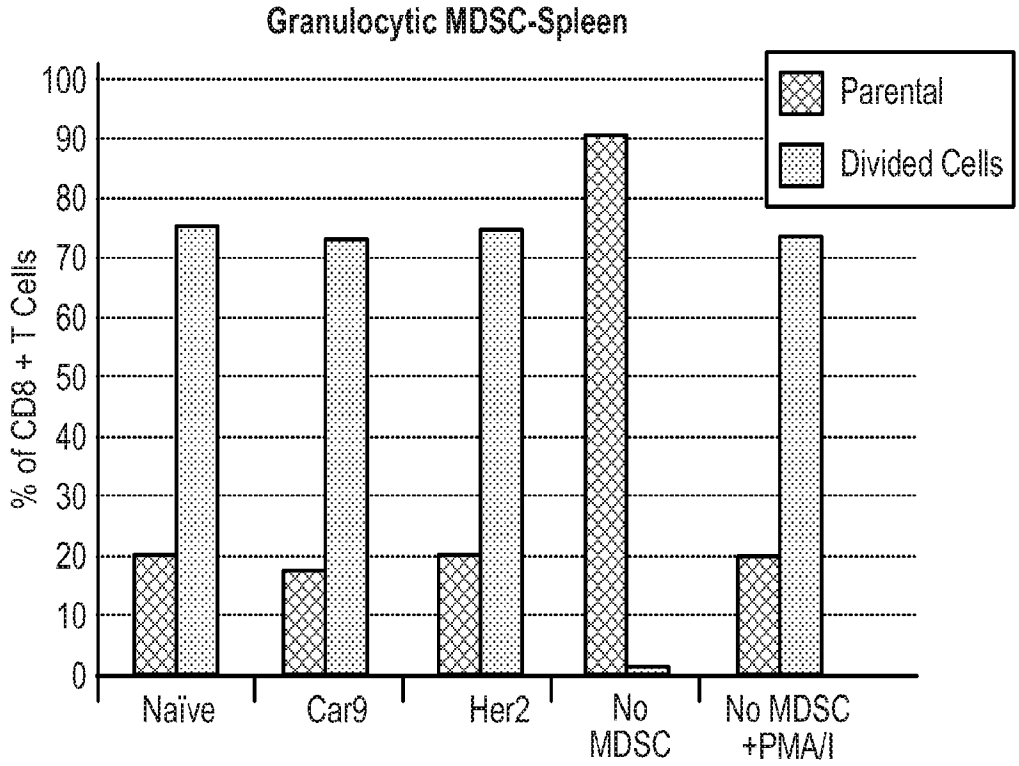


Figure 22B

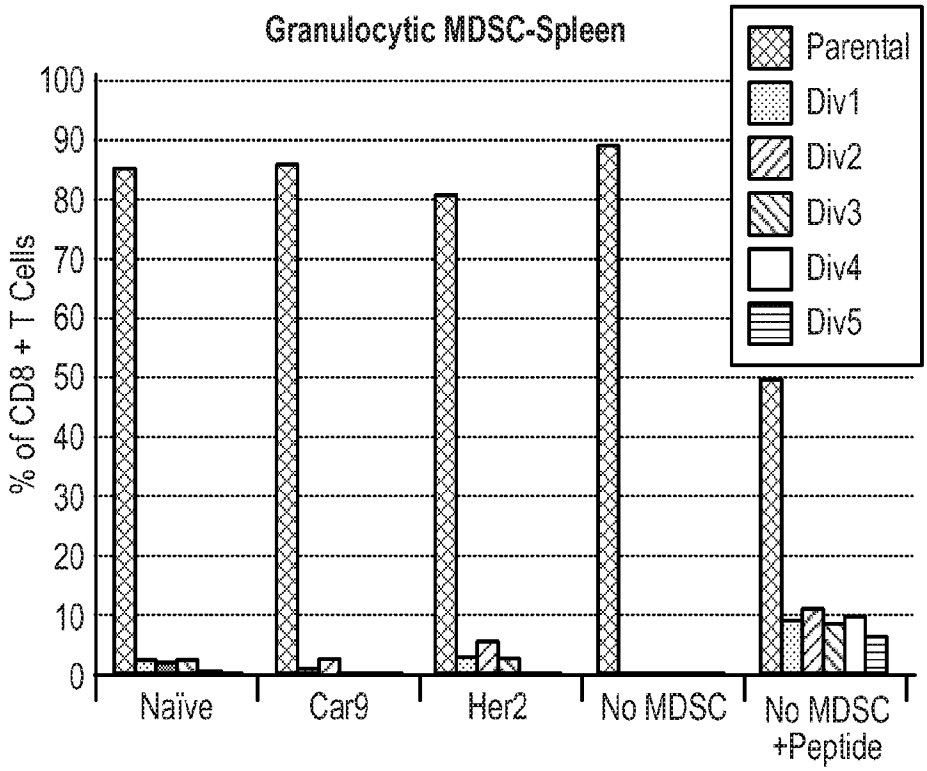


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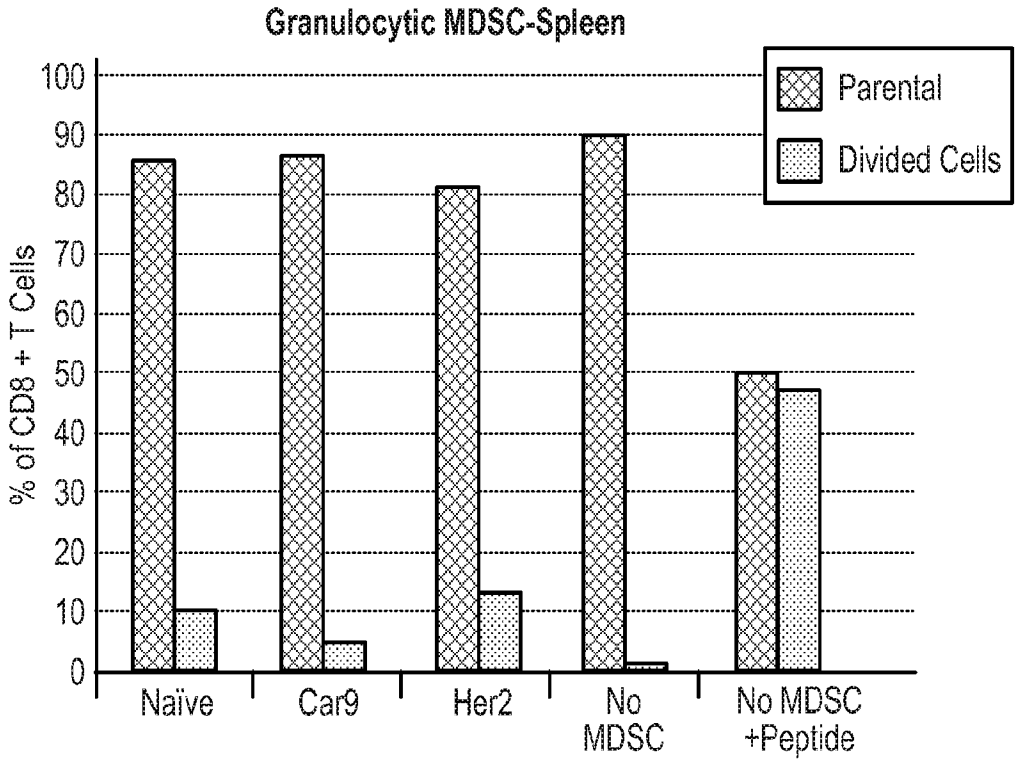


Figure 22D

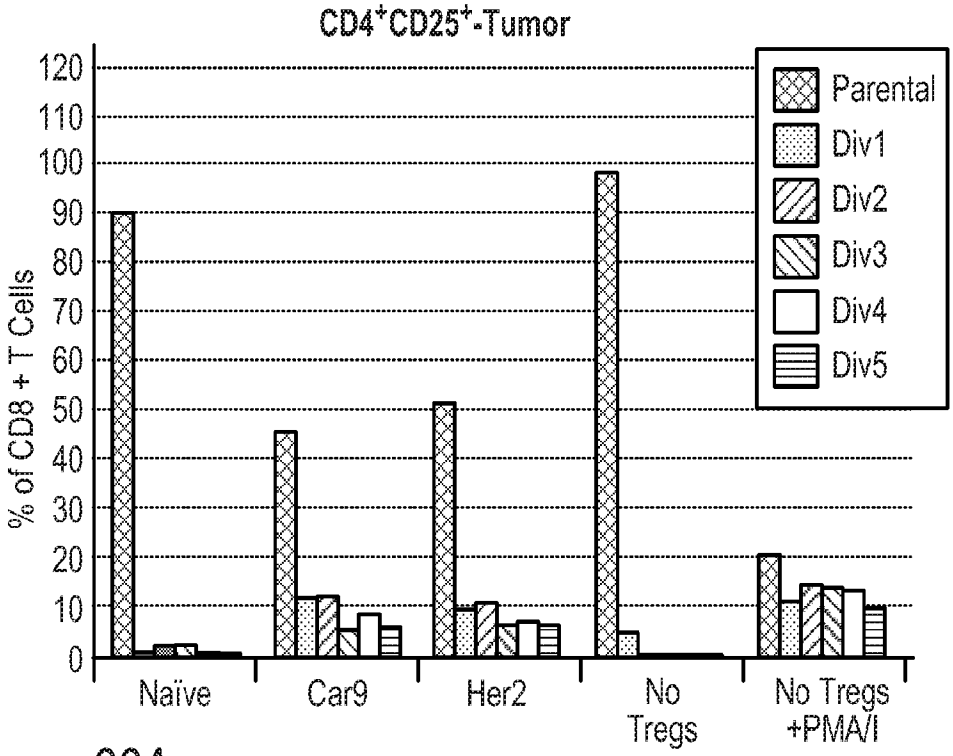


Figure 23A

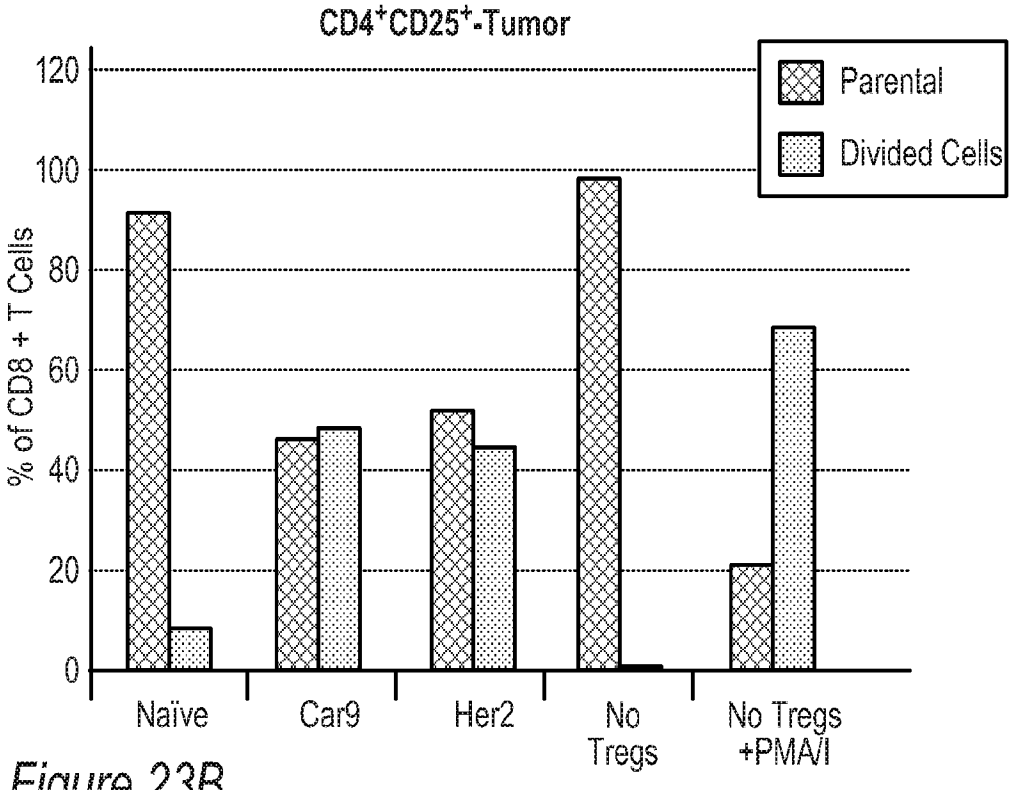


Figure 23B

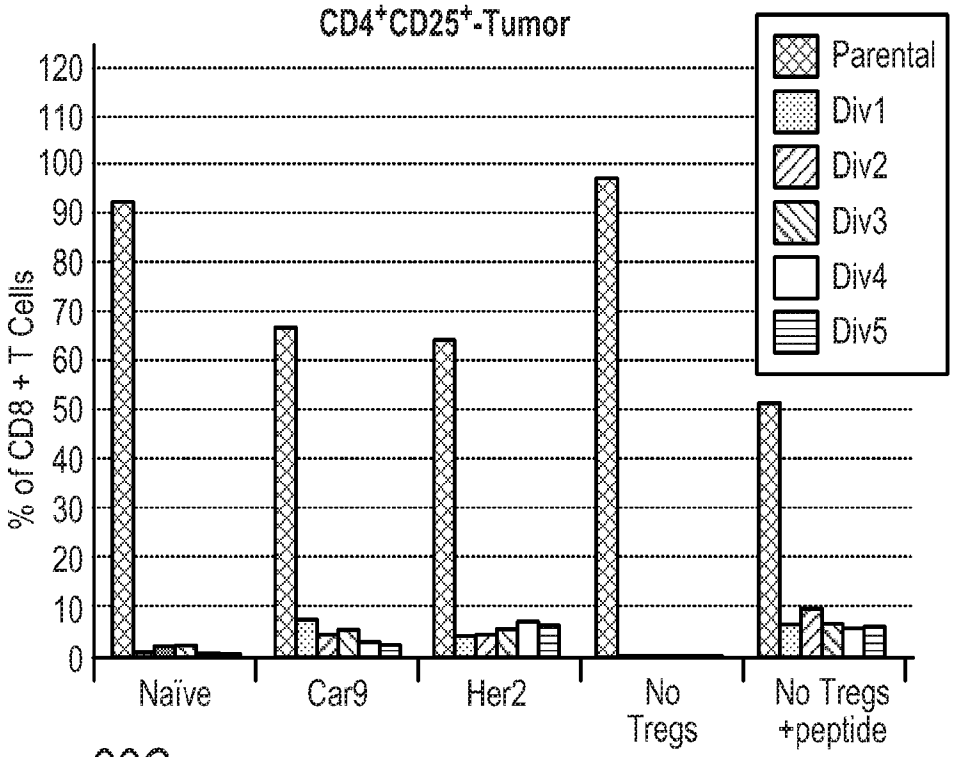


Figure 23C

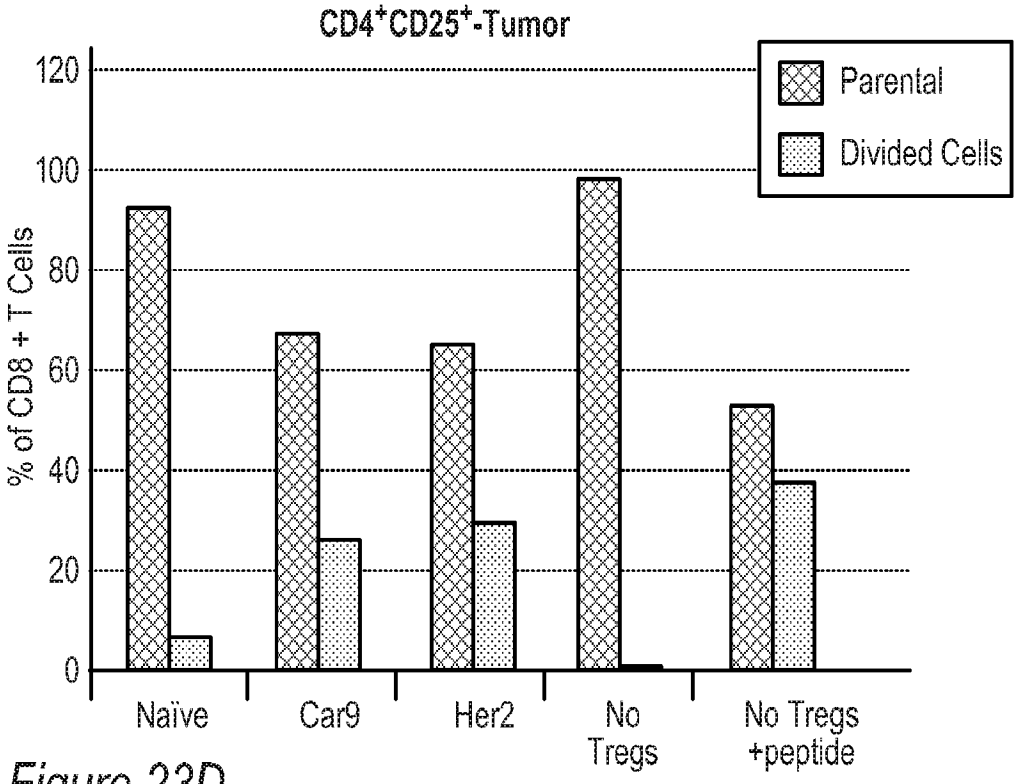


Figure 23D

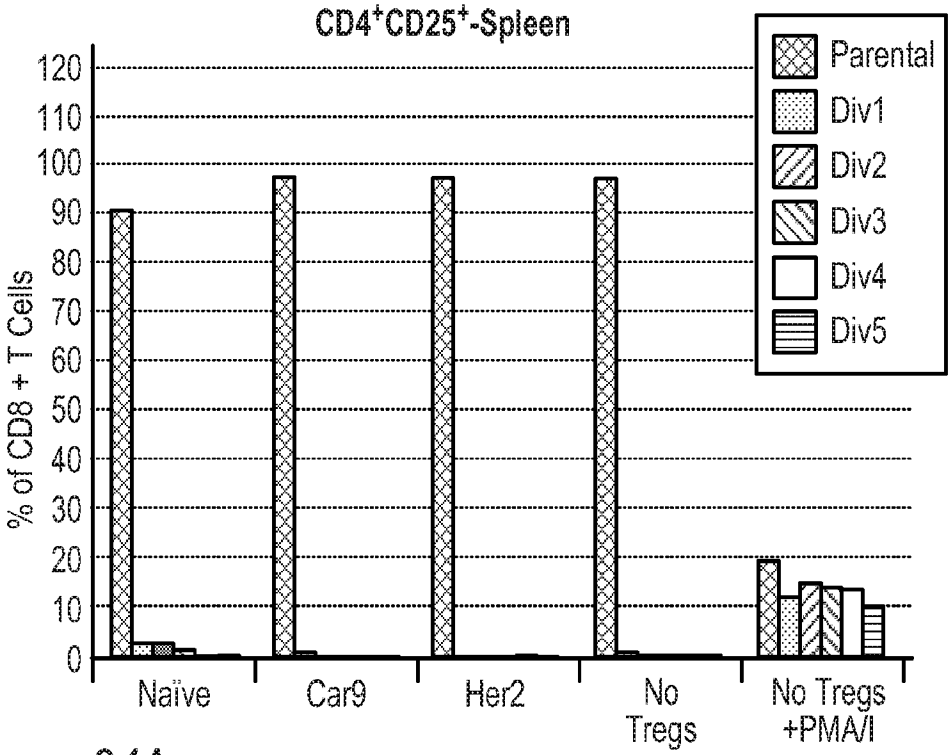


Figure 24A

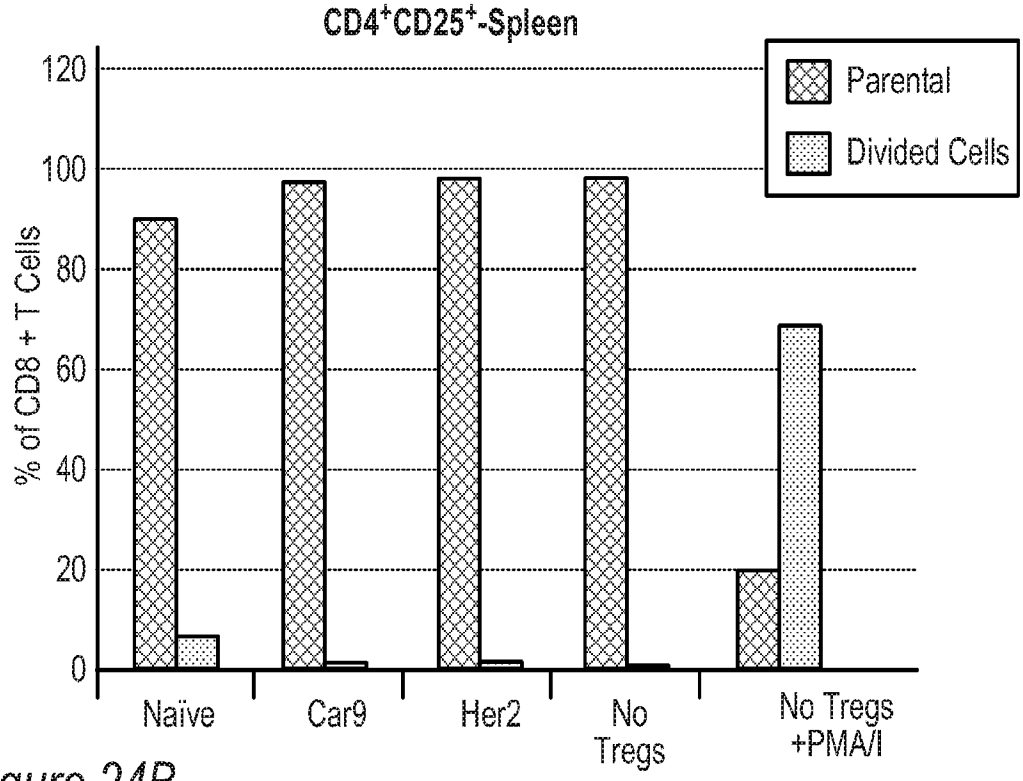


Figure 24B

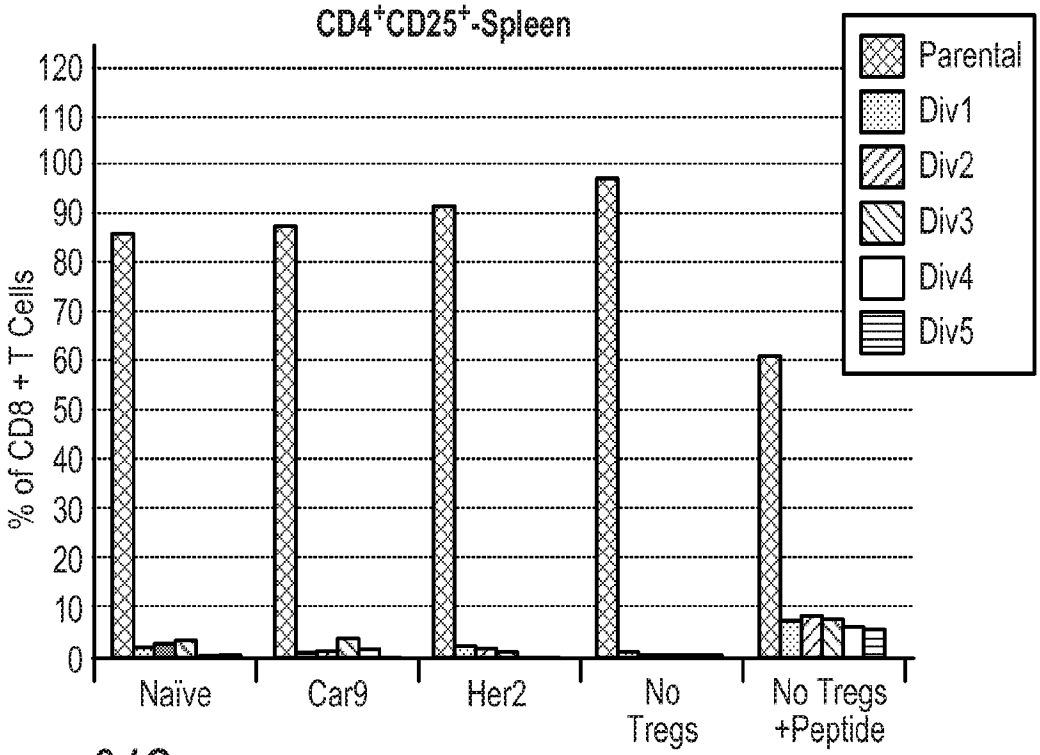


Figure 24C

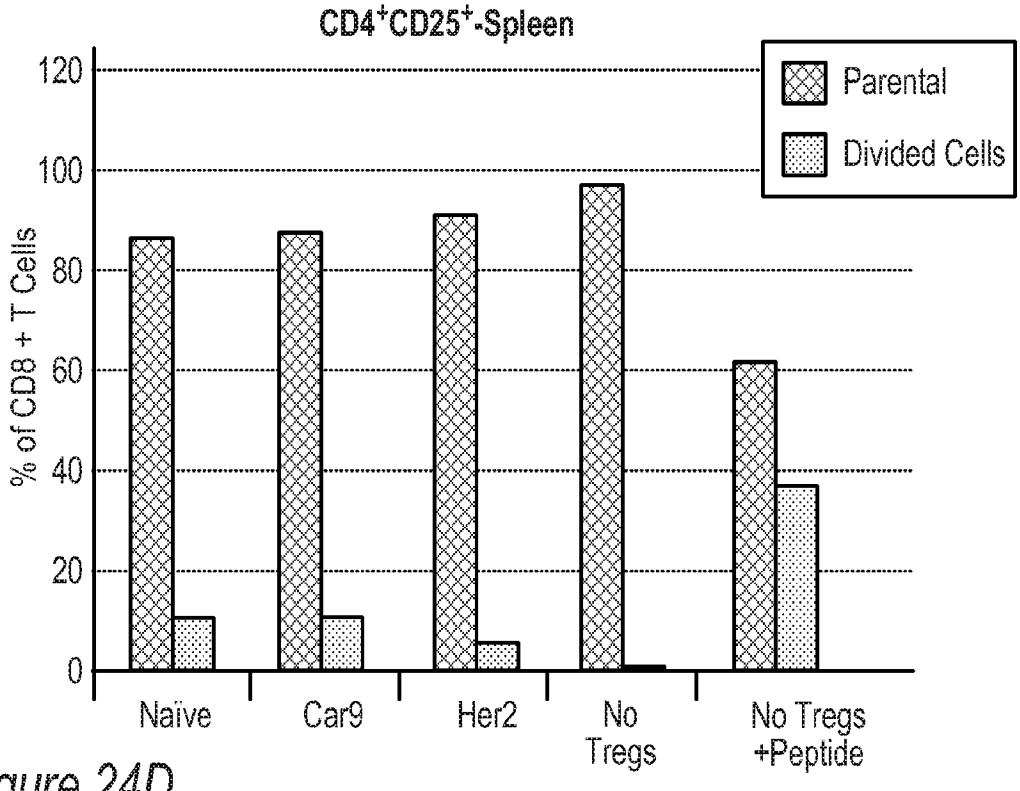


Figure 24D

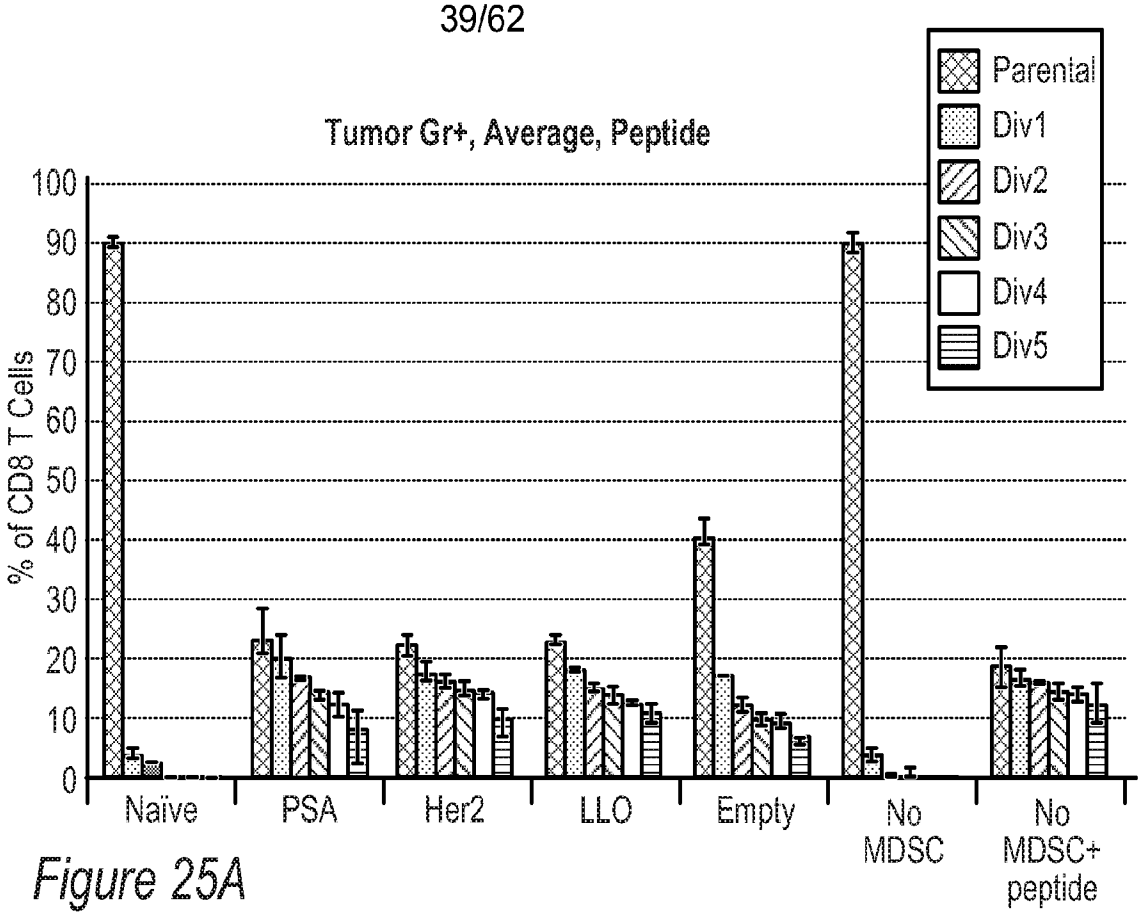


Figure 25A

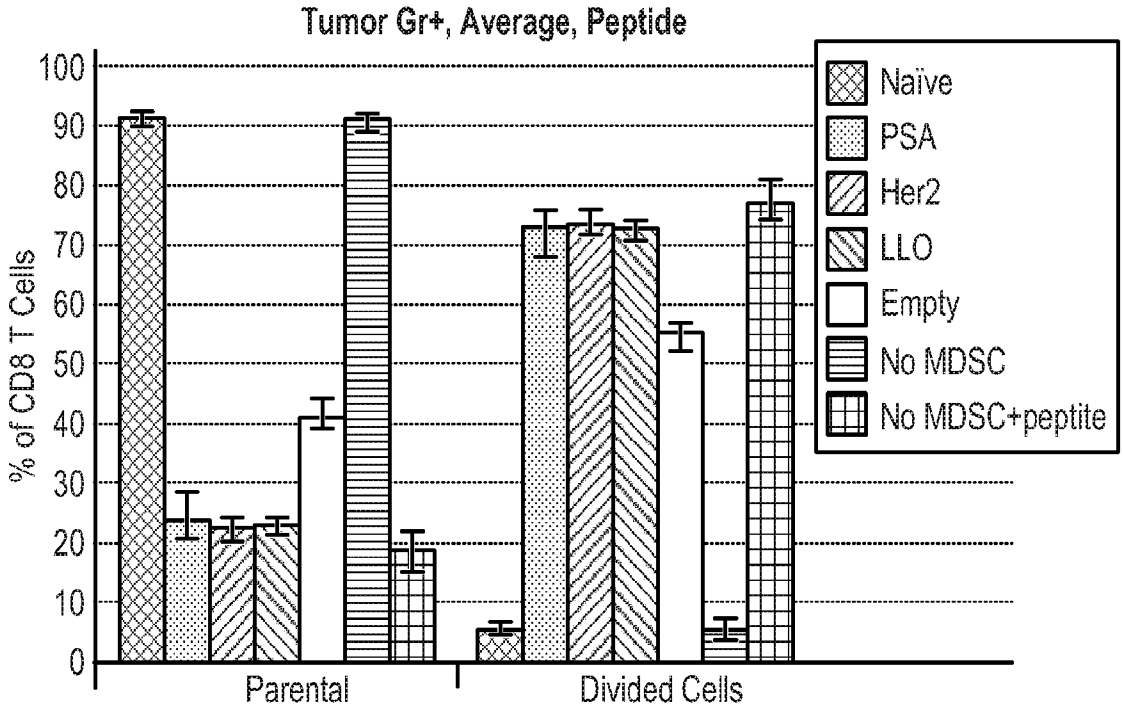


Figure 25B

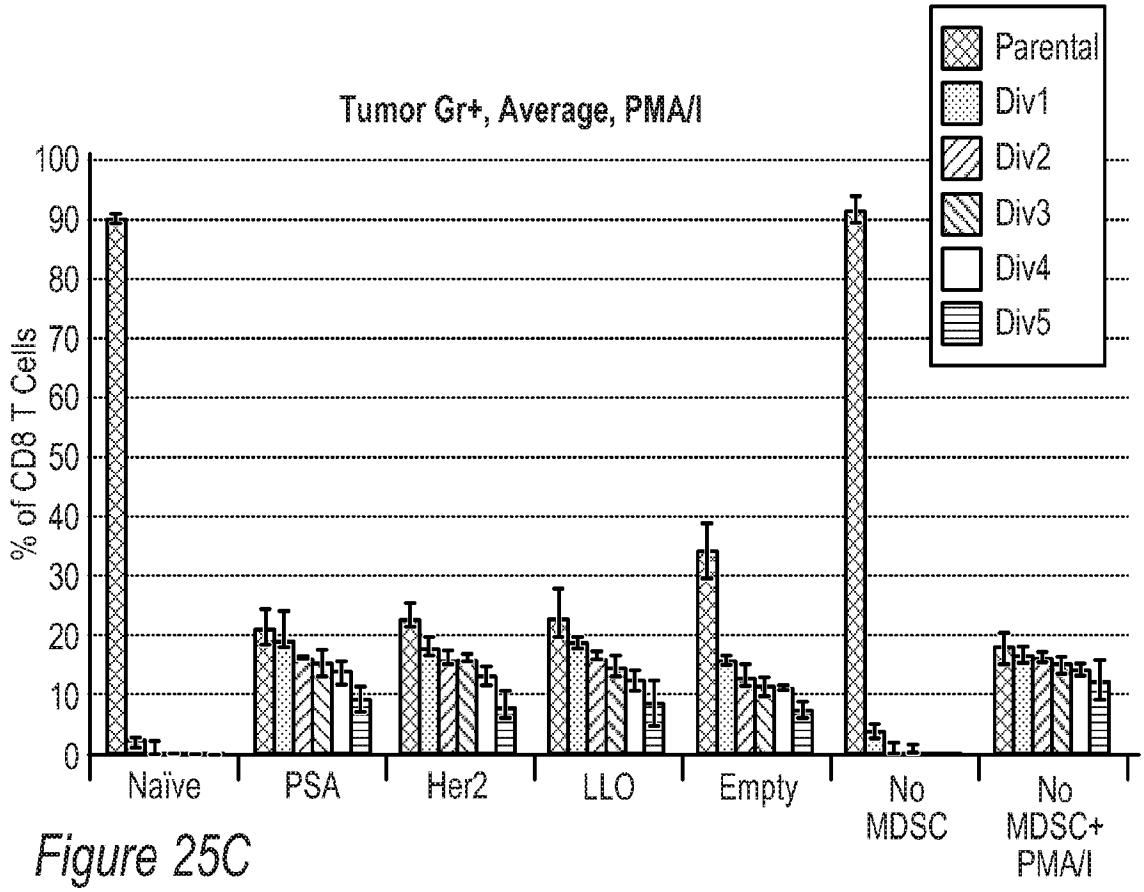


Figure 25C

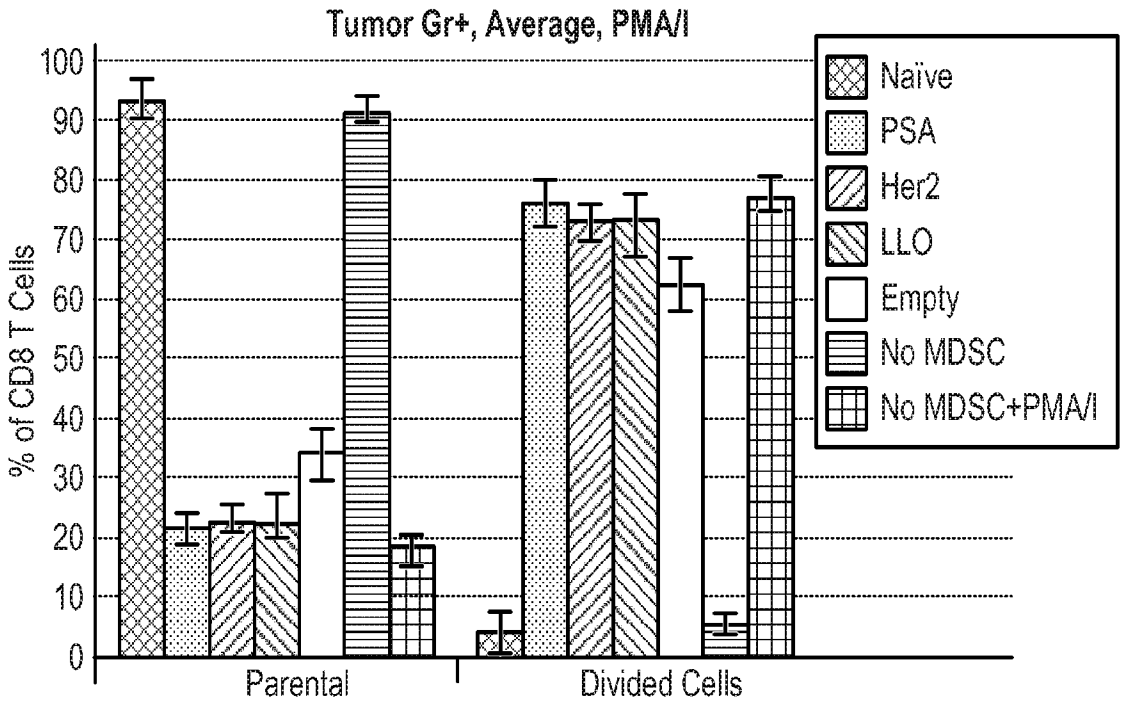


Figure 25D

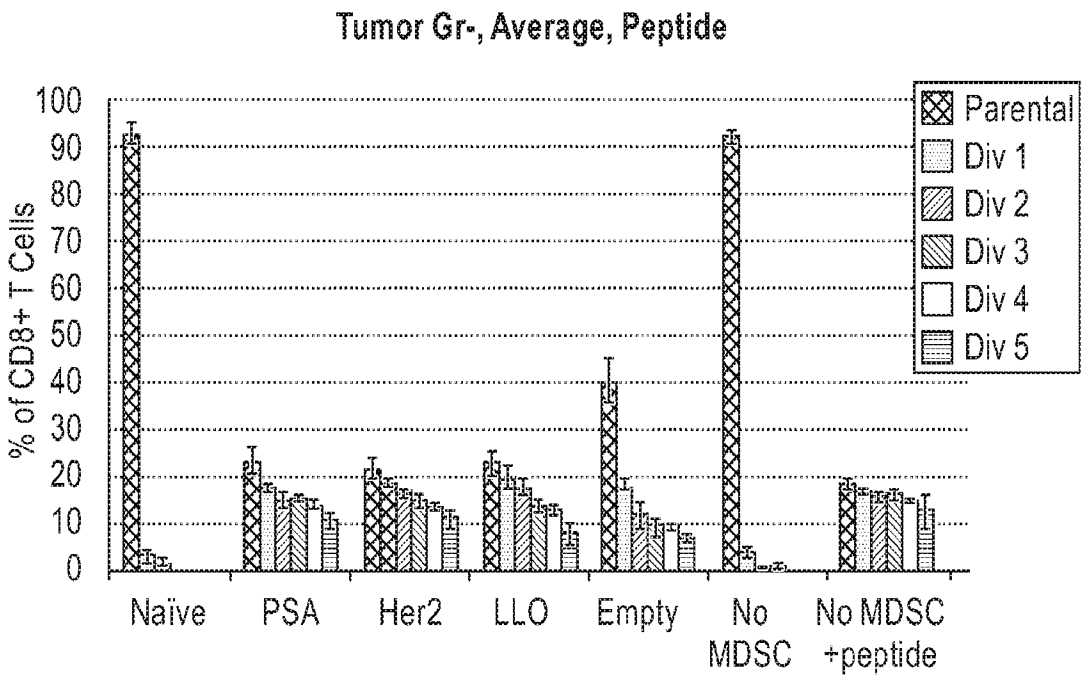


Figure 26A

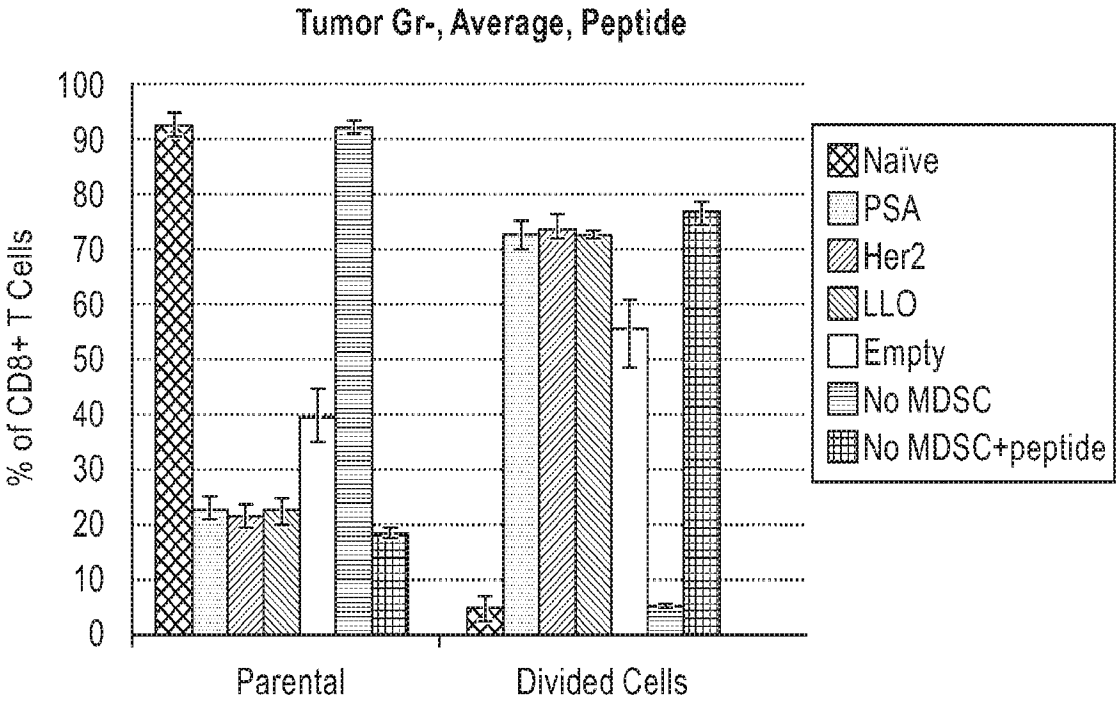


Figure 26B

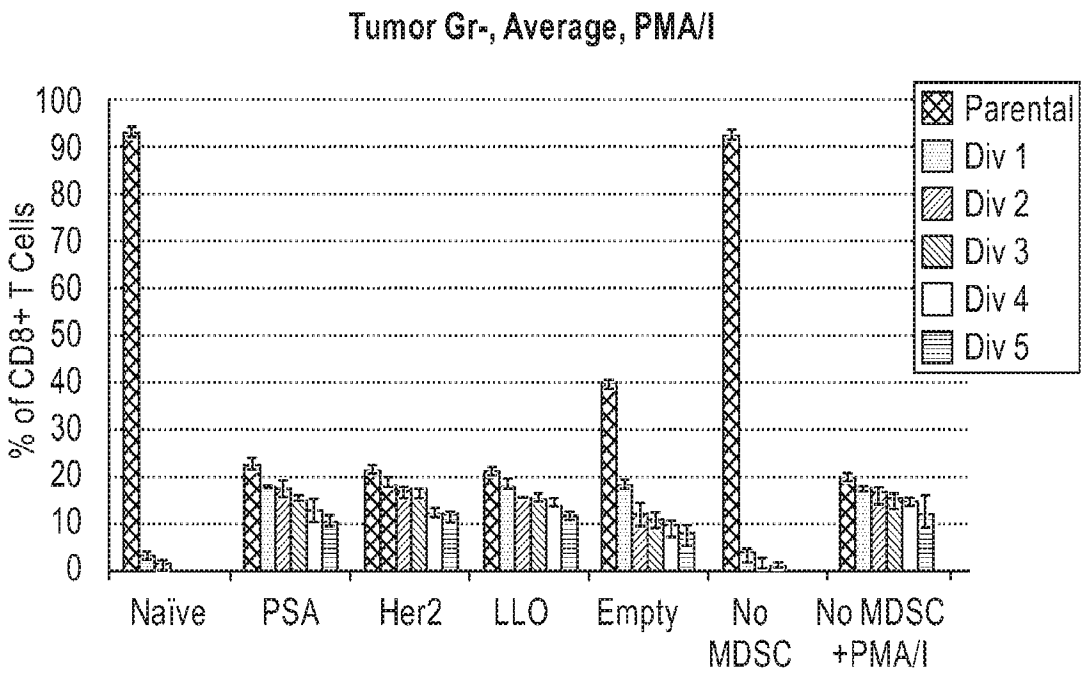


Figure 26C

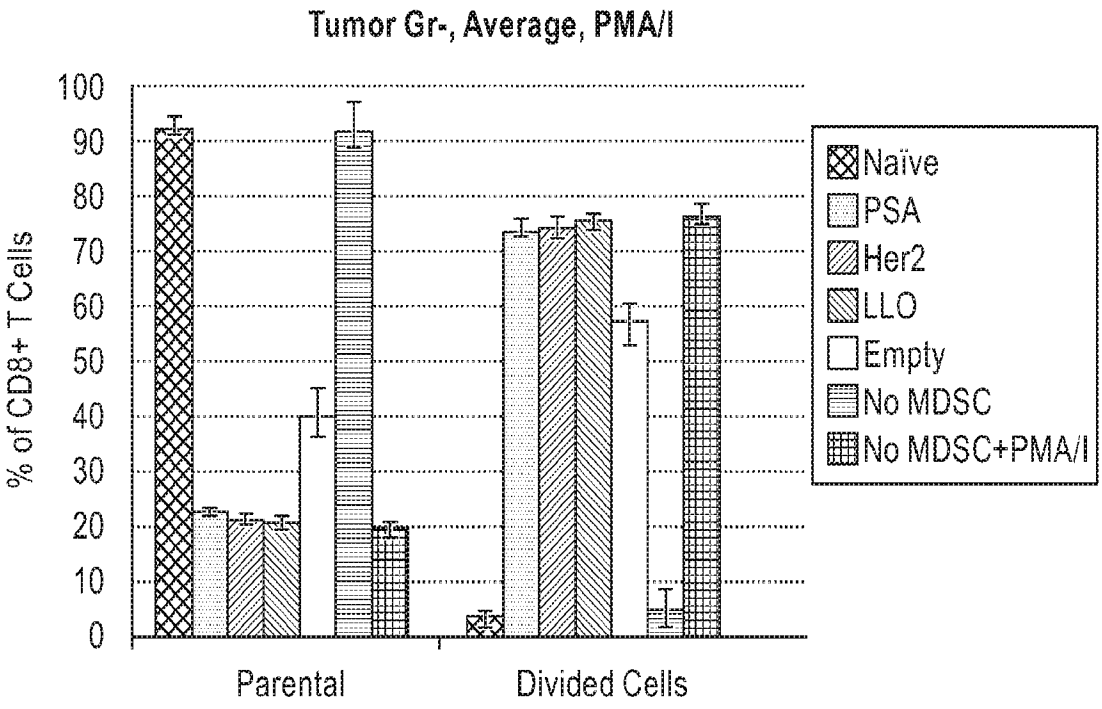


Figure 26D

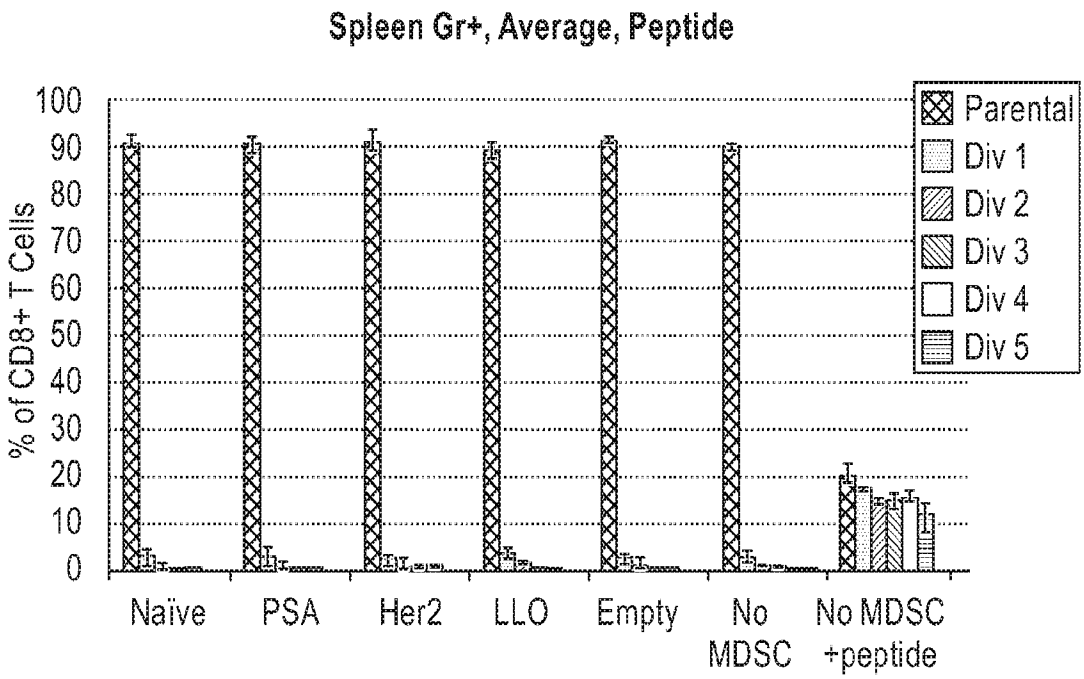


Figure 27A

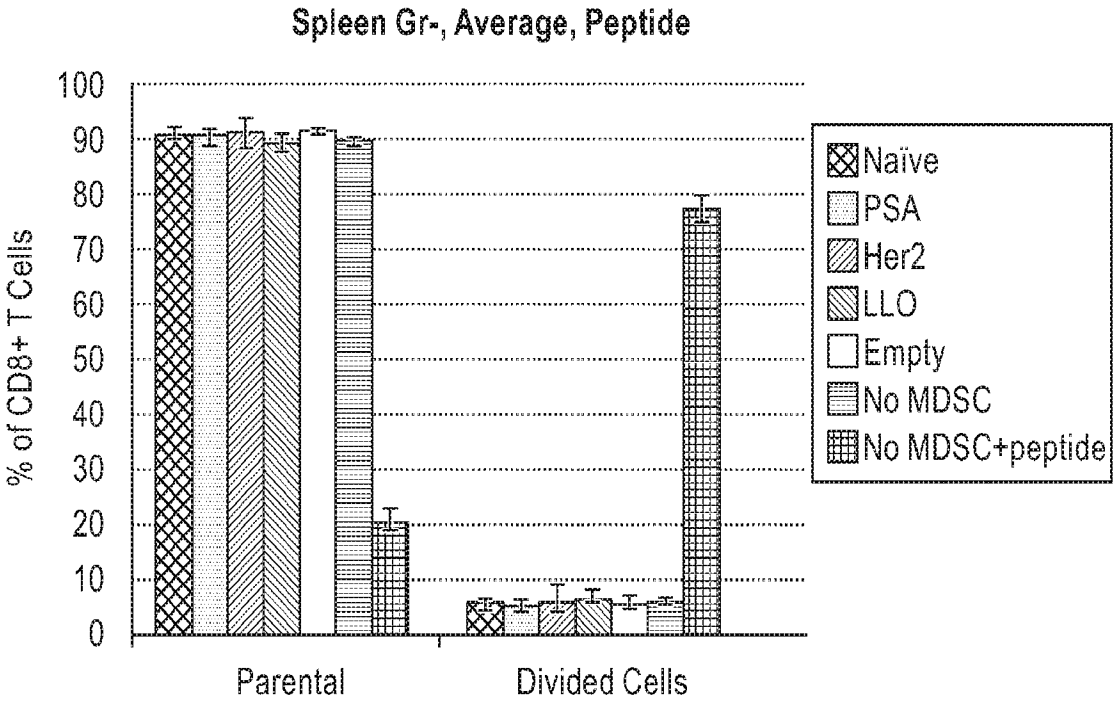


Figure 27B

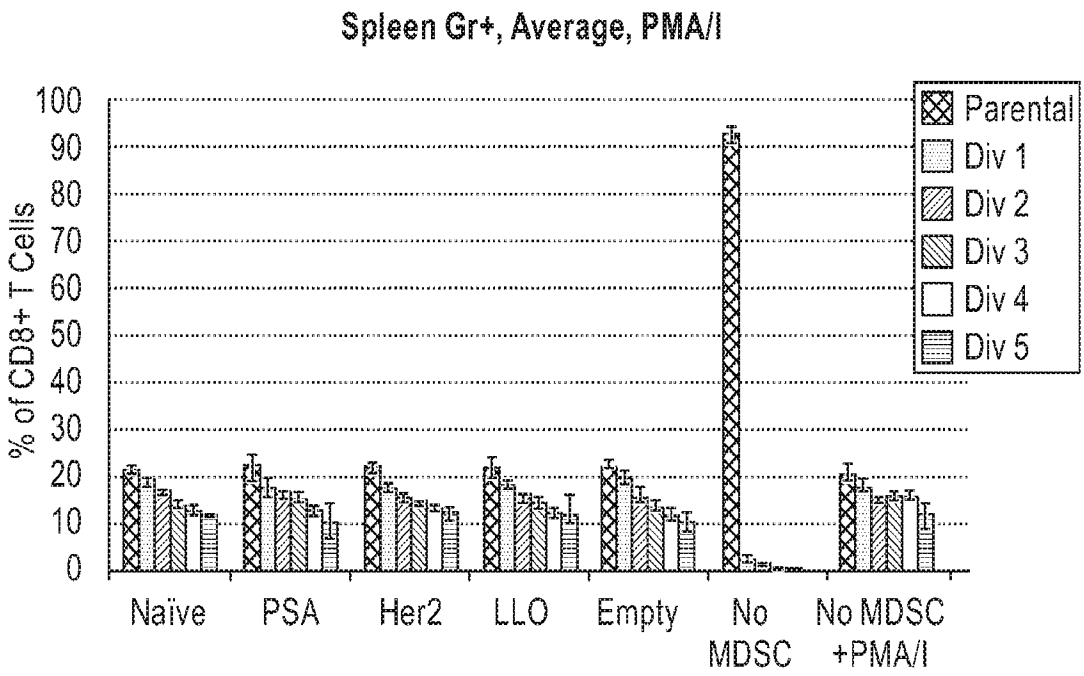


Figure 27C

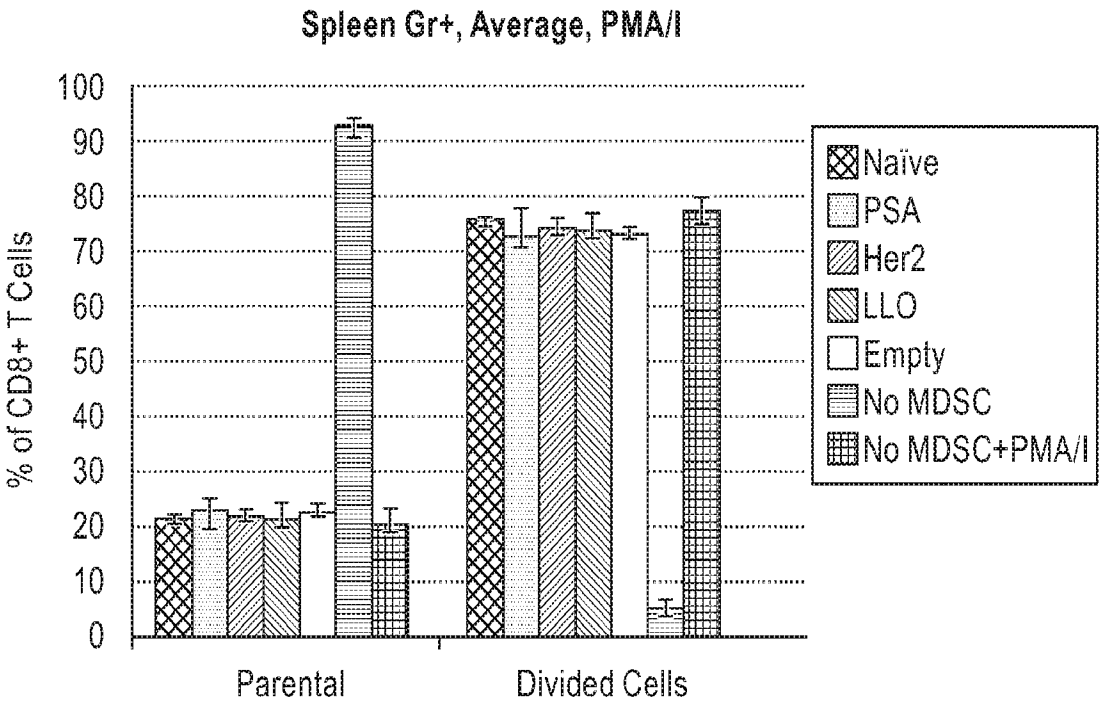


Figure 27D

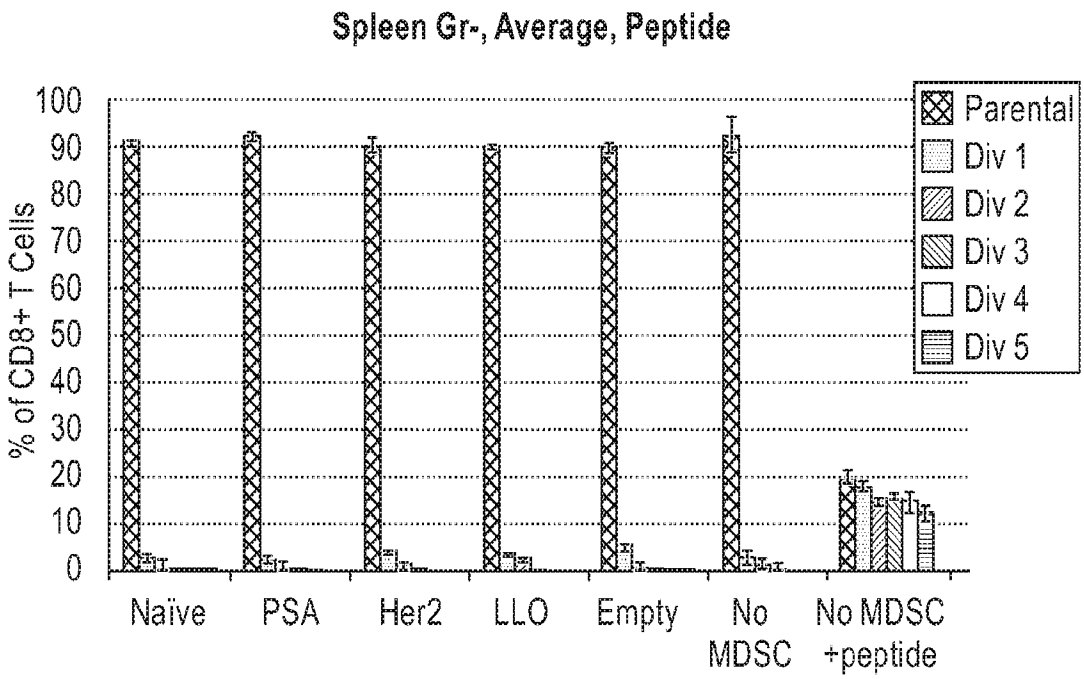


Figure 28A

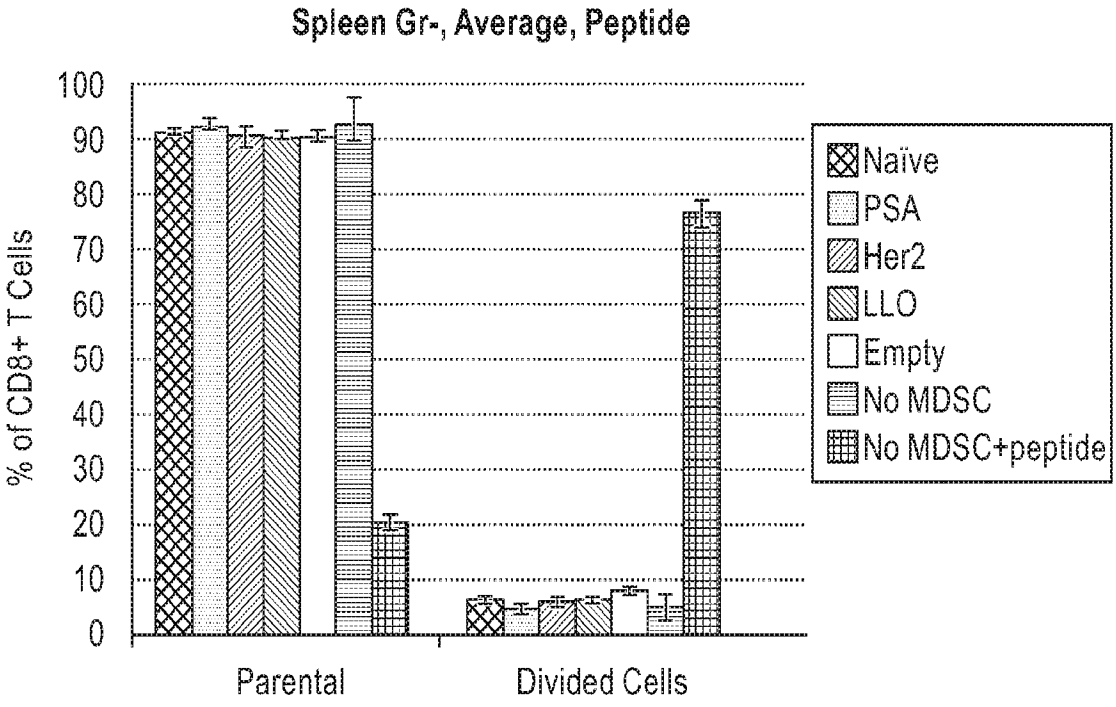


Figure 28B

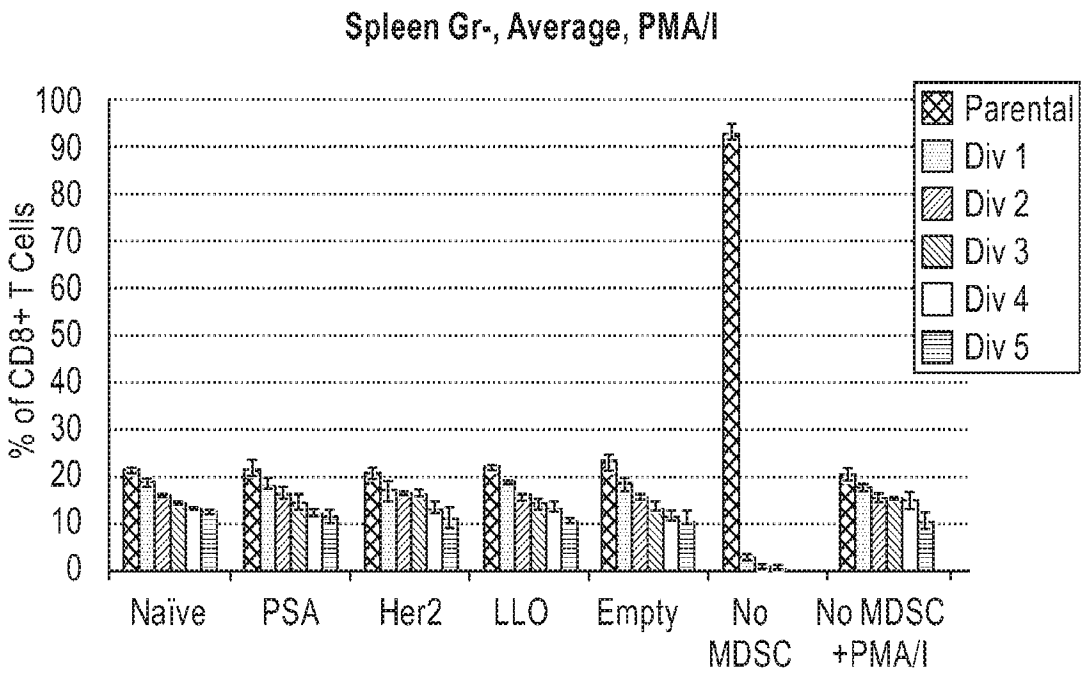


Figure 28C

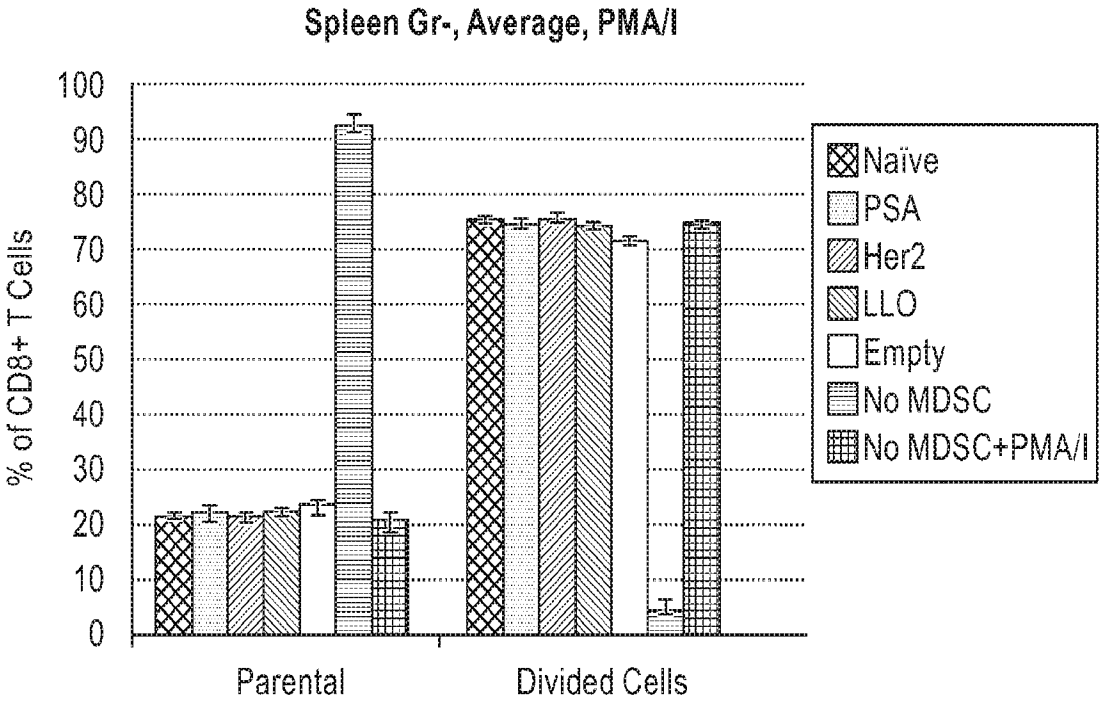


Figure 28D

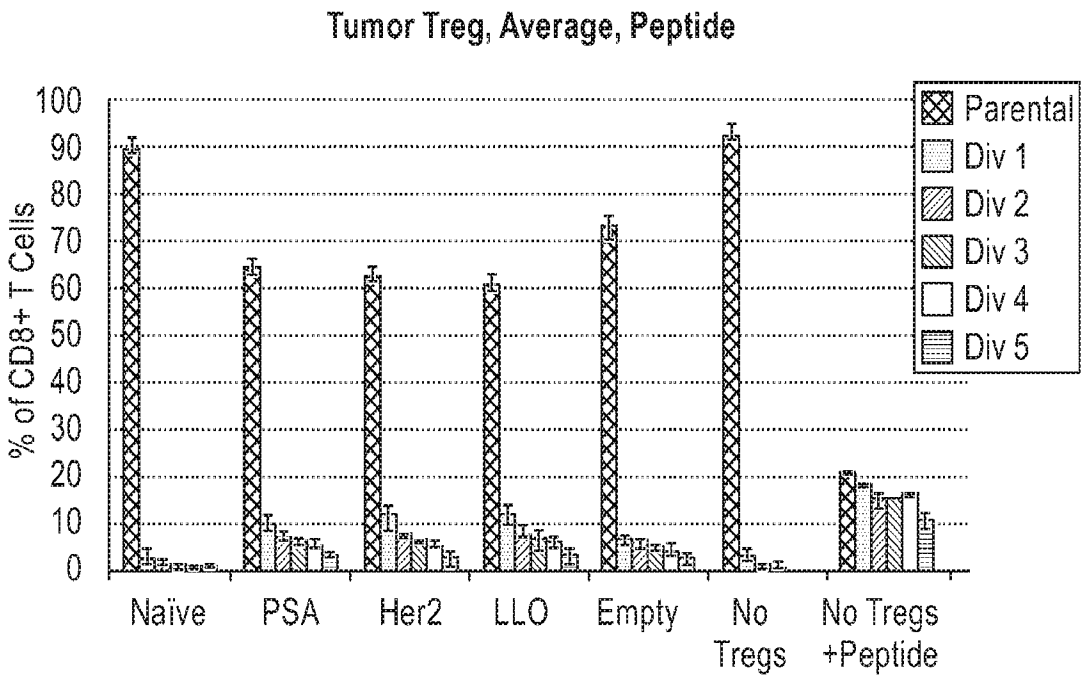


Figure 29A

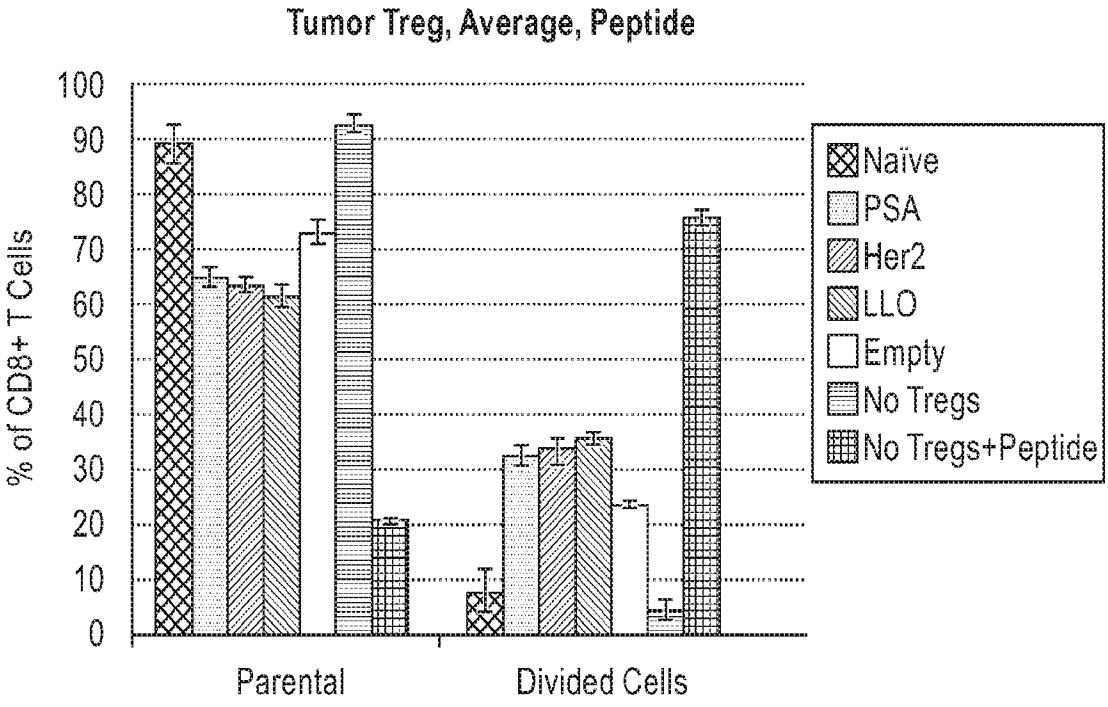


Figure 29B

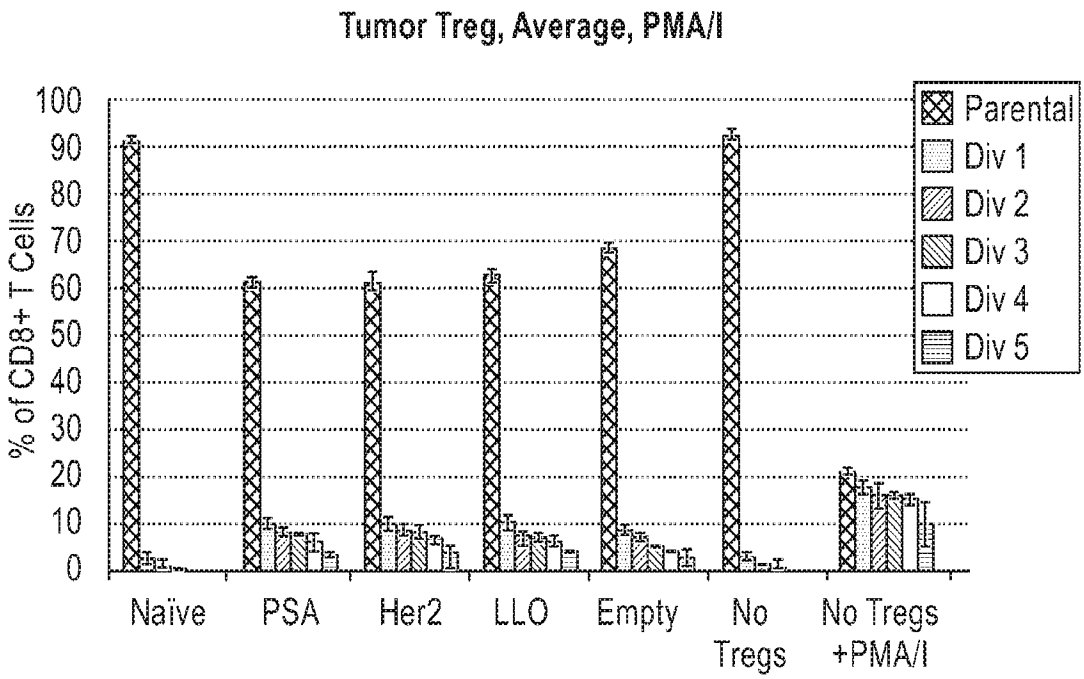


Figure 29C

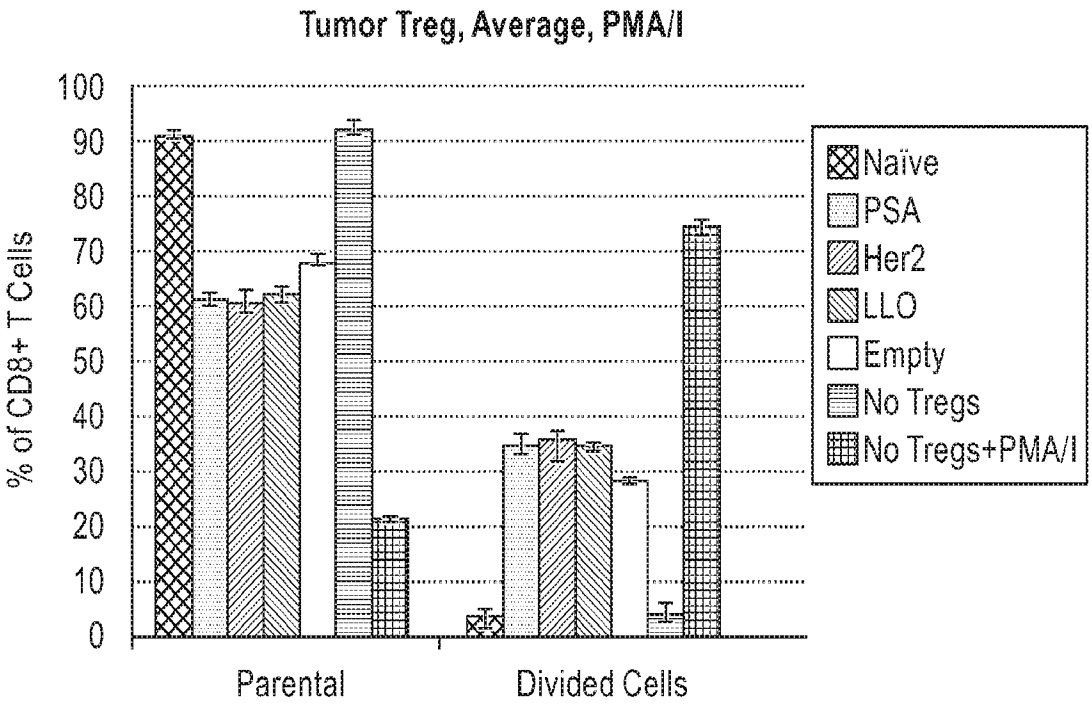


Figure 29D

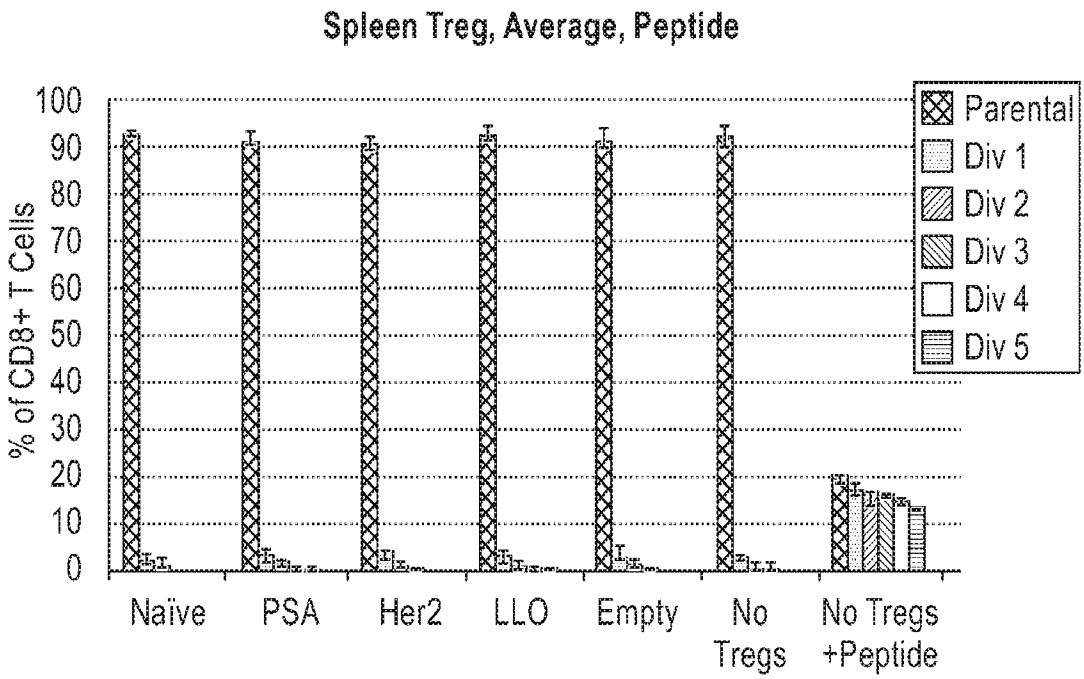


Figure 30A

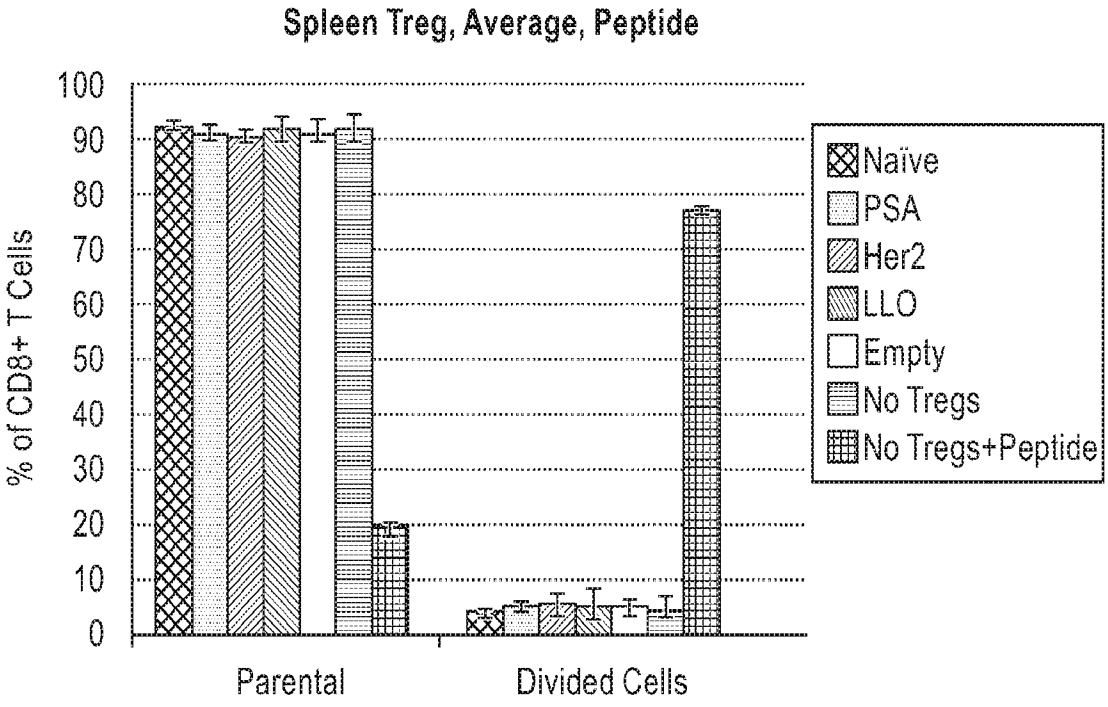


Figure 30B

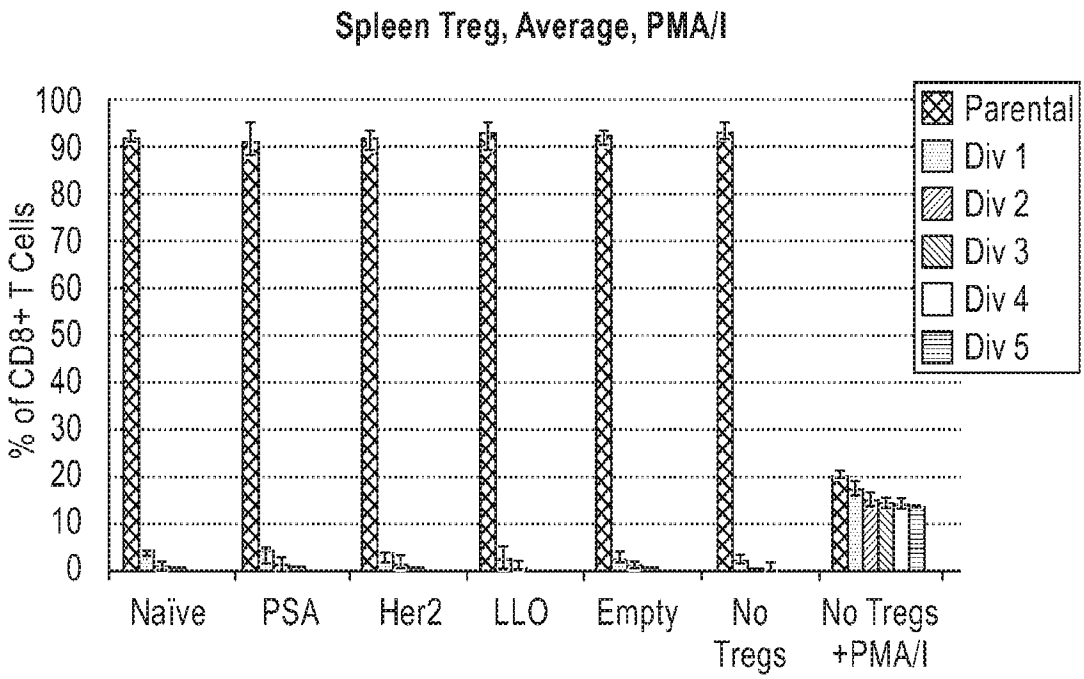


Figure 30C

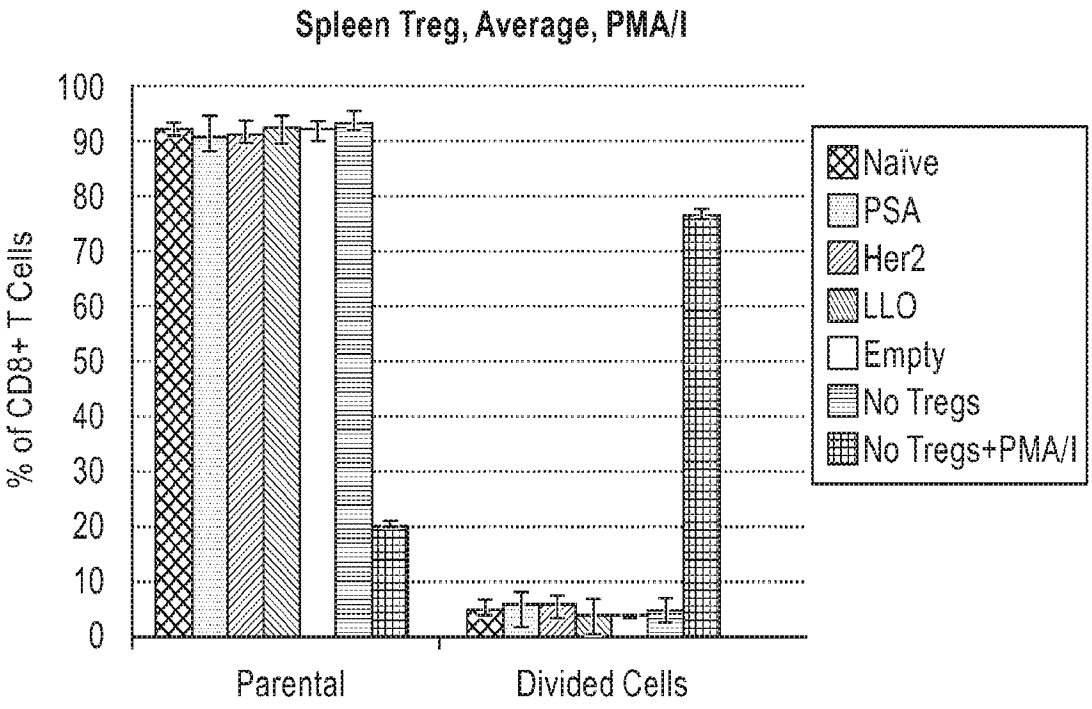


Figure 30D

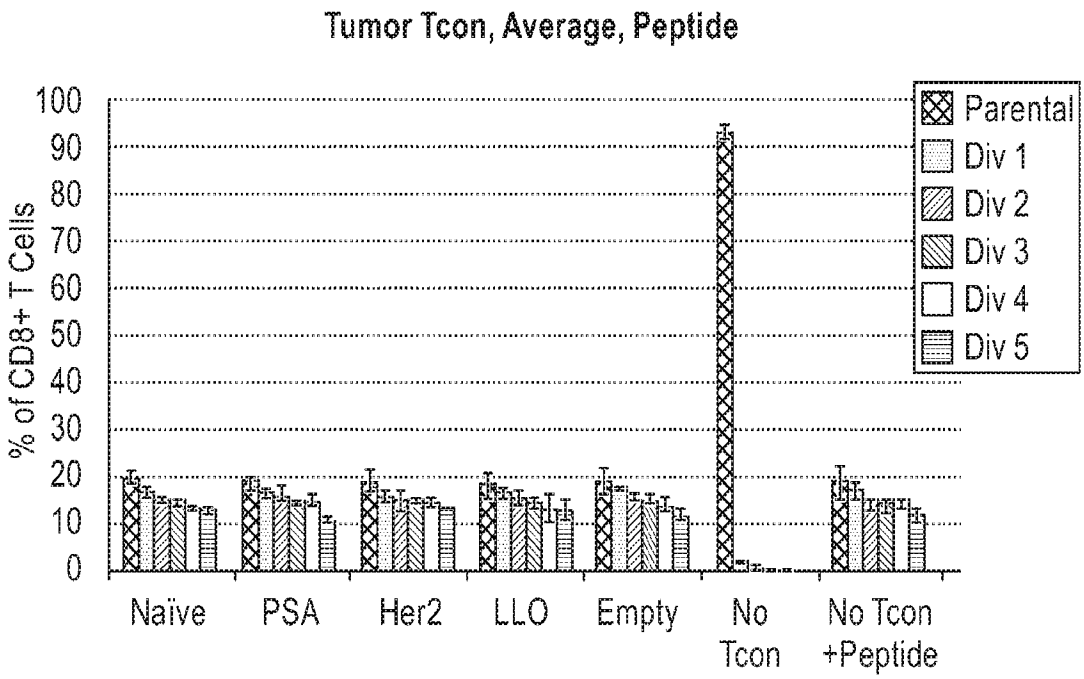


Figure 31A

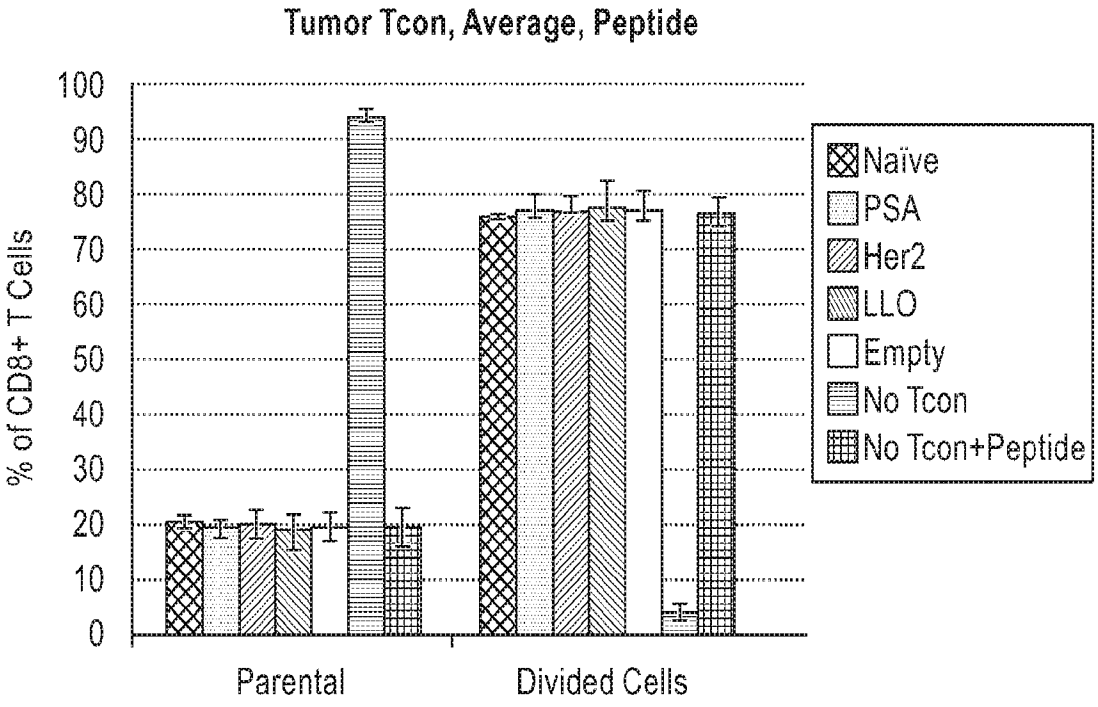


Figure 31B

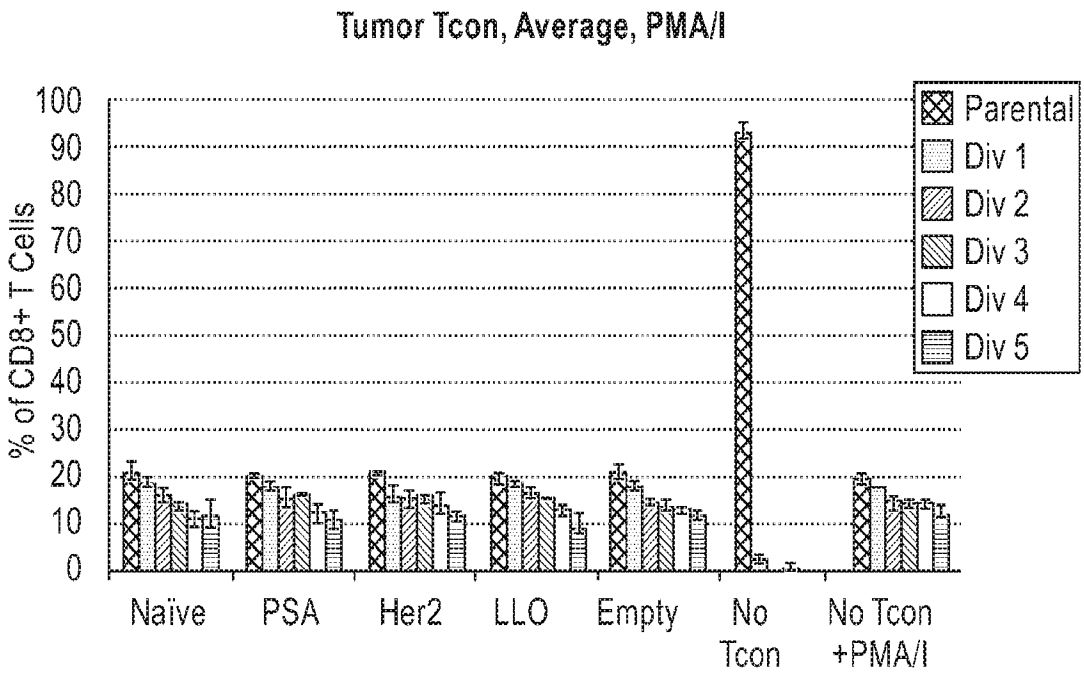


Figure 31C

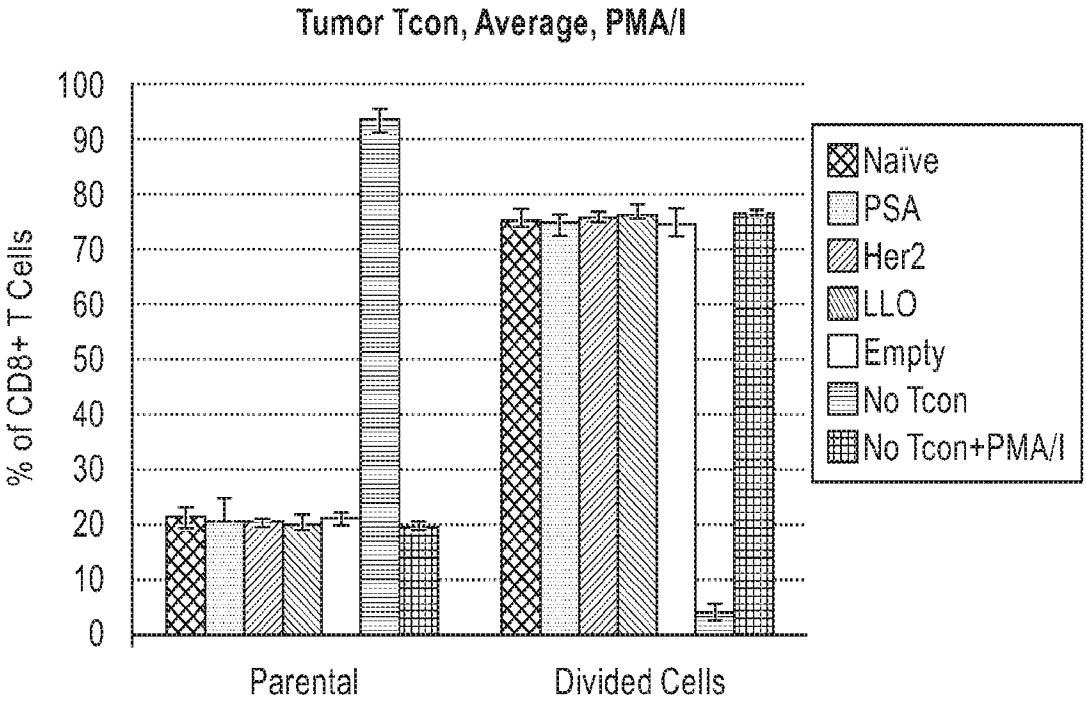


Figure 31D

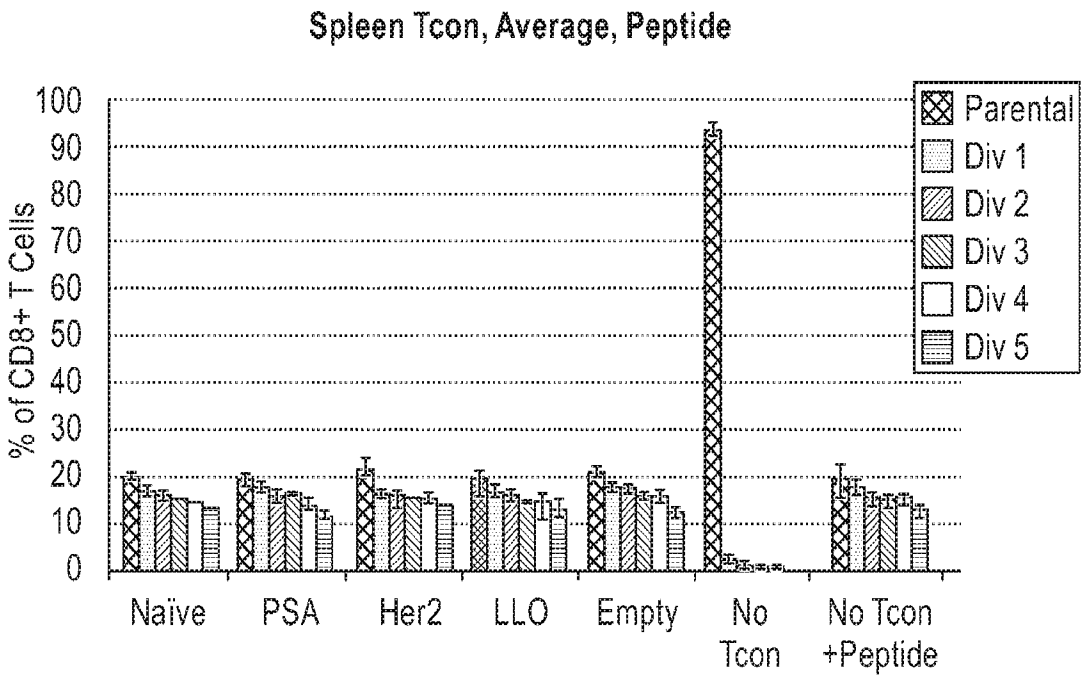


Figure 32A

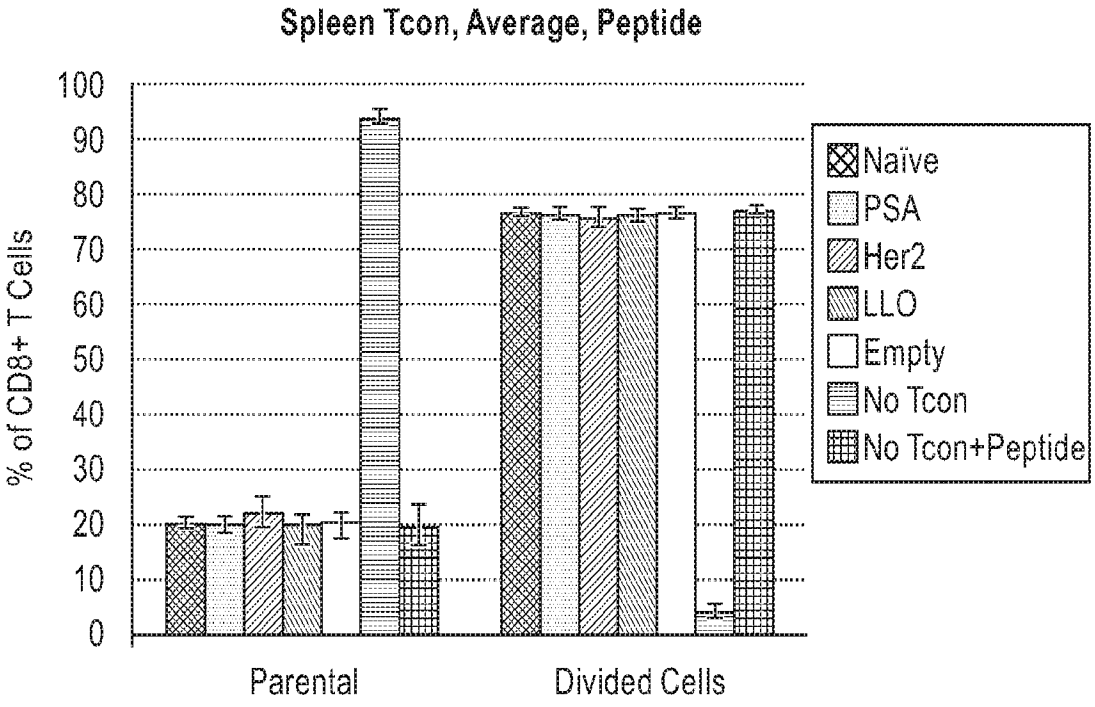


Figure 32B

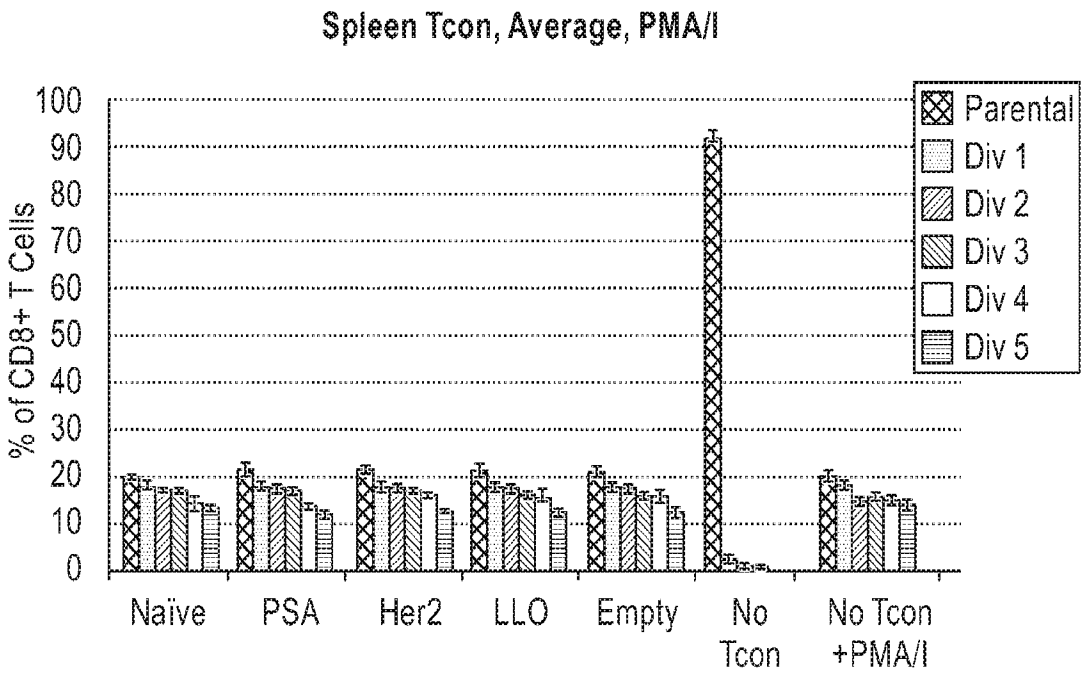


Figure 32C

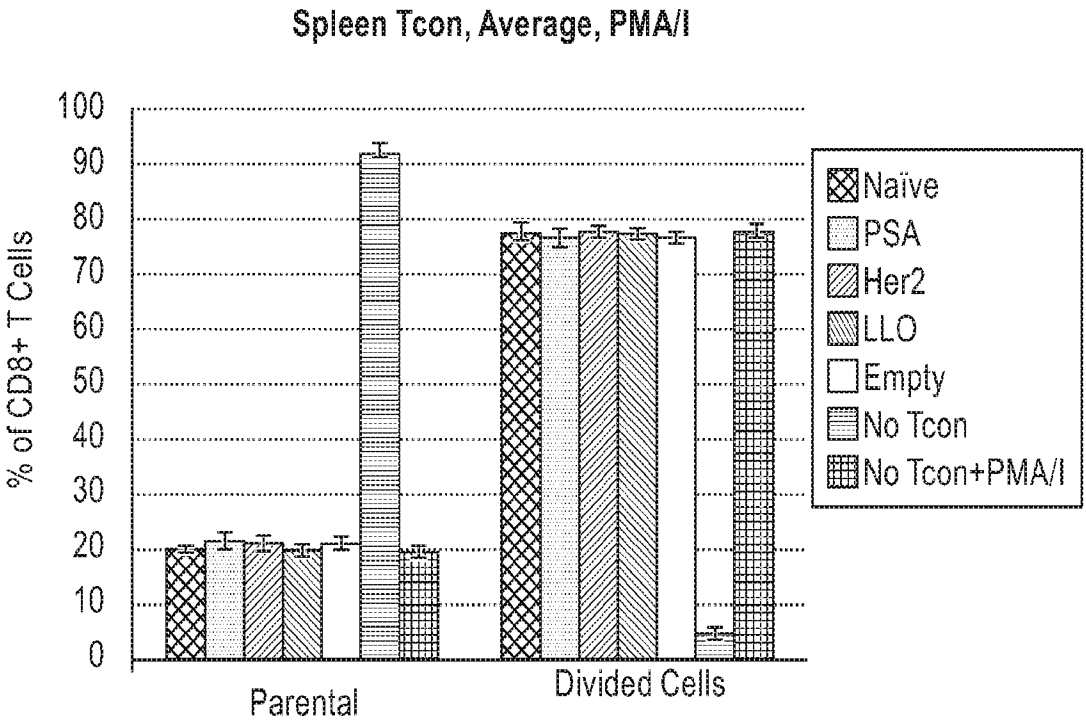


Figure 32D

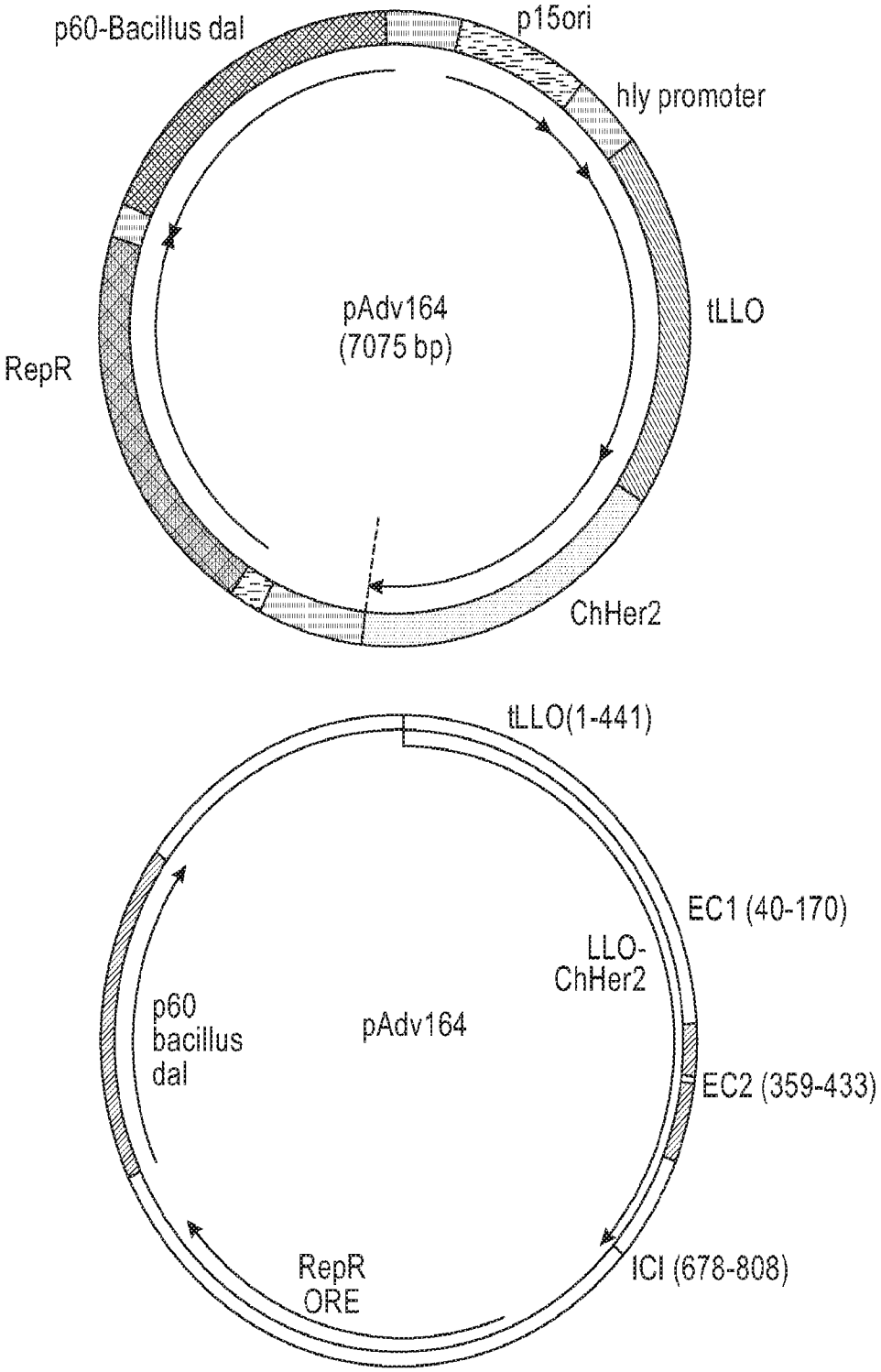

p60-Bacillus dal
p15ori
hly promoter
tLLO
pAdv164
(7075 bp)
RepR
ChHer2
tLLO(1-441)
EC1 (40-170)
LLO-
ChHer2
p60
bacillus
dal
pAdv164
EC2 (359-433)
RepR
ORE
ICI (678-808)

Figure 33A

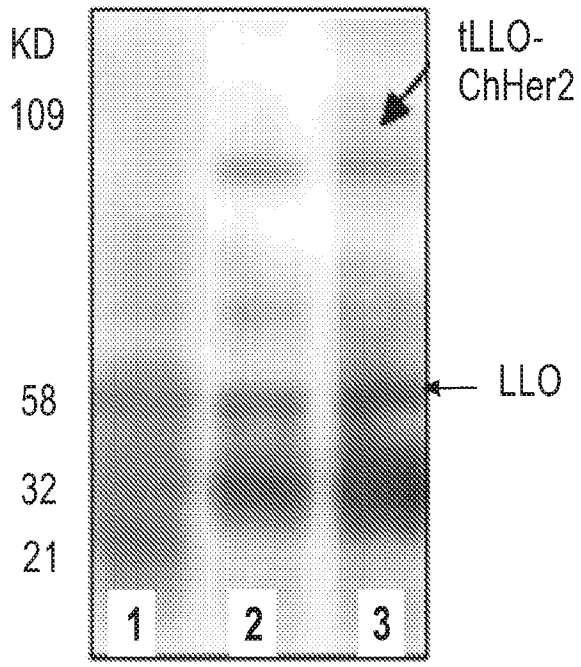

KD
109
58
32
21
tLLO-
ChHer2
LLO
1
2
3
1-Negative Lm-control
2-Lm-LLO-ChHer2
3-ADXS31-164

Figure 33B

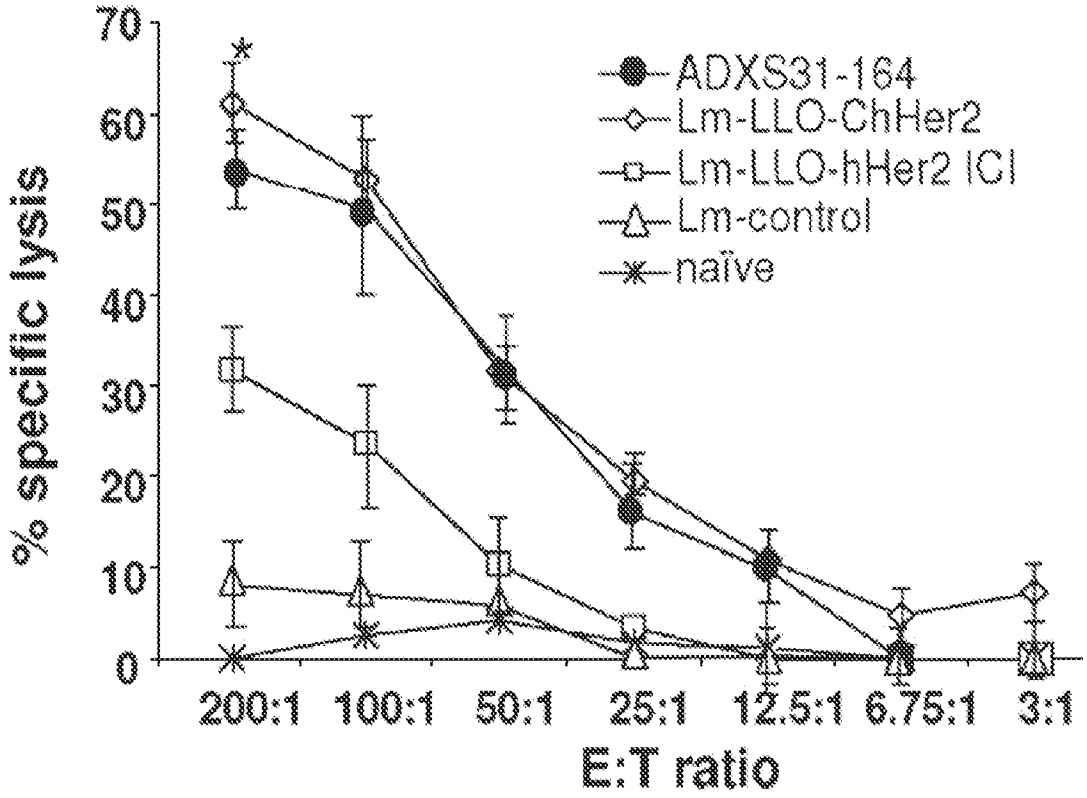
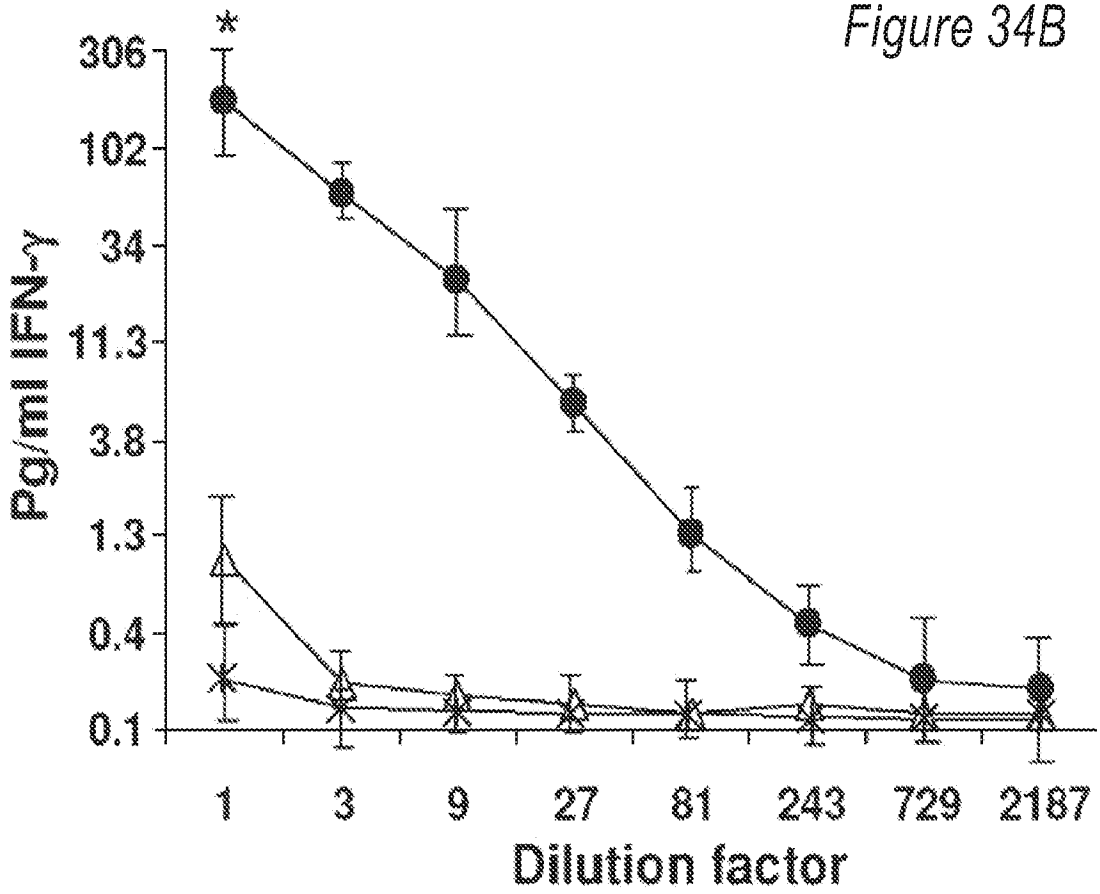


Figure 34A

Figure 34B



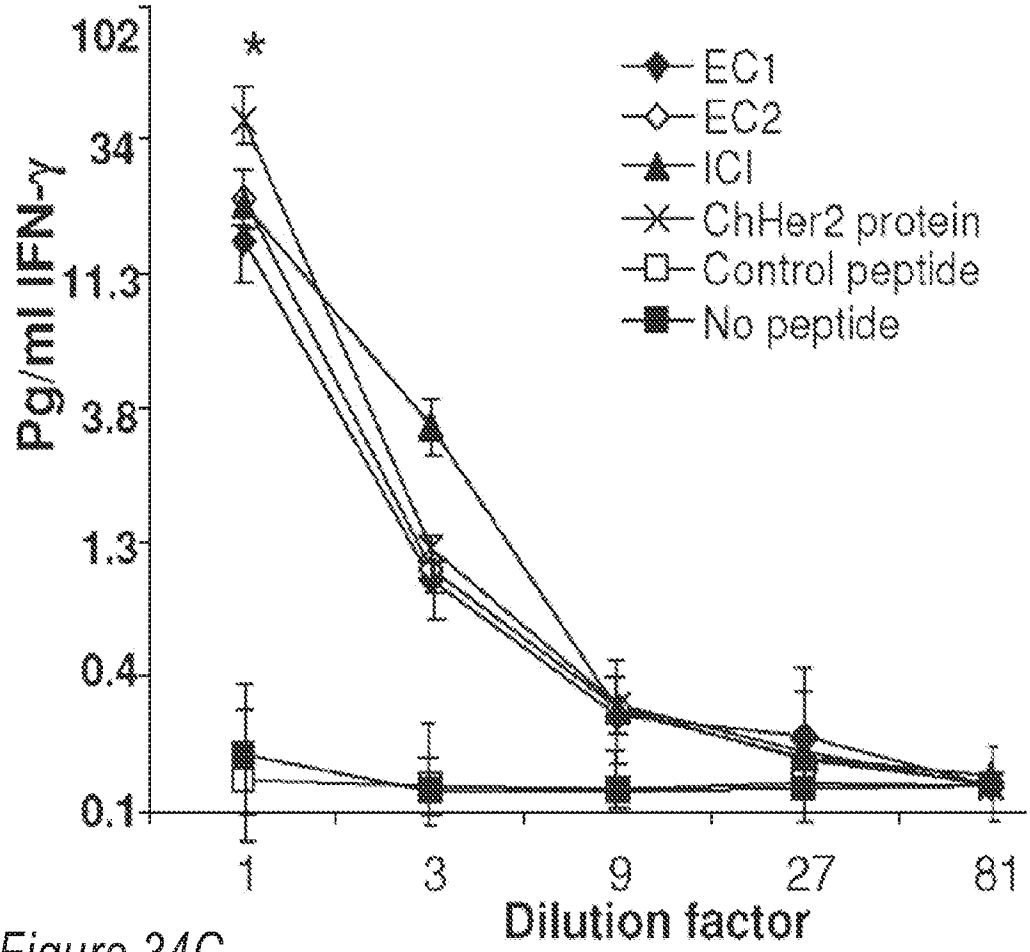


Figure 34C

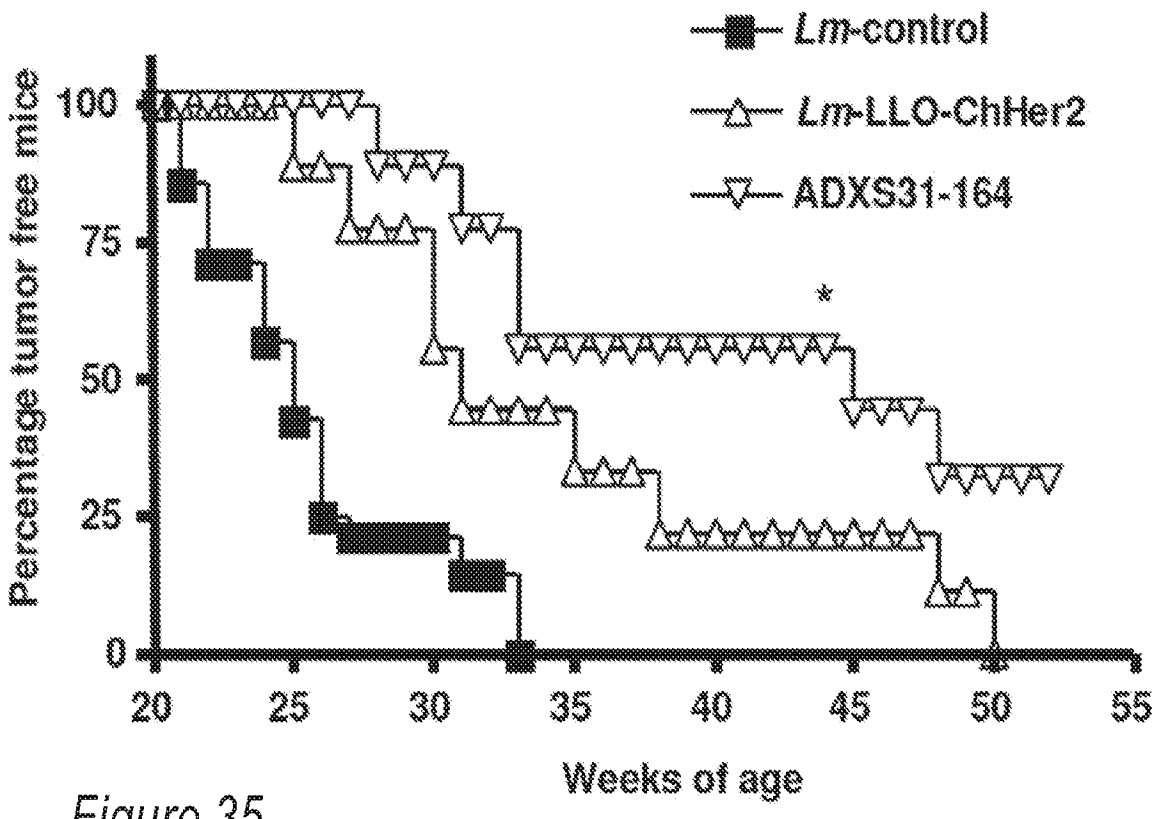


Figure 35

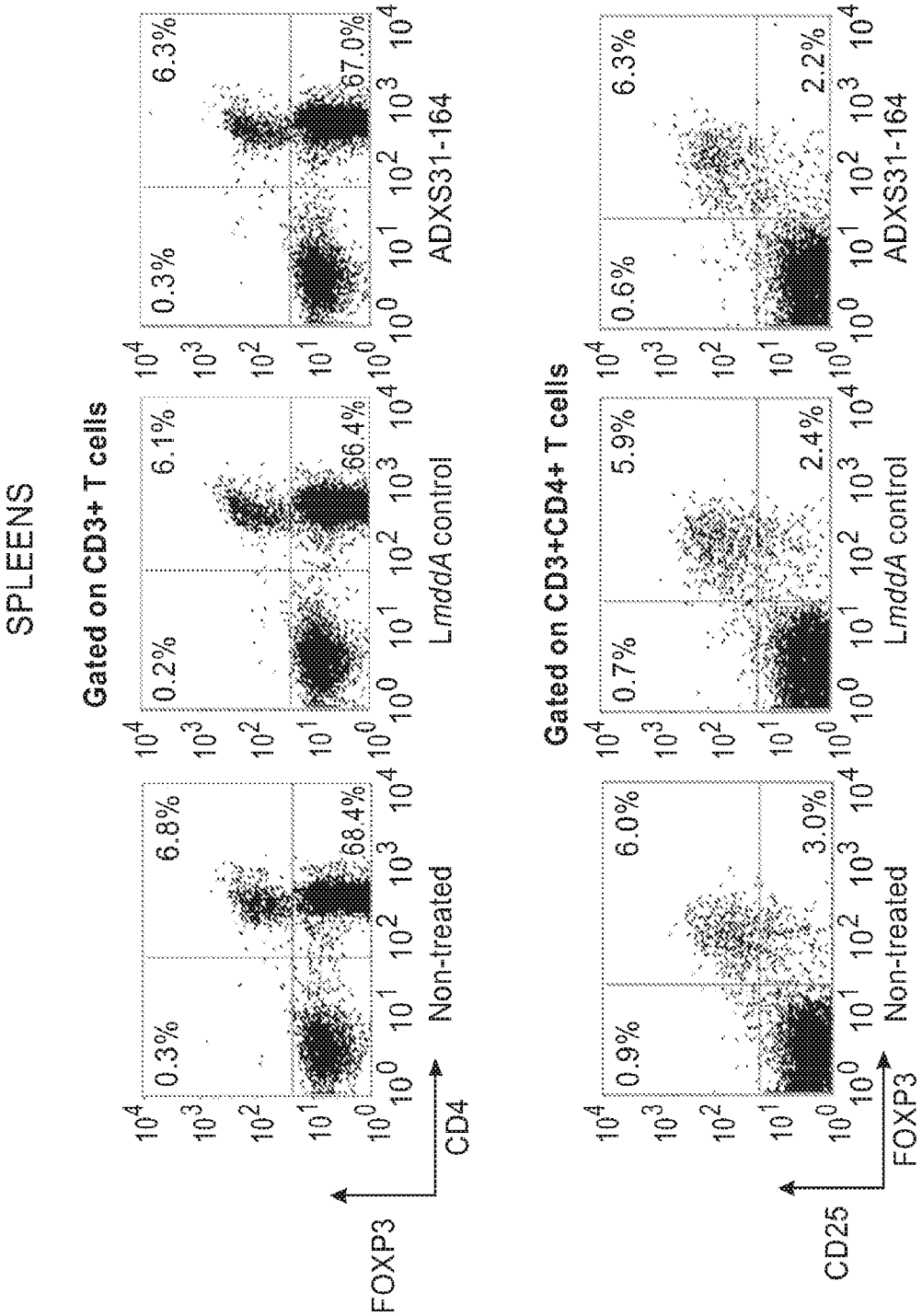

SPLEENS
Gated on CD3+ T cells
Gated on CD3+CD4+ T cells
0.3%
6.8%
68.4%
0.2%
6.1%
66.4%
0.3%
6.3%
67.0%
0.9%
6.0%
3.0%
0.7%
5.9%
2.4%
0.6%
6.3%
2.2%
FOXP3
CD4
CD25
FOXP3
Non-treated
LmddA control
ADXS31-164

Figure 36

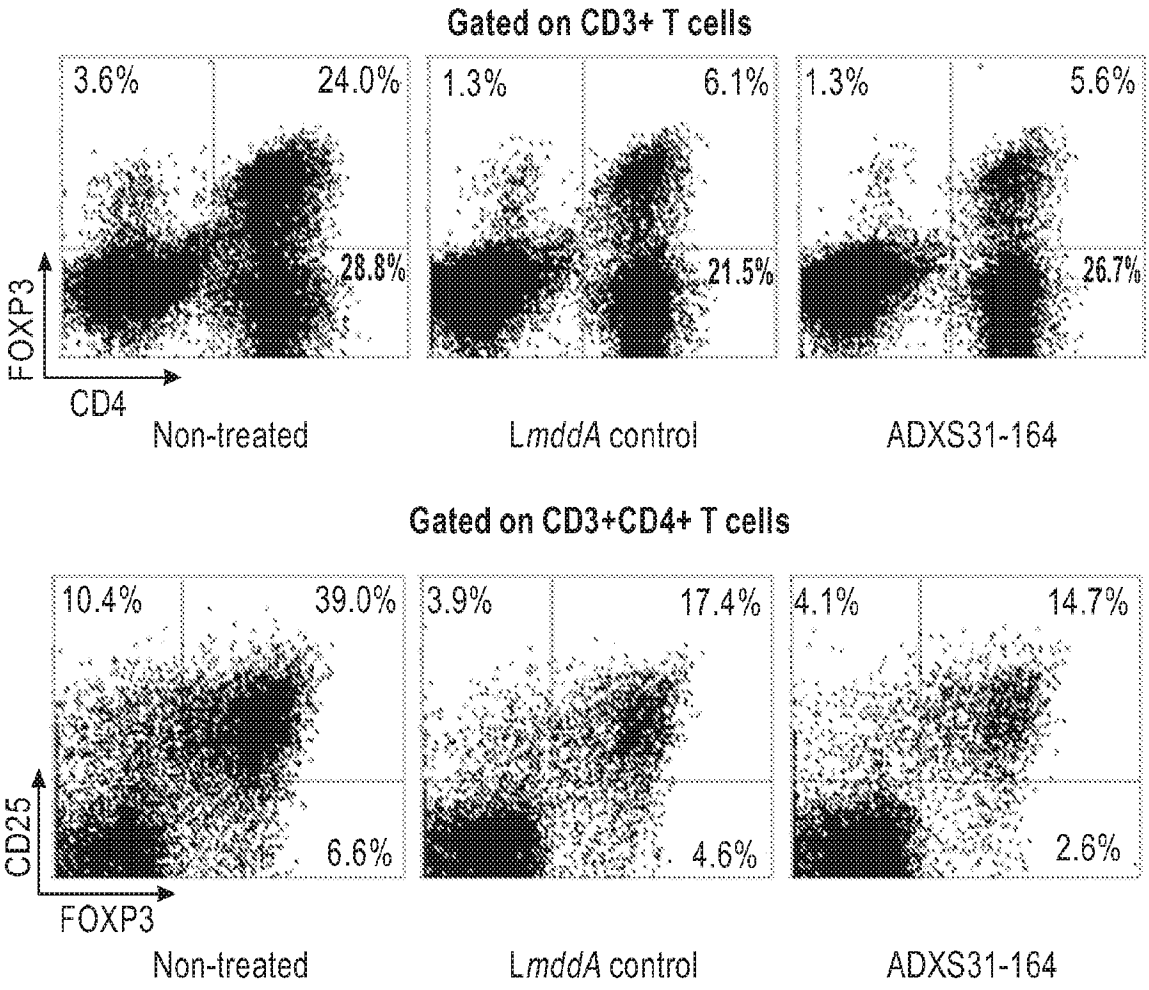

Gated on CD3+ T cells
3.6%
24.0%
28.8%
1.3%
6.1%
21.5%
1.3%
5.6%
26.7%
FOXP3
CD4
Non-treated
LmddA control
ADXS31-164
Gated on CD3+CD4+ T cells
10.4%
39.0%
6.6%
3.9%
17.4%
4.6%
4.1%
14.7%
2.6%
CD25
FOXP3
Non-treated
LmddA control
ADXS31-164

Figure 37A

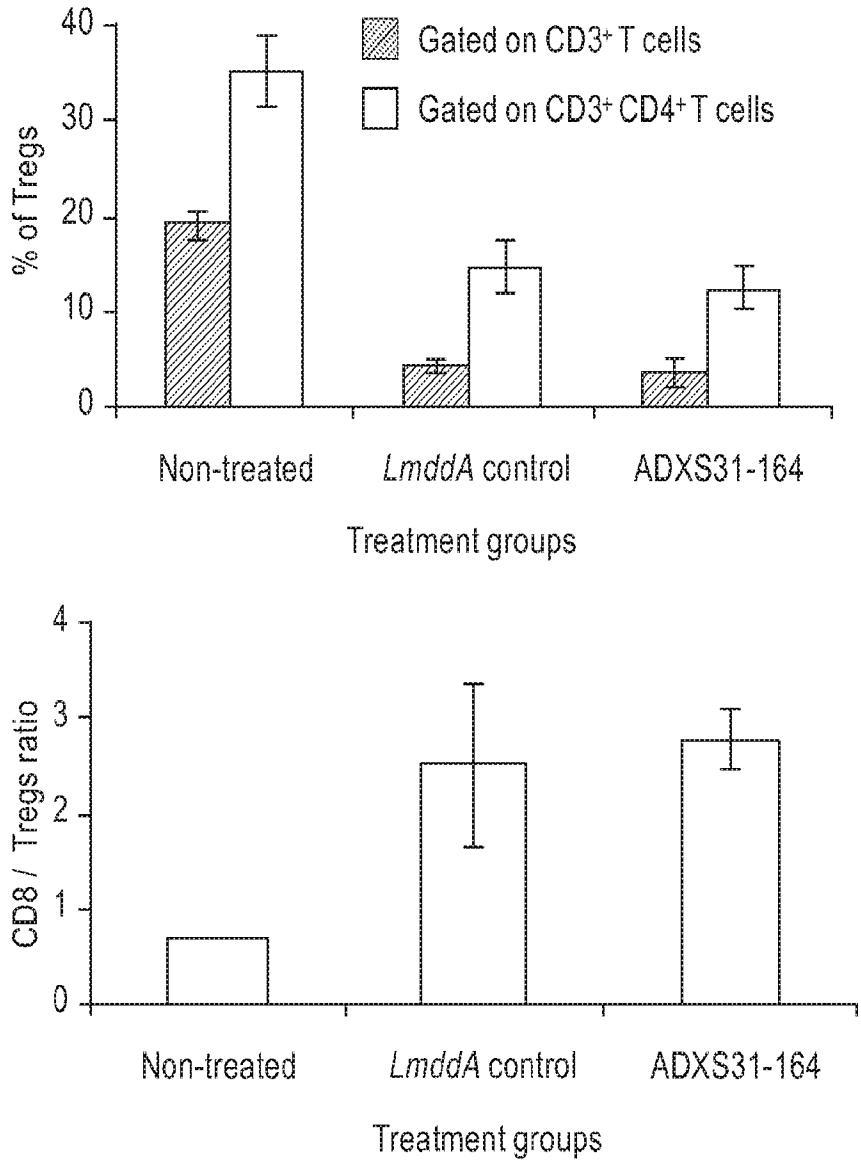

40
30
20
10
0
% of Tregs
Gated on CD3+ T cells
Gated on CD3+ CD4+ T cells
Non-treated
LmddA control
ADXS31-164
Treatment groups
4
3
2
1
0
CD8 / Tregs ratio
Non-treated
LmddA control
ADXS31-164
Treatment groups

Figure 37B

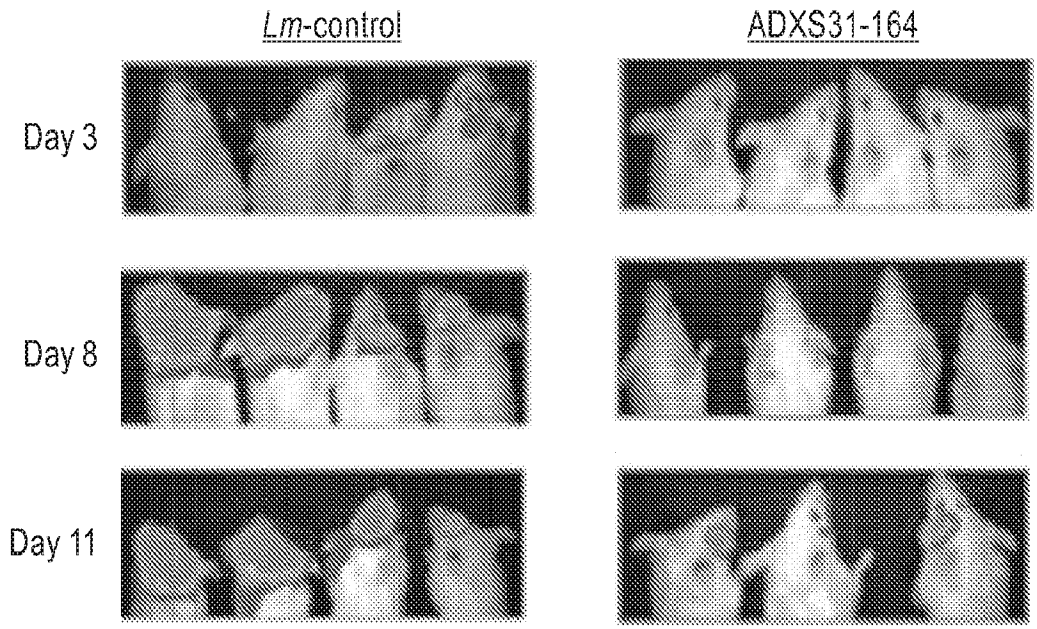


Figure 38A

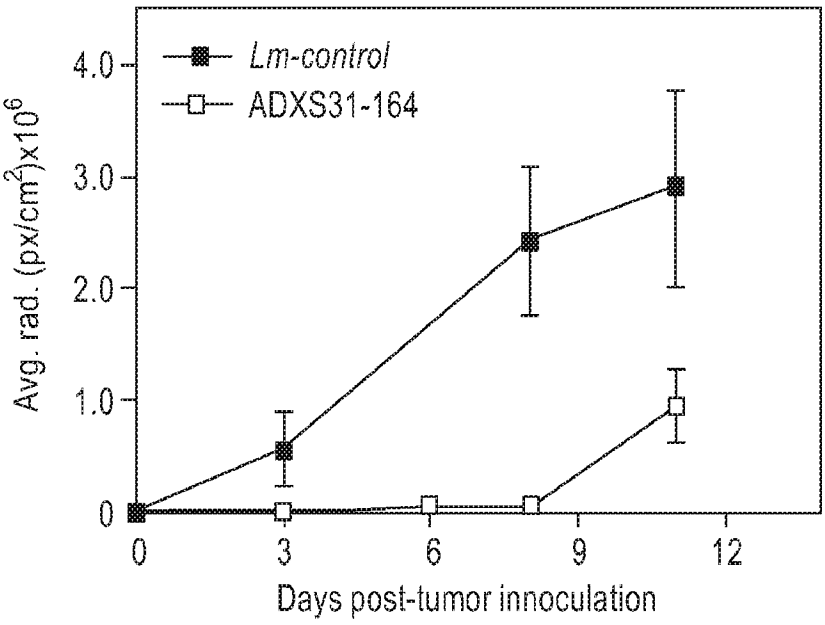


Figure 38B

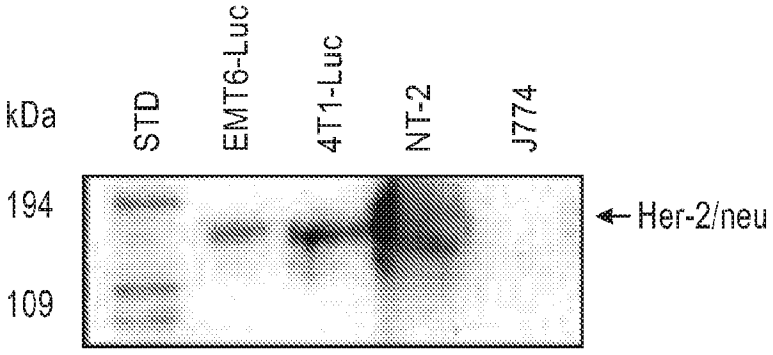


Figure 38C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066885

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/70(2006.01)i, C12N 15/62(2006.01)i, C07K 14/195(2006.01)i, C07K 14/47(2006.01)i, C07K 14/025(2006.01)i, C07K 14/705(2006.01)i, C07K 16/28(2006.01)i, A61K 39/00(2006.01)i, A61K 39/02(2006.01)i, A61K 39/39(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/70; A61K 39/02; A01N 65/00; A61P 35/00; A61K 47/48; C12N 15/62; C07K 14/195; C07K 14/47; C07K 14/025; C07K 14/705; C07K 16/28; A61K 39/00; A61K 39/39

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: Listeria, fusion polypeptide, truncated LLO, ActA, PEST, antigen, oncolytic virus, chimeric antigen receptor engineered T cell, antibody, thymidine kinase inhibitor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MKRTICHYAN, MIKAYEL et al., 'Anti-PD-1 antibody significantly increases the rapeutic efficacy of Listeria monocytogenes (Lm)-LLO immunotherapy', Journal for Immunotherapy of Cancer, 29 August 2013, Vol. 1, Article No. 15, Internal pp. 1-9 See abstract; page 2, left column, paragraph 1; page 2, left column, paragraph 6 - right column, paragraph 1.	1-2,5
Y		3-4,6-13
Y	WO 2014-047350 A1 (MORNINGSIDE TECHNOLOGY VENTURES LTD.) 27 March 2014 See abstract; paragraph [0047]; claims 1 and 3-4.	3-4
Y	CARTELLIERI, MARC et al., 'Chimeric antigen receptor-engineered T Cells for immunotherapy of cancer', Journal of Biomedicine & Biotechnology, Epub. 5 May 2010, Vol. 2010, Article ID 956304, pp. 1-13 See abstract; figures 1-2.	6-9,11-12
Y	US 2014-0356930 A1 (PANACEA PHARMACEUTICALS) 4 December 2014 See abstract; paragraph [0026]; claim 1.	10

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 2016 (11.04.2016)	**12 April 2016 (12.04.2016)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea
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Authorized officer
HEO, Joo Hyung
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066885

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WIERNIK, PETER H. et al., 'Survival more than 19 years after the diagnosis of accelerated phase of chronic myelocytic leukemia', Clinical Advances in Hematology & Oncology, March 2011, Vol. 9, No. 3, pp. 242-248 See abstract; table 3.	13
A	US 2011-0223187 A1 (SHAHABI, VAFA et al.) 15 September 2011 See abstract; claims 1-4 and 14-15.	1-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066885

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

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

1. ☒ Claims Nos.: 39-87
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 39-87 pertain to a method for treatment of the human body by therapy or surgery, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

2. ☒ Claims Nos.: 16-17,19-26,28-29,33-35,37,54-55,57-64,66-67,71-73,75,82
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 16-17, 19-26, 28-29, 33-35, 37, 54-55, 57-64, 66-67, 71-73, 75 and 82 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 14-15,18,27,30-32,36,38,52-53,56,65,68-70,74,76-81,83-87
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/066885

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014-047350 A1	27/03/2014	US 2015-0250837 A1	10/09/2015
US 2014-0356930 A1	04/12/2014	WO 2014-197350 A2	11/12/2014
		WO 2014-197350 A3	05/11/2015
US 2011-0223187 A1	15/09/2011	WO 2011-100754 A1	18/08/2011