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(54) Title: METHODS OF USING IL-2/CD25 FUSION PROTEIN

(57) Abstract: The present disclosure relates to a methods of using an IL-2/CD25 fusion protein in combination with at least one neo-antigen for promoting anti-tumor immunity in a subject of need. The disclosure further provides a method of treating cancer. The method comprises administering to mammalian subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.



WO 2020/146773 A1

METHODS OF USING IL-2/CD25 FUSION PROTEIN

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/791,713, filed on January 11, 2019, the entire contents of which is fully incorporated herein by reference.

STATEMENT OF FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant number R21CA195334 awarded by the National Institute of Health. The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] Incorporated by reference in its entirety is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: 53839_Seqlisting.txt; Size: 153,694_bytes; Created: January 10, 2020.

FIELD OF THE INVENTION

[0004] The present disclosure relates to a method of using an IL-2/CD25 fusion protein in combination with at least one neo-antigen.

BACKGROUND

[0005] Immunotherapy of cancer by checkpoint blockade results in a substantial remission in approximately 50% of patients (1). This therapy depends on releasing anti-tumor T cells from their non-responsive state through stimulation of immune checkpoints, such as CTLA4 or PD-1 (2,3). This therapy points to the critical role tumor-specific T cells play in responding to and eliminating tumor cells. However, many patients still do not benefit from this type of immunotherapy. One prerequisite for successful immunotherapy by checkpoint blockade is a sufficient number of pre-existing tumor-specific T cells that respond to checkpoint blockade to become activated and reject the tumor. However, for a number of reasons, e.g., the immunosuppressive environment of the tumor or the lack to tumor-specific antigens, an immune response against the tumor may not occur. Thus, strategies are required to induce tumor-specific T cells to directly eliminate tumors or increase the efficacy of checkpoint blockade.

SUMMARY

[0006] The disclosure provides methods of using an IL-2/CD25 fusion protein in combination with at least one neo-antigen for promoting anti-tumor immunity in a subject in need thereof.

[0007] In various aspects, the disclosure provides a method of increasing neo-antigen tumor-specific T cells in a mammalian subject. The method comprises administering to a subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen. In various aspects, the disclosure provides a method of enhancing immunogenicity to tumor neo-antigens in a mammalian subject. The method comprises administering to the subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen. In related aspects, the disclosure provides a method of enhancing tumor-specific T cell response in a mammalian subject. The method comprises administering to the subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen. In various aspects, the disclosure provides a method of treating cancer, the method comprises administering to mammalian subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.

[0008] Aspects of the disclosure are defined or summarized in the following numbered paragraphs:

[0009] 1. A method of increasing neo-antigen-specific T cells in a mammalian subject, the method comprising administering to a subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.

[0010] 2. A method of enhancing immunogenicity to a neo-antigen in a mammalian subject, the method comprising administering to the subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.

[0011] 3. The method of paragraph 2, wherein enhancing immunogenicity comprises enhancing cancer-specific T cell response in a mammalian subject.

[0012] 4. A method of treating cancer, the method comprising administering to mammalian subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.

[0013] 5. The method of any one of the preceding paragraphs, wherein administration of one or more doses of neo-antigen is followed by administration of one or more doses of the IL-2/CD25 fusion protein.

[0014] 6. The method of any one of the preceding paragraphs, wherein the IL-2/CD25 fusion protein is delivered within 7 days of administration of the neo-antigen peptide.

[0015] 7. The method of any one of the preceding paragraphs, wherein a second dose of the IL-2/CD25 fusion protein is delivered within 7 days after administration of the neo-antigen peptide.

[0016] 8. The method of any one of the preceding paragraphs, wherein one or more doses of neo-antigen peptide is administered no more than 10 days prior to administration with the IL-2/CD25 fusion protein.

[0017] 9. The method of any one of the preceding paragraphs, wherein the subject is administered a priming dose comprising at least one neo-antigen no more than 10 days prior to administration of the IL-2/CD25 fusion protein.

[0018] 10. The method of any one of the preceding paragraphs, wherein the subject is administered one or more boosting doses comprising at least one neo-antigen 1-30 days after administration of the priming dose comprising at least one neo-antigen.

[0019] 11. The method of any one of the preceding paragraphs, wherein the subject is administered a dose comprising the IL-2/CD25 fusion protein within 7 days of administration of the boosting dose comprising at least one neo-antigen.

[0020] 12. The method of any one of the preceding paragraphs, wherein the subject is administered a second dose comprising the IL-2/CD25 fusion protein within 7 days after administration of the initial dose comprising the IL-2/CD25 fusion protein.

[0021] 13. The method of any one of claims 1-4, wherein the subject is administered a second dose comprising the IL-2/CD25 fusion protein within 10 days after administration of the initial dose comprising the IL-2/CD25 fusion protein.

[0022] 14. The method of any one of paragraphs 1-4 further comprising the steps of (a) administering a priming dose comprising at least one neo-antigen; (b) administering a first boosting dose comprising at least one of the neo-antigen(s) 1-30 days after step (a); (c)

administering a first dose comprising the IL-2/CD25 fusion protein within 7 days after step (b); (d) administering a second or more boosting doses comprising at least one neo-antigen(s) within 30 days after step (c); and (e) administering a second dose comprising the IL-2/CD25 fusion protein within 7 days after step (d).

[0023] 15. The method of any one of the preceding paragraphs, wherein the IL-2/CD25 fusion protein comprises (a) a first polypeptide comprising Interleukin-2 (IL-2) or a functional variant or fragment thereof and (b) a second polypeptide, comprising CD25 or a functional variant or fragment thereof, fused in frame to said first polypeptide, wherein said fusion protein has IL-2 activity.

[0024] 16. The method of any one of paragraphs 1-15, wherein the administration step comprises administering to the subject a neo-antigen peptide.

[0025] 17. The method of any one of paragraphs 1-15, wherein the administration step comprises administering to the subject a nucleic acid molecule that encodes the neo-antigen peptide.

[0026] 18. The method of any one of paragraphs 1-15, wherein the administration step comprises administering to the subject a vector comprising a nucleic acid molecule that encodes the neo-antigen peptide.

[0027] 19. The method of any one of the preceding paragraphs, wherein the neo-antigen comprises a mutation specific to a tumor of the subject.

[0028] 20. The method of any one of the preceding paragraphs, wherein the neo-antigen is administered with an adjuvant.

[0029] 21. The method of paragraph 20, wherein the adjuvant is Polyinosinic:polycytidylic acid (poly-IC), MPL, GLA, imiquimod, CpG ODN, LPS, Polyinosinic-Polycytidylic Acid stabilized with Polylysine and Carboxymethylcellulose (poly-IC LC) gardiquimod, aluminum, resiquimod, sodn-dsRNA, flagellin, or SMP-105.

[0030] 22. The method of paragraph 20, wherein the adjuvant is STING agonists or liposomes.

[0031] 23. The method of any one of the preceding paragraphs, wherein administration of the IL-2/CD25 fusion protein increases the number of CD4⁺ and CD8⁺ T effector and memory cells.

[0032] 24. The method of any one of the preceding paragraphs, wherein the IL-2/CD25 fusion protein is administered in combination with one or more checkpoint inhibitors.

[0033] 25. The method of paragraph 24, wherein the checkpoint inhibitor is selected from the group consisting of an anti-PD-1 antibody, an anti-PDL-1 antibody, an anti-CTLA-4 antibody, a PD-1 inhibitor, a PDL-1 inhibitor or a CTLA-4 inhibitor, anti-Tim-3 antibody, anti-LAG3 antibody, and anti-TIGIT antibody.

[0034] 26. The method of any one of the preceding paragraphs, wherein said mammalian subject is a human.

[0035] 27. The method of any one of the preceding paragraphs, wherein said subject is suffering from cancer.

[0036] 28. The method of paragraph 27, wherein the cancer is melanoma, cutaneous melanoma, ocular melanoma, cervical cancer, follicular B cell non-Hodgkin's lymphoma, kidney cancer, prostate cancer, and multiple myeloma, breast cancer, lung cancer, colon cancer, ovarian cancer, bladder cancer, pancreatic cancer, endometrial cancer, liver cancer, thyroid cancer, or leukemia.

[0037] It is understood that each aspect, feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the invention and, as such, is meant to be combinable with any other aspect, feature or embodiment, or combination, described herein. For example, where features are described with language such as “one aspect,” “some aspects,” “various aspects,” “related aspects,” each of these types of aspects of the disclosure is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination. Such features or combinations of features apply to any of the aspects of the invention.

[0038] The headings herein are for the convenience of the reader and not intended to be limiting. Additional aspects, embodiments, and variations of the disclosure will be apparent from the Detailed Description and/or drawings and/or claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] **Figures 1A-1C** demonstrate that IL-2/CD25 amplifies the response of CD8⁺ Pmel-1 T cells against a tumor-related antigen. C57BL/6 mice adoptively transferred with Pmel-1 T cells were immunized with hgp100 and LPS and mouse IL-2/CD25 fusion protein (mIL-2/mCD25), as shown. Pmel-1 T cells were identified by expression of Thy-1.1. Mice were bled as indicated and Pmel-1 T cells were enumerated in the PBMCs.

[0040] **Figures 2A-2C** demonstrate that IL-2/CD25 amplifies CD8⁺ T memory cells. The figure illustrates the distribution of memory subsets of Pmel-1 T cells in the PBMCs from mice.

[0041] **Figures 3A-3D** demonstrate that IL-2/CD25 amplifies the response of TRP-1 CD4⁺ T cells against a tumor-related antigen. C57BL/6^{CD45.1} congenic were adoptively transferred with TRP-1 T cells and immunized and treated with mIL-2/mCD25, as shown. TRP-1 T cells were enumerated in the PBMCs.

[0042] **Figures 4A-4C** show the effect of IL-2/CD25-induced memory Pmel-1 cells reject the B16 melanoma. Sixty five days after vaccination with or without IL-2/CD25, mice were challenged with B16.F10 s.c. Tumor growth was monitored over time.

[0043] **Figures 5A-5F** show that IL-2/CD25 promotes a favorable ratio of CD8⁺ tumor-specific T cells in the tumor microenvironment. For the experiment described in Fig. 4, tumor growth was monitored to approximately 500 mm³. The tumors were excised and T cells within the tumor were enumerated by FACS. **Figures 5G-5K** show that IL-2/CD25 increases the number of polyclonal CD4⁺ and CD8⁺ T cells and NK cells in the tumor microenvironment while the number of B cells and myeloid lineage cells are unaffected.

[0044] **Figures 6A-6E** show that IL-2/CD25-amplified Pmel-1 cells reject pre-existing B16 melanoma. The experimental design is shown in **Figure 6A**. **Figures 6A-6C**: Mice were inoculated with B16.F10 and 3 days later were immunized with hgp100 and LPS, with and without IL-2/CD25. Tumor growth was monitored over time. Initial experiments (**Figures 6B-6C**) showed no anti-tumor activity with neoantigen treatment alone. **Figures 6D-6E**: Subsequent experiments showed that mice immunized with hgp100 and LPS generate anti-tumor responses. All experiments showed enhanced anti-tumor responses when IL-2/CD25 was administered with hgp100 and LPS.

[0045] **Figure 7** describes mutated B16.F10 melanoma peptides for inducing CD4⁺ and CD8⁺ tumor-specific T cells. The peptides exhibit the following properties: 27 amino acid (aa) long synthetic peptides, not expressed in healthy mouse tissue, and MHC class I and II restricted with moderate binding capacity. The reactive T cell subtype response after vaccination was obtained with either mutated RNA or peptide sequence as indicated (15).

[0046] **Figures 8A-8E** show the primed and boost immunization scheme using a mixture of 4 neo-antigens from B16.F10 and IL-2/CD25 to induce anti-neo-peptide specific T cell responses. The days (D) when the peptides and Poly (I:C) (MP) and mIL-2/mCD25 were administered are indicated. On day 12, spleen cells were cultured with a mixture of neo-antigen peptides and IFN γ ELISpots were determined 24 hr later. **Figure 8B** shows representative ELISpot results and data from individual mice are shown in **Figure 8C**. **Figure 8E** includes additional replicate determinations. Also, the last four groups on this figure represent additional data compared with **Figure 8C**. These data show a second boost immunization maintains high frequency of tumor-peptide reactive T cells.

[0047] **Figures 9A-9B** show that the tumor-specific IFN γ by CD4⁺ T cells response is amplified by IL-2/CD25. C57BL/6 mice were immunized with neo-antigen peptides and Poly (I:C) (MP) and mIL-2/mCD25 (FP). CD4⁺ and CD8⁺ T cells were isolated using magnetic beads from the spleen cells and IFN γ ELISpots were determined 24 hr later after challenge with a mixture of neo-antigen peptides.

[0048] **Figures 10A-10C** show that IL-2/CD25 amplified neo-antigen-specific T cells protect mice after challenge with B16.F10. C57BL/6 mice were immunized with a mixture of neo-antigen peptides as shown, and then the mice were challenged with B16.F10 subcutaneously (s.c.) 2 days after the boost injection. Tumor growth was measured over time.

[0049] **Figures 11A-11C** show that IL-2/CD25 amplified neo-antigen-specific T cells delay the growth of pre-established B16.F10. C57BL/6 mice were s.c. inoculated with B16.F10 and 3 days later the mice were immunized with a mixture of neo-antigen peptides. Tumor growth was measured over time.

[0050] **Figures 12A-12I** show that IL-2/CD25 and neo-antigen peptide vaccine amplified the T cell and NK response in the tumor microenvironment. C57BL/6 mice were inoculated and immunized as described in Figure 5. On day 15, tumors were excised, and tumor-associated

lymphoid cells were determined, as indicated. **Figures 12J-12R** show that IL-2/CD25 amplifies neo-antigen vaccine responses to B16 tumors. C57BL/6 mice were inoculated and immunized as described in Figure 11. On day 18, tumors were excised, and the frequencies of tumor-associated lymphoid cells were determined, as indicated. **Figures 12J-12R** contain additional replicate determinations compared with **Figures 12A-12I**.

[0051] **Figures 13A-13C** show that IL-2/CD25 and neo-antigen peptide vaccine increased the proportion of activated CD4⁺ and CD8⁺ T effector cells, but reduced the frequency of activated Tregs in the tumor micro-environment. C57BL/6 mice were inoculated and immunized as described in Figure 5. On day 15, tumors were excised and the expression of CD44 was determined for the indicated T cell populations, as indicated.

[0052] **Figures 14A-14L** show the varied effect of IL-2/CD25-amplified tumor-specific T cells on immune checkpoints while granzyme B levels uniformly increase on T cells in the tumor micro-environment. C57BL/6 mice were inoculated and immunized as described in Figure 5. On day 15, tumors were excised and the expression of the indicated molecules was determined for the indicated tumor-associated T cells. **Figures 14M-14X** show the varied effects of IL-2/CD25-amplified tumor-specific T cells on immune checkpoints while granzyme B levels uniformly increase on T cells in the tumor micro-environment. C57BL/6 mice were inoculated and immunized as described in Figure 11. On day 18, tumors were excised, and expression of the indicated molecules was determined for the indicated tumor-associated T cells. **Figures 14M-14X** contain more replicate determinations compared with **Figures 14A-14L**.

[0053] **Figures 15A-15G** show that IL-2/CD25 fusion protein-amplified tumor-specific T cells promote an increase in T effector and memory cells in the tumor micro-environment. C57BL/6 mice were inoculated and immunized as described in Figure 5. On day 15, tumor was excised, and the expression of the indicated molecules was determined for the indicated tumor-associated T cells.

[0054] **Figure 16** describes mutated 4T1 mammary carcinoma peptides for inducing CD8⁺ tumor-specific T cells.

[0055] **Figures 17A-17B** show the testing of different TLR agonists in the prime and boost immunization scheme using a mixture of 5 neo-antigens from 4T1 and IL-2/CD25 fusion protein to induce anti-neo-peptide specific T cell responses. The basic experimental scheme is shown.

On day 12, spleen cells were cultured with a mixture of neo-antigen peptides and IFN γ ELISpots were determined 24 hours later.

[0056] **Figures 18A-18D** show that IL-2/CD25 is superior to IL-2 at expanding CD8⁺ tumor-reactive Pmel-1 T cells. The basic experimental scheme is shown. Pmel-1 cells were adoptively transferred into C57BL/6 mice and primed 24 h later. **Figures 18A-18B**, expansion of Pmel-1 cells after a single dose of IL-2/CD25 or IL-2, or after 5 doses of IL-2, as indicated. Relative to IL-2/CD25, this represents an equivalent or 5-fold more molecules of IL-2, respectively. **Figures 18C-18D**, expansion of Pmel-1 cells after a single dose of IL-2/CD25 or 5 doses of IL-2, as indicated, over three days. Relative to IL-2/CD25, this represents 10- to 20-fold more molecules of IL-2. PBMCs were collected to show Pmel-1 expansion.

[0057] **Figures 19A-19B** show that a single dose of IL-2/CD25 leads to a frequency of melanoma neo-antigen-specific T cells similar to that induced by multiple administrations of a 10-fold higher dose of IL-2 (**Figure 19B**). C57BL/6 mice were immunized with a mixture of B16 neo-antigen peptides and Poly (I:C) as shown in **Figure 19A** and either IL-2/CD25 or IL-2 were administered after the 1st and 2nd boost immunization. Neo-antigen-specific T cells were enumerated through ELISpot on day 18.

[0058] **Figures 20A-20B** show that IL-2/CD25 promotes a favorable ratio of Pmel-1 CD8⁺ tumor-specific T cells to Tregs in the tumor microenvironment. Also, the overall immune response is amplified. C57BL/6 mice received B16.F10 tumor cells. Thirteen days later, Pmel-1 T cells were adoptively transferred into mice, treated as shown. Tumors were excised and analyzed 4 days after IL-2/CD25 administration by FACS.

[0059] **Figures 21A-21C** show that IL-2/CD25-amplified TRP-1 cells reject pre-existing B16 melanoma. The experimental design is shown in **Figure 21A**. Mice were inoculated with the B16.F10 tumor and 3 days later were immunized with the trp-1 antigen and LPS with and without IL-2/CD25. Tumor growth was monitored over time (**Figures 21A and 21C**).

[0060] **Figures 22A-22B** show that IL-2/CD25 promotes a favorable ratio of TRP-1 CD4⁺ tumor-specific T cells to Tregs in the tumor microenvironment and enhances immune infiltration. C57BL/6^{CD45.1} congenic mice received the B16.F10 tumor. 13 days later, TRP-1 T cells were adoptively transferred into mice, treated as in **Figure 22A**. Tumors were excised and analyzed 4 days after IL-2/CD25 administration by FACS.

[0061] **Figures 23A-23B** show that IL-2/CD25 induced neo-antigen-specific T cells after a prime and boost immunization using a mixture of 5 neo-antigens (nAg) (shown in Figure 16) from 4T1 breast tumor. The basic experimental scheme is shown in Figure 23A. The days (D) when the peptides and poly (I:C) (nAg/Poly(I:C)) and IL-2/CD25 were administered are indicated. On day 12, splenocytes were cultured with the mixture of neo-antigen peptides and IFN γ ELISpots were enumerated 24 h later. Significance is shown for most relevant groups.

DETAILED DESCRIPTION

[0062] The disclosure provides methods of using of an IL-2/CD25 fusion protein in combination with at least one neo-antigen for promoting anti-tumor immunity. In this regard, the disclosure provides methods of administering to a subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen. In various aspects, the materials and methods described herein are employed in a method for treating cancer in a mammalian subject.

Neo-antigens

[0063] Tumors harbor mutations in a number of their genes that yield neo-antigens (4). Neo-antigens are antigens (e.g., proteins or peptides) encoded by genes which are mutated in cancer cells, such as tumor-specific mutated genes (16). A neo-antigen may arise as a result of genetic change (e.g., inversions, translocations, deletions, missense mutations, splice site mutations, etc. that alter amino acid coding sequences). The mutations that result in neo-antigens are not restricted to genes that encode proteins that promote tumor growth; genes that encode non-tumorigenic proteins may produce neo-antigens useful in the context of the disclosure. Preferably, the mutations are unique to a tumor or cancer cell type, and provide a source of non-self-antigens that can be recognized by the immune system. Work in mouse preclinical studies have identified tumor neo-antigens associated with several distinct tumor types; immunization with such tumor neo-antigens can result in an anti-tumor T cell response (5). Recently, this approach has been translated to patients with melanoma, where some, but not all, exhibited an objective response (6,7). These limited data point to the usefulness of this strategy, but also highlight the deficiencies associated with current immunization approaches.

[0064] In various aspects, whole genome/exome sequencing may be used to identify neo-antigens that are present in a cancer (e.g., tumor) of an individual patient. Optionally, the method

comprises administering multiple neo-antigens, thereby providing (in various aspects) a tailored cocktail of neo-antigens for use as a cancer vaccine. Thus, providing “one or more neo-antigens” to a subject entails administering a composition comprising one type of neo-antigen (i.e., a pool of peptides comprising the same amino acid sequence) and administering a composition comprising multiple, different neo-antigens comprising different amino acid sequences (e.g., a composition comprising at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or more different neo-antigens). Methods of identifying neo-antigens is further described in International Patent Application Publication No. WO 2015/095811, which is incorporated by reference in its entirety and particularly with respect to its description of neo-antigens and methods of production.

[0065] The neo-antigen may be administered to a subject as a peptide, or the neo-antigen may be administered to a subject in the form of nucleic acid molecule or a complex of nucleic acid molecules that encode one or more neo-antigen peptide(s), which is expressed *in vivo* to produce the neo-antigen. In various aspects, the neo-antigen is encoded by vector that is administered to the subject; the vector comprises a nucleic acid molecule that encodes one or more neo-antigen peptide(s). A nucleic acid (DNA or RNA), vector, or peptide may be synthesized and purified by any suitable method. Optionally, the peptide is pulsed on an antigen presenting cells (APC), such as dendritic cells or antigen presenting cells (APC), by culture of bone marrow cells after culture with Granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), and or FMS-like tyrosine kinase 3 ligand (FLT3L). (See, for example, (17)). The peptide-pulsed APCs are then administered to the mammalian subject as the source of neo-antigen by, e.g., subcutaneous administration, intravenous administration, or intra-lymph node injections.

[0066] The neo-antigen may be an antigen associated with a cancer of the subject being treated, or may be a cancer-associated antigen taken from a different individual. In some aspects, the disclosure provides a method of treating cancer in a subject. In this respect, the neo-antigen may result from genetic abnormalities specific to the particular cancer being treated, or may result from genetic abnormalities that are not specific to the particular cancer being treated.

[0067] In various aspects, the neo-antigen is administered with an adjuvant. Suitable adjuvants include, but are not limited to, Polyinosinic:polycytidylic acid (poly-IC), MPL, GLA,

imiquimod, CpG ODN, LPS, Polyinosinic-Polycytidylic Acid stabilized with Polylysine and Carboxymethylcellulose (poly-IC LC) gardiquimod, aluminum, resiquimod, sodn-dsRNA, flagellin, STING agonists, liposomes, and SMP-105. The disclosure contemplates administration of more than one adjuvant.

IL-2/CD25 fusion protein

[0068] Interleukin-2 (IL-2) has distinct and opposing roles in the immune system. On the one hand, IL-2 is essential for immune tolerance based on its roles in promoting thymic development and peripheral homeostasis of CD4⁺ Foxp3⁺ regulatory T cells (Tregs) (8). On the other hand, IL-2 contributes to the induction of optimal immune responses through its roles in promoting T cell growth as well as development of T effector and memory cells (9). Several drawbacks related to IL-2 therapy are: 1) IL-2 has a very short half life *in vivo* (< 15 minutes when administered systemically, and requires frequent administrations at high doses to stimulate T effector cells); 2) at high doses, IL-2 exhibits very severe and potentially lethal side effects; and 3) IL-2 also expands Tregs, which may limit anti-tumor responses. As disclosed herein, however, a fusion protein comprising IL-2 and CD25 enhances an immune response to neo-antigen administration to a subject, thereby providing a strategy for effectively inducing anti-tumor immunity in a subject in need thereof.

[0069] In various aspects, the IL-2/CD25 fusion protein (also referred to as "IL-2/CD25") comprises (a) a first polypeptide comprising IL-2 or a functional variant or fragment thereof and (b) a second polypeptide comprising CD25 or a functional variant or fragment thereof, fused in frame to the first polypeptide. Compositions of IL-2/CD25 fusion proteins contemplated for use in the instant disclosure are further disclosed in International Patent Application Publication number WO2016022671, which is incorporated by reference in its entirety.

[0070] As used herein, "fusion protein" refers to the in frame genetic linkage of at least two heterologous polypeptides. Upon transcription/translation, a single protein is made. In this way, multiple proteins or fragments thereof can be incorporated into a single polypeptide. "Operably linked" is intended to mean a functional linkage between two or more elements. For example, an operable linkage between two polypeptides fuses both polypeptides together in frame to produce a single polypeptide fusion protein. In a particular aspect, the fusion protein further comprises a

third polypeptide which, as discussed in further detail below, can comprise a linker sequence (although the third polypeptide may comprise a different sequence, and need not be a linker).

[0071] The fusion protein exhibits IL-2 activity. In this regard, the IL-2/CD25 fusion protein, or an active variant or fragment thereof, can have one or more the following properties/activities: (1) increasing activity of regulatory T cells (Tregs) and/or increasing immune tolerance in low dose IL-2 based therapies; (2) increasing immune response and memory in higher dose therapies; (3) increasing IL-2 availability when compared to recombinant IL-2; and/or (4) increasing persistent IL-2 stimulation of IL-2R bearing lymphocytes *in vivo*. Methods of characterizing these properties/activities are described in the examples herein and include measuring the levels of CD4⁺ and CD8⁺ T effector cells. Tumor-specific T effector cells can be detected, for example, by enumerating the numbers of T cells that bind to MHC-tetramers containing the respective neo-antigen peptide after flow cytometry or by re-stimulation of T cells from the immunized subject and determining the numbers of T cells that respond to the neo-antigen peptide(s) and produce cytokines, e.g., IFN γ , IL-2, IL-4, IL-17, as measured by intracellular flow cytometry or an ELIspot assay.

[0072] In specific aspects, the fusion protein has an improved activity over the native or recombinant IL-2. For example, the effect of the IL-2/CD25 fusion protein can increase tolerogenic Tregs at about 2 fold, 5 fold, 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold, 100 fold 150 fold, 200 fold or lower level IL-2 activity in comparison to native or recombinant IL-2. In other aspects, the IL-2/CD25 fusion protein is more effective than native or recombinant IL-2 in inducing persistent augmentation of Tregs and related properties.

[0073] Various IL-2 and CD25 fragments and variants (including the CD25 extracellular domain) from a variety of organisms can be used to generate the IL-2/CD25 fusion proteins provided herein. Examples of non-limiting unprocessed IL-2/CD25 fusion proteins are set forth in SEQ ID NOs: 17, 19, 21, 23, 25, 27, 29, 36, 38, 44, 46, 54, 55, 56, 58, 59, 60, 61, 62, and 64, while non-limiting examples of mature forms of the IL-2/IL-Ra fusion proteins are set forth in SEQ ID NOs: 16, 18, 20, 22, 24, 26, 37, 39, 43, 45, and 57. Non-limiting examples of polynucleotides encoding such fusion proteins are set forth in SEQ ID NOs: 29, 30, 31, 32, 33, 34, 42, 47, 48, 49, 63, and 65. The "unprocessed" form of the fusion protein retains the secretory peptide sequence (i.e., a polypeptide sequence that directs the polypeptide through a secretory

pathway of a cell). A "mature" form of a fusion protein or polypeptide comprises the processed form of the polypeptide that has had the secretory peptide removed.

[0074] Biologically active fragments and variants of the mature and unprocessed form of the IL-2/CD25 fusion proteins, and the polynucleotide encoding the same, are also provided. Such a functional polypeptide fragment can comprise at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500 or more continuous amino acids of any one of SEQ ID NO: 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 36, 37, 38, 39, 43, 44, 45, 46, 54, 55, 56, 57, 58, 59, 60, 61, 62, or 64. Alternatively, a functional polypeptide variant can comprise at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the sequence set forth in SEQ ID NO: 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 36, 37, 38, 39, 43, 44, 45, 46, 54, 55, 56, 57, 58, 59, 60, 61, 62, or 64.

[0075] Active variants and fragments of polynucleotides encoding the IL-2/IL-R α fusion proteins are further provided. Such polynucleotides can comprise at least 100, 200, 300, 400, 500, 600, 700, 800, 1000, 1100, 1200, 1300, 1500, 1800, 2000 continuous nucleotides of SEQ ID NO: 29, 30, 31, 32, 33, 34, 42, 47, 48, 49, 63 or 65 or the polynucleotide encoding the polypeptides set forth in SEQ ID NO: 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 36, 37, 38, 39, 43, 44, 45, 46, 54, 55, 56, 57, 58, 59, 60, 61, 62, or 64 and continue to encode a functional IL-2/CD25 fusion protein. Alternatively, a functional polynucleotide can comprise at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the sequence set forth in SEQ ID NO: 29, 30, 31, 32, 33, 34, 42, 47, 48, 49, 63 or 65 or the polynucleotide encoding the polypeptides set forth in SEQ ID NO: 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 36, 37, 38, 39, 43, 44, 45, 46, 54, 55, 56, 57, 58, 59, 60, 61, 62, or 64 and continue to encode a functional IL-2/CD25 domain fusion proteins.

[0076] It is further recognized that the components of the IL-2/CD25 fusion protein can be found any order. In one aspect, the IL-2 polypeptide is at the N-terminus and the extracellular domain of CD25 is at the C-terminus of the fusion protein. Alternatively, the IL-2 portion may be located at the C-terminus of the fusion polypeptide, and the CD25 portion may be located at the N-terminus.

Interleukin-2

[0077] As used herein, "Interleukin-2" or "IL-2" refers to any native or recombinant IL-2 from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), and domesticated or agricultural mammals unless otherwise indicated. The term encompasses unprocessed IL-2, as well as any form of IL-2 that results from processing in the cell (i.e, the mature form of IL-2). The term also encompasses naturally occurring variants and fragments of IL-2, e.g., splice variants or allelic variants, and non-naturally occurring variants. The amino acid sequence of an exemplary mature form of human IL-2 (having the 20 amino acid signal sequence) is shown in SEQ ID NO: 2. Unprocessed human IL-2 additionally comprises an N-terminal 20 amino acid signal peptide (SEQ ID NO: 1), which is absent in the mature IL-2 molecule. The amino acid sequence of an exemplary mature form of mouse IL-2 (having the 20 amino acid signal sequence) is shown in SEQ ID NO: 4. Unprocessed mouse IL-2 additionally comprises an N-terminal 20 amino acid signal peptide (SEQ ID NO: 3), which is absent in the mature IL-2 molecule. By a "native IL-2", also termed "wild-type IL-2", is meant a naturally occurring or recombinant IL-2.

[0078] Additional nucleic acid and amino acid sequences for IL-2 are known. See, for example, GenBank Accession Nos: Q7JFM2 (Aotus lemurinus (Gray-bellied night monkey)); Q7JFM5 (Aotus nancymae (Ma's night monkey)); P05016 (Bos taurus (Bovine)); Q29416 (Canis familiaris (Dog) (Canis lupus familiaris)); P36835 (Capra hircus (Goat)); and, P37997 (Equus caballus (Horse)).

[0079] Biologically active variants of IL-2 are known. See, for example, US Application Publications 20060269515 and 20060160187 and WO 99/60128, each of which is herein incorporated by reference.

[0080] Biologically active fragments and variants of IL-2 can be employed in the fusion proteins disclosed herein. Such a functional fragment can comprise at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 75, 100, 125, 150 or more continuous amino acids of SEQ ID NO: 1, 2, 3, or 4. Alternatively, a functional variant can comprise at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the sequence set forth in SEQ ID NO: 1, 2, 3, or 4.

[0081] Active variants and fragments of polynucleotides encoding the IL-2 proteins are further provided. Such polynucleotide can comprise at least 100, 200, 300, 400, 500, 600, 700 continuous nucleotides of polypeptide encoding SEQ ID NO: 1, 2, 3, or 4, and continue to encode a protein having IL-2 activity. Alternatively, a functional polynucleotide can comprise at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide encoding the amino sequence set forth in SEQ ID NO: 1, 2, 3, or 4 and continue to encode a functional IL-2 polypeptide.

CD25 (Interleukin-2 Receptor Alpha)

[0082] The term "CD25" or "IL-2 receptor α " or "IL-2R α " as used herein, refers to any native or recombinant CD25 or CD25 extracellular domain from any vertebrate source, including mammals such as primates (e.g., humans) and rodents (e.g., mice and rats) and domesticated or agricultural mammals unless otherwise indicated. The term also encompasses naturally occurring variants of CD25, e.g., splice variants or allelic variants, or non-naturally occurring variants. Human IL-2 exerts its biological effects via signaling through its receptor system, IL-2R. IL-2 and its receptor (IL-2R) are required for T-cell proliferation and other fundamental functions which are crucial of the immune response. IL-2R consists of 3 noncovalently linked type I transmembrane proteins which are the alpha (p55), beta (p75), and gamma (p65) chains. The human IL-2R alpha chain contains an extracellular domain of 219 amino acids, a transmembrane domain of 19 amino acids, and an intracellular domain of 13 amino acids. The secreted extracellular domain of IL-2R alpha (IL-2R-a) can be employed in the fusion proteins described herein.

[0083] The amino acid sequence of an exemplary mature form of human CD25 is shown in SEQ ID NO: 6. Unprocessed human CD25 is shown in SEQ ID NO: 5. The extracellular domain of SEQ ID NO: 6 is set forth in SEQ ID NO: 7. The amino acid sequence of an exemplary mature form of mouse CD25 is shown in SEQ ID NO: 9. Unprocessed mouse CD25 is shown in SEQ ID NO: 8. The extracellular domain of SEQ ID NO: 9 is set forth in SEQ ID NO: 10. By a "native CD25", also termed "wild-type CD25", is meant a naturally occurring or recombinant CD25. The sequence of a native human CD25 molecule is shown in SEQ ID NO: 5 and 6.

[0084] Nucleic acid and amino acid sequences for CD25 are known. See, for example, GenBank Accession Nos: NP 001030597.1 (*P. troglodytes*); NP 001028089.1 (*M. mulatta*); NM

001003211.1 (*C. lupus*); NP 776783.1 (*B.taurus*); NP 032393.3 (*M.musculus*); and, NP 037295.1 (*R.norvegicus*), each of which is herein incorporated by reference.

[0085] Biologically active fragments and variants of the extracellular domain of CD25 are also provided for use in IL-2/CD25 fusion proteins. Such CD25 extracellular domain active variants or fragments will retain the CD25 extracellular domain activity. The phrase "biological activity of the CD25 extracellular domain" refers to one or more of the biological activities of extracellular domain of CD25, including but not limited to, the ability to enhance intracellular signaling in IL-2 receptor responsive cells. Non-limiting examples of biologically active fragments and variants of the CD25 are disclosed, for example, in Robb et al., *Proc. Natl. Acad. Sci. USA*, 85:5654-5658, 1988, which is herein incorporated by reference.

[0086] Biologically active fragments and variants of the extracellular domain of CD25 can be employed in the fusion proteins disclosed herein. Such a functional fragment can comprise at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 215 or greater continuous amino acids of the extracellular domain of any one of SEQ ID NO: 6, 9, 7, 10, 5, or 8. Alternatively, a functional variant can comprise at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the sequence set forth in SEQ ID NO: 6, 9, 7, 10, 5, or 8.

[0087] In one aspect, the fusion proteins provided herein can comprise at least one mutation within the extracellular domain of CD25. In a specific aspect, the arginine at position 35 of CD25 can be mutated to a threonine and/or the arginine at position 36 of CD25 can be mutated to a serine. Such a fusion protein can have increased IL-2 activity compared to a fusion protein not comprising these mutations in the extracellular domain of CD25 and/or compared to native or recombinant IL-2. The amino acid sequences of exemplary fusion proteins comprising CD25 with mutations within the extracellular domain of CD25 are set forth in SEQ ID NOS: 62 and 64. In one aspect, the fusion protein comprises the amino acid sequence of any one of SEQ ID NO: 62 or 64; or a sequence having at least 80%, 85%, 90%, or 95% to any one of SEQ ID NO: 62 or 64.

[0088] Active variants and fragments of polynucleotides encoding the extracellular domain of CD25 are further provided. Such polynucleotide can comprise at least 100, 200, 300, 400, 500, 600 or greater continuous nucleotides of polypeptide encoding SEQ ID NO: 6, 9, 7, 10, 5, or 8

and continue to encode a protein having the extracellular domain activity of CD25. Alternatively, a functional polynucleotide can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide encoding the amino sequence set forth in SEQ ID NO: 6, 9, 7, 10, 5, or 8 and continue to encode a protein having the extracellular domain activity of CD25.

Additional Components

[0089] The IL-2/CD25 fusion proteins can further comprise additional elements. Such elements can aid in the expression of the fusion protein, aid in the secretion of the fusion protein, improve the stability of the fusion protein, allow for more efficient purification of the protein, and/or modulate the activity of the fusion protein.

[0090] "Heterologous" in reference to a polypeptide or polynucleotide is a polypeptide or polynucleotide that originates from a different protein or polynucleotide. The additional components of the fusion protein can originate from the same organism as the other polypeptide components of the fusion protein, or the additional components can be from a different organism than the other polypeptide components of the fusion protein.

[0091] In one aspect, the IL-2/CD25 fusion protein comprises a linker sequence located between the IL-2 polypeptide and the CD25 polypeptide. The linker can be of any length and can comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 50 or 60 or more amino acids. In one aspect, the linker sequence comprises glycine amino acid residues. In other instances, the linker sequence comprises a combination of glycine and serine amino acid residues. Such glycine/serine linkers can comprise any combination of the amino acid residues, including, but not limited to, the peptide GGGS (SEQ ID NO: 15) or GGGGS (SEQ ID NO: 52) or repeats of the same, including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more repeats of these given peptides. For example, linker sequences can comprise GGGSGGGSGGGS (SEQ ID NO: 13) (also noted as (Gly3Ser)₃); GGGSGGGSGGGSGGGS (SEQ ID NO: 11) (also noted as (Gly3Ser)₄); or (Gly3Ser)₅ (SEQ ID NO: 14); (Gly3Ser)₆ (SEQ ID NO: 66); (Gly3Ser)₇ (SEQ ID NO: 67), etc. Linker sequences can further comprise (Gly4Ser)₃ as set forth in SEQ ID NO: 50; GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 40) (also noted as (Gly4Ser)₄); GGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 41) (also noted as (Gly4Ser)₅); (Gly4Ser)₂ (SEQ ID NO: 51); (Gly4Ser)₁ (SEQ ID NO: 52); (Gly4Ser)₆

(SEQ ID NO: 68); (Gly4Ser)₇ (SEQ ID NO: 69); (Gly4Ser)₈ (SEQ ID NO: 70), etc. In addition, active variants and fragments of any linker can further be employed in the fusion protein disclosed herein. In various aspects, the linker is not cleavable by an enzyme found in the human body, such that the IL-2 and CD25 components remain connected *in vivo*.

[0092] It is further recognized that the polynucleotide encoding the IL-2/CD25 fusion protein can comprise additional elements that aid in the translation of the fusion protein. Such sequences include, for example, Kozak sequences attached to the 5' end of the polynucleotide encoding the fusion protein. The Kozak consensus sequence is a sequence which occurs on eukaryotic mRNA that plays a role in the initiation of the translation process and has the consensus (gcc)gccRccAUGG (SEQ ID NO: 35); wherein (1) a lower case letter denotes the most common base at a position where the base can nevertheless vary; (2) upper case letters indicate highly-conserved bases, i.e., the 'AUGG' sequence is constant or rarely, if ever, changes, with the exception being the IUPAC ambiguity code 'R' which indicates that a purine (adenine or guanine) is normally observed at this position; and (3) the sequence in brackets ((gcc)) is of uncertain significance. In one aspect, the Kozak sequence comprises the sequence set forth in SEQ ID NO: 53.

[0093] In one non-limiting aspect, the IL-2/CD25 fusion protein comprises an IL-2 leader optimized Kozak sequence as set forth in SEQ ID NO: 28 or a functional variant or fragment thereof. A functional variant or fragment of a Kozak sequence will retain the ability to increase translation of the protein when compared to the level of translation from a sequence lacking the leader. Such a functional fragment can comprise at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40 continuous nucleotides of a kozak sequence or the sequence set forth in SEQ ID NO: 28 or 53. Alternatively, a functional variant can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the kozak sequence or the sequence set forth in SEQ ID NO: 28 or 53.

[0094] In still further aspects, the IL-2/CD25 fusion protein comprises one or more tags at the C-terminus to aid in the purification of the polypeptide. Such tags are known and include, for example, a Histidine tag. In specific aspects a 6X His tag is employed. It is further recognized that an additional linker sequence can be employed between the fusion protein and the His tag.

Variants and Fragments

[0095] Fragments and variants of the polynucleotides encoding the IL-2/CD25 fusion protein or the various components contained therein (i.e., the CD25 extracellular domain, the CD25 polypeptides, the linker sequences and/or Kozak sequences) can be employed in the various methods and compositions of the disclosure. By "fragment" is intended a portion of the polynucleotide and hence the protein encoded thereby or a portion of the polypeptide. Fragments of a polynucleotide may encode protein fragments that retain the biological activity of the native protein and hence have IL-2 activity, CD25 extracellular domain activity, IL-2/CD25 fusion protein activity, or if encoding a linker sequence, provide for the desired activity of the IL-2/CD25 fusion protein.

[0096] A biologically active portion of a CD25 extracellular domain, IL-2 polypeptide, IL-2/CD25 fusion protein, Kozak sequence, or linker sequence can be prepared by isolating a portion of one of the polynucleotides encoding the portion of the CD25 extracellular domain or IL-2 polypeptide and expressing the encoded portion of the polypeptide (e.g., by recombinant expression *in vitro*), and assessing the activity of the portion of the CD25 extracellular domain or/and IL-2 polypeptide or the activity of the IL-2/CD25 fusion protein.

[0097] "Variant" sequences have a high degree of sequence similarity. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the CD25 extracellular domain polypeptides, IL-2 polypeptides, IL-2/CD25 fusion proteins, or hybridization techniques. Variant polynucleotides also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode an CD25 extracellular domain, IL-2 polypeptide, IL-2/CD25 fusion protein, a Kozak sequence, or the linker sequence. linker sequences. Variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, polymerase chain reaction (PCR) and hybridization techniques. Variant polynucleotides also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode an CD25 extracellular domain, IL-2 polypeptide, IL-2/CD25 fusion protein, a Kozak sequence, or the linker sequence.

[0098] By "variant" protein is intended a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins are biologically active, that is they continue to possess the desired biological activity, that is, IL-2/CD25 fusion protein activity. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a IL-2/CD25 fusion protein or any one of its components (i.e., an CD25 extracellular domain polypeptide, a IL-2 polypeptide, or a linker sequence) will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

[0099] Proteins may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the IL-2/CD25 fusion protein or linker sequences can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel 30 (1985) Proc. Natl. Acad. Sci. USA 82:488-492; Kunkel et al. (1987) Methods in Enzymol. 154:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) Techniques in Molecular Biology (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff et al. (1978) Atlas of Protein Sequence and Structure (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferable.

[00100] Thus, the polynucleotides described herein can include the naturally occurring sequences, the "native" sequences, as well as mutant forms. Likewise, the proteins used in the methods of the invention encompass naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the ability to implement a

recombination event. Generally, the mutations made in the polynucleotide encoding the variant polypeptide should not place the sequence out of reading frame, and/or create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

Dosing and administration

[00101] "Therapeutically effective amount" refers, e.g., to an amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen which is effective, upon single or multiple dose administration, to increase the number of cancer neo-antigen-specific T cells in a subject or enhance an immune response against a cancer neo-antigen. Where the subject is suffering from a disorder, a therapeutically effective amount optionally prolongs the survivability of the subject, reduces one or more signs or symptoms of the disorder, prevents or delays onset or progression of the disorder, and the like, beyond that expected in the absence of such treatment. The dose of the fusion protein and/or neo-antigen need not remain consistent over the course of a treatment period, but can be varied until a desired effect is achieved.

[00102] In various aspects, an IL-2/CD25 fusion protein is administered in an amount sufficient to increase the number of neo-antigen -specific T cells in a subject. In general, a dose of IL-2/CD25 fusion protein employed for mammalian subject treatment is in the range of about 0.01 mg/kg to about 5 mg/kg per administration. In related aspects, a dose of IL-2/CD25 fusion protein is between 0.05-0.1 mg/kg, 0.1-1 mg/kg, 2-3 mg/kg, 3-4 mg/kg, or 4-5 mg/kg.

[00103] In various aspects, the neo-antigen peptide is administered in an amount sufficient to increase the number of neo-antigen -specific T cells in a subject. In general, a dose of neo-antigen peptide employed for mammalian subject treatment is in the range of about 10 µg to about 5000 µg per administration. In related aspects, the dose of the neo-antigen peptide is between 10-100 µg, 100-500 µg, 500-1000 µg, or 1000-5000 µg per administration.

[00104] In various aspects, a nucleic acid encoding the neo-antigen peptide is administered in an amount sufficient to increase the number of neo-antigen -specific T cells in a subject. In general, a dose of nucleic acid encoding the neo-antigen peptide employed for mammalian subject treatment is in the range of about 50 µg to about 5000 µg per administration. In related aspects, a dose of nucleic acid encoding the neo-antigen peptide is between 50-100 µg, 100-500 µg, 500-1000 µg, or 1000-5000 µg per administration.

[00105] In various aspects, a vector comprising the nucleic acid encoding the neo-antigen peptide is administered in an amount sufficient to increase the number of neo-antigen -specific T cells in a subject. In general, a dose of vector comprising the nucleic acid encoding the neo-antigen peptide employed for mammalian subject treatment is in the range of about 10 µg to about 5000 µg per administration. In related aspects, the dose of vector comprising the nucleic acid encoding the neo-antigen peptide is between 10-100 µg, 100-500 µg, 500-1000 µg, or 1000-5000 µg per administration.

[00106] In various aspects, the neo-antigen (or fusion protein) is administered in combination with an adjuvant. In general, a dose of adjuvant employed for mammalian subject treatment is in the range of about 10 µg to about 5000 µg per administration. In related aspects, the dose of adjuvant peptide is between 10-100 µg, 100-500 µg, 500-1000 µg or 1000-5000 µg per administration.

[00107] The neo-antigen peptide (or nucleic acid or vector encoding the neo-antigen peptide) and the IL-2/CD25 fusion protein (as well as the adjuvant, if employed) may be administered to a subject using any suitable route of administration. Suitable routes of administration include, but are not limited to, subcutaneous, intramuscular, intravenous, intraperitoneal, intratumoral, intradermal, intra-lymph node administration.

[00108] The method comprises administering IL-2/CD25 fusion protein in combination with at least one neo-antigen (and optionally with an adjuvant). “In combination with” does not require concurrent administration, i.e., within the same composition or administration in separate compositions close in time, although concurrent administration is contemplated. In various aspects, the IL-2/CD25 fusion protein and at least one neo-antigen are given sequentially, where administration of one or more doses of neo-antigen(s) is followed by administration of one of more doses of IL-2/CD25 fusion protein. Alternatively, administration of one or more doses of IL-2/CD25 fusion protein may be followed by administration of one of more doses of neo-antigen. The sequential administration may occur within minutes of each other, within hours of each other, or within days of each other. In various aspects, the IL-2/CD25 fusion protein is delivered within 7 days of administration of the neo-antigen peptide. In various aspects, the IL-2/CD25 fusion protein is delivered the same day (within 4, 8, 12, or 24 hours) or within 1, 2, 3, 4, 5, 6, or 7 days after (or before) administration of the neo-antigen peptide.

[00109] The disclosure contemplates administration of multiple doses of neo-antigen and/or IL-2/CD25 fusion protein over the course of a treatment period. In this regard, optionally, a second dose of the IL-2/CD25 fusion protein is administered within 7 days after administration of the neo-antigen peptide (e.g., a second dose of the IL-2/CD25 fusion protein is delivered the same day (within 4, 8, 12, or 24 hours) or within 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 days after (or before) administration of the neo-antigen. In various aspects, one or more doses of neo-antigen is administered no more than 10 days prior to administration of the IL-2/CD25 fusion protein. In various aspects, a one or more doses of neo-antigen peptide is administered the same day (within 4, 8, 12, or 24 hours) or 1, 2, 3, 4, 5, 6, 7, 8 9, or 10 days prior to administration of the IL-2/CD25 fusion protein.

[00110] In various aspects, the subject is administered a priming dose of at least one neo-antigen no more than 10 days prior to administration of the IL-2/CD25 fusion protein. In various aspects, a priming dose comprising at least one neo-antigen is delivered the same day (within 4, 8, 12, or 24 hours) of administration or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days prior to administration with the IL-2/CD25 fusion protein. The priming dose, as used herein, refers to the initial dose administered prior to administration of a boosting dose.

[00111] In various aspects, the subject is administered one or more boosting doses comprising at least one neo-antigen 1-30 days after administration of the priming dose. In various aspects, one or more boosting doses is administered 1, 5, 10, 15, 20, 25, or 30 days after the priming dose is administered. The boosting dose, as used herein, refers to the second or subsequent dose administered following administration of a priming dose.

[00112] In various aspects, the subject is administered a dose of the IL-2/CD25 fusion protein within 7 days of administering a boosting dose of at least one neo-antigen. In various aspects, the IL-2/CD25 fusion protein is delivered the same day (within 4, 8, 12, or 24 hours) or 1, 2, 3, 4, 5, 6, or 7 days after administration of the boosting dose of neo-antigen(s).

[00113] In various aspects, the subject is administered one or more boosting doses comprising at least one neo-antigen 1- 30 days after administration of the priming dose comprising at least one neo-antigen. In various aspects, one or more boosting doses comprising at least one neo-antigen is delivered 1, 5, 10, 15, 20, 25, or 30 days after administration of the priming dose.

[00114] In various aspects, the subject is administered a second dose comprising the IL-2/CD25 fusion protein within 10 days after administration of the initial dose comprising the IL-2/CD25 fusion protein. In various aspects, the IL-2/CD25 fusion protein is delivered the same day (within 4, 8, 12, or 24 hours) or 1, 2, 3, 4, 5, 6, or 7 days after administration of the initial dose comprising the IL-2/CD25 fusion protein.

Methods of use

[00115] The disclosure provides a method for increasing neo-antigen tumor-specific T cells in a mammalian subject. The method comprises administering to a subject in need thereof a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen. As used herein, the term "increases" includes inducing, potentiating, elevating, or enhancing the levels of neo-antigen tumor-specific T cells in a mammalian subject.

[00116] The disclosure further provides a method of enhancing immunogenicity of tumor neo-antigens in a mammalian subject. The method comprises administering to the subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen. As used herein, the term "enhancing immunogenicity of tumor neo-antigens" includes (but is not limited to) inducing, potentiating, elevating, or increasing an immune response. In related aspects, administration of the IL-2/CD25 fusion protein in combination with at least one neo-antigen increases the number of CD4⁺ and CD8⁺ T effector and memory cells.

[00117] The subject of the disclosed methods is optionally a mammalian subject, such as primates, humans, agricultural and domesticated animals such as, but not limited to, dogs, cats, cattle, horses, pigs, sheep, and the like. In one aspect, the subject is a human. Optionally, the subject is suffering from cancer.

[00118] In various aspects, the disclosure provides a method of treating cancer, the method comprising administering a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen to a mammalian subject in need thereof.

[00119] In certain aspects, the cancer is selected from the group consisting of esophageal cancer, pancreatic cancer, metastatic pancreatic cancer, metastatic adenocarcinoma of the pancreas, bladder cancer, stomach cancer, fibrotic cancer, glioma, malignant glioma, diffuse intrinsic pontine glioma, recurrent childhood brain neoplasm renal cell carcinoma, clear-cell

metastatic renal cell carcinoma, kidney cancer, prostate cancer, metastatic castration resistant prostate cancer, stage IV prostate cancer, metastatic melanoma, melanoma, malignant melanoma, cutaneous melanoma, ocular melanoma, melanoma brain metastases, malignant melanoma of head and neck, lung cancer, non small cell lung cancer (NSCLC), squamous cell non-small cell lung cancer, breast cancer, recurrent metastatic breast cancer, hepatocellular carcinoma, hodgkin's lymphoma, follicular lymphoma, non-hodgkin's lymphoma, advanced B-cell NHL, HL including diffuse large B-cell lymphoma (DLBCL) including DLBCL following autologous stem cell transplantation, multiple myeloma, chronic myeloid leukemia, adult acute myeloid leukemia in remission; adult acute myeloid leukemia alkylating agent-related acute myeloid leukemia, chronic lymphocytic leukemia, richter's syndrome; waldenstrom macroglobulinemia, adult glioblastoma; adult gliosarcoma, recurrent glioblastoma, recurrent childhood rhabdomyosarcoma, recurrent ewing sarcoma/ peripheral primitive neuroectodermal tumor, recurrent neuroblastoma; recurrent osteosarcoma, colorectal cancer, MSI positive colorectal cancer; MSI negative colorectal cancer, nasopharyngeal nonkeratinizing carcinoma; recurrent nasopharyngeal undifferentiated carcinoma, cervical adenocarcinoma; cervical adenosquamous carcinoma; cervical squamous cell carcinoma; cervical cancer, anal canal squamous cell carcinoma; metastatic anal canal carcinoma; recurrent anal canal carcinoma, recurrent head and neck cancer; head and neck squamous cell carcinoma (HNSCC), ovarian carcinoma, colon cancer, gastric cancer, advanced GI cancer, gastric adenocarcinoma; gastroesophageal junction adenocarcinoma, thyroid cancer, liver cancer, endometrial cancer, bone neoplasms, soft tissue sarcoma; bone sarcoma, thymic carcinoma, urothelial carcinoma, recurrent merkel cell carcinoma; stage III merkel cell carcinoma; stage IV merkel cell carcinoma, myelodysplastic syndrome and recurrent mycosis fungoides and Sezary syndrome. In various aspects, the cancer is melanoma, cutaneous melanoma, ocular melanoma, cervical cancer, follicular B cell non-Hodgkin's lymphoma, kidney cancer, prostate cancer, and multiple myeloma, breast cancer, lung cancer, colon cancer, ovarian cancer, bladder cancer, pancreatic cancer, endometrial cancer, liver cancer, thyroid cancer, leukemia.

[00120] In various aspects, the IL-2/CD25 fusion protein and neo-antigen is administered in combination with one or more checkpoint inhibitors. The checkpoint inhibitor may include, but is limited to, an anti-PD-1 antibody, an anti-PDL-1 antibody, an anti-CTLA-4 antibody, a PD-1

inhibitor, a PDL-1 inhibitor, a CTLA-4 inhibitor, anti-Tim-3 antibody, anti-LAG3 antibody, or anti-TIGIT antibody.

[00121] In various aspects of the disclosure, an IL-2/CD25 fusion protein and at least one neo-antigen are administered in an amount and for a time sufficient to reduce tumor volume, reduce tumor burden, and/or reduce metastasis in the mammalian subject. Tumor volume can be measured using methods such as, for example, computed tomographic (CT) scan or positron emission tomography (PET) imaging. Tumor burden can be determined by, e.g., measuring tumor markers in biological samples. In various aspects, the method reduces tumor volume or tumor burden by at least 1%, 3%, 5%, 10%, 20%, 30%, 40%, 50% or more. In various aspects, the method reduces tumor volume or tumor burden by at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100%. Ranges containing any of the aforementioned integers as lower and upper ends are contemplated (e.g., 10%-50% or 3%-50%). It will be appreciated that complete eradication of the tumor or tumor burden is not required to achieve a beneficial response; any level of reduction of tumor burden, tumor volume, or metastasis is contemplated.

[00122] The terms "treat," "treated," "treating," "treatment," and the like are meant to refer to reducing or ameliorating a disorder and/or symptoms associated therewith (e.g., a neoplasia or tumor). "Treating" may refer to administration of the combination therapy to a subject after the onset, or suspected onset, of a cancer. "Treating" includes the concepts of "alleviating", which refers to lessening the frequency of occurrence or recurrence, or the severity, of any symptoms or other ill effects related to a cancer and/or the side effects associated with cancer therapy. The term "treating" also encompasses the concept of "managing" which refers to reducing the severity of a particular disease or disorder in a patient or delaying its recurrence, e.g., lengthening the period of remission in a patient who had suffered from the disease. It is appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition, or symptoms associated therewith be completely eliminated.

[00123] It is further contemplated that other adjunct therapies may be administered, where appropriate. For example, the subject may also be administered additional cytotoxic agents, photodynamic therapy and/or radiation therapy, or have undergone a surgical procedure (e.g.,

tumor resection). Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

[00124] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

EXAMPLES

General Methods

Mice and cell lines

[00125] C57BL/6, C57BL/6^{CD45.1} congenic, Pmel-1, and TRP-1 mice (7-to-8-week-old females and males) were purchased from The Jackson Laboratory. C57BL/6^{CD45.1} congenic, Pmel-1, and TRP-1 mouse colonies were maintained in our laboratory. B16.F10 melanoma was purchased from the American Type Culture Collection and maintained as recommended. For tumor inoculations, cell lines were maintained in culture for less than 2 weeks and only early passage stocks were used for each experiment.

Synthetic peptides and reagents

[00126] Peptides for monoclonal experiments were purchased from Anaspec and CHI Scientific. Peptides for polyclonal studies were obtained from CHI Scientific and reconstituted based on their solubility properties. Stock solutions were reconstituted as advised by the vendors and maintained at -20°C. All peptides were >95% pure as determined by high-performance liquid chromatography. The peptides used in studies described herein are as follows: Monoclonal peptides included: KVPRNQDWL (human gp100₂₅₋₃₃) (SEQ ID NO: 71) and SGHNCGTCRPGWRGAACNQKILTVR (trp-1₁₀₆₋₁₃₀) (SEQ ID NO: 72). Polyclonal neo-antigen peptides included: REGVELCPGKNKYEMRRHGTTHSLVIHD (B16-M27) (SEQ ID NO: 73), PSKPSFQEFVDWENVSPELNSTDQPFL (B16-M30) (SEQ ID NO: 74), EFKHIKAFDRTFANNPGPMVVFATPGM (B16-M44) (SEQ ID NO: 75), SHCHWNDLAVIPAGVVHNWDFEPRKVS (B16-M48) (SEQ ID NO: 76), QGVTVLAVSAVYDIFVFHRLKMKQILP (4T1-M8) (SEQ ID NO: 77), NDEPDLDPVQELIYDLRSQCDAIRVTK (4T1-M20) (SEQ ID NO: 78), AVKEEDSLHWQRPEDVQKVKALSFYQP (4T1-M26) (SEQ ID NO: 79), FAICFSCLLAHALNLIKLVGRGRKPLSW (4T1-M27) (SEQ ID NO: 80),

KELLQFKKLKKQNLQQMQAESGFVQHV (4T1-M35) (SEQ ID NO: 81) and sequences were obtained from Kreiter et al., 2015 (15). LPS was purchased from Invitrogen (cat # 12880) and Poly (I:C) was purchased from Sigma Aldrich (cat # P9582). PD-1 antibody was obtained from Bristol Myers Squibb.

Preparation of mIL-2/mCD25 and hIL-2 for injection

[00127] IL-2/CD25 used for the study was synthesized in our laboratory and hIL-2 was purchased from Peprotech. Stocks were stored at -70°C and injected in 100µl total intraperitoneally (i.p.)

Immunization protocols for monoclonal studies

[00128] Pmel-1 cells were adoptively transferred i.v. into C57BL/6 mice. 24 hours later, mice were primed with human gp100 (100µg) and LPS (10µg) i.v. A day after priming, mIL-2/mCD25 (100µg) was administered. In studies where hIL-2 was used; this protein was also injected i.p. To measure TRP-1 responses, TRP-1 cells from male TRP-1 mice were adoptively transferred into C57BL/6^{CD45.1} congenic mice. 24 h later, mice were primed with trp-1 peptide (100µg) and LPS (10µg) i.v. A day after priming, mIL-2/mCD25 (100µg) was administered.

Immunization protocols for polyclonal studies

[00129] For initial studies to determine the best immunization regimen, mice were immunized with a pool of the neo-antigen peptides (50 µg each) and Poly (I:C) (50 µg) s.c. in 200 µl volume and mIL-2/mCD25 i.p. (50 µg) at different time points. Upon determination of the best regimen, mice were primed on day 0 and boosted on day 7 with neo-antigen/Poly (I:C) pool followed by mIL-2/mCD25 administration a day after the boost. A second boost followed on day 13 with neo-antigen/Poly (I:C) pool followed by mIL-2/mCD25 on day 14.

ELISpot assay

[00130] Splenocytes were co-cultured with syngeneic bone marrow-derived dendritic cells and stimulated with the neo-antigen pool (2µg/ml) overnight at 37°C in anti-IFN-γ coated Multiscreen 96-well plate and cytokine secretion was detected with an anti-IFN-γ antibody. ImmunoSpot analyzer was used to count the IFN-γ-producing cells.

Flow cytometric analysis

[00131] Blood or single cell suspensions from lymphoid and non-lymphoid tissues were obtained. Red blood cells were lysed using an ACK solution. Cells were stained in staining buffer and flow cytometry was performed using Fortessa. Data analysis was performed using FACS DIVA software (version 8.1).

In vivo tumor model

[00132] C57BL/6 mice were inoculated with B16.F10 melanoma (1×10^5 / mouse) on the rear flank. In recall studies, tumors were inoculated 65 days post immunization in the Pmel-1 model or 1 day post end of therapy in polyclonal studies. In therapeutic studies, tumors were inoculated 3 days prior (day -3) to immunization and mice with visible, darkly-pigmented tumors were subsequently immunized. Tumor growth was monitored every 2-3 days by measuring two opposing diameters with a set of calipers. Results are presented as the mean tumor volume (mm^3) $\pm$ SEM. In survival studies, mice were sacrificed when tumor volume reached 2000 mm^3 .

Analysis of tumor infiltrating lymphocytes (TILs)

Tumor infiltrating lymphocytes were prepared from subcutaneous B16.F10 tumors on day 18 after immunization in the polyclonal setting and when the tumor reached $500\text{-}1000 \text{ mm}^3$ in the monoclonal setting. In other monoclonal studies, mice were inoculated with B16.F10 13 days prior to adoptive transfer of Pmel-1 or TRP-1 T cells. Tumors were harvested, weighed, and minced into 1-2mm pieces in a collagenase D (1mg/ml) solution prepared in RPMI complete media. Single cell suspensions were obtained using the gentleMACS tissue Dissociator followed by 20 min incubation in a 37°C warm room with gentle shaking. The lymphocytes were then purified using CD45 magnetic beads in magnetic columns and stained in staining buffer or stained without CD45 enrichment.

[00133] Additional aspects and details of the disclosure will be apparent from the following examples, which are intended to be illustrative rather than limiting.

Example 1: Using mIL-2/mCD25 to enhance the immune response to melanoma tumor neo-antigens

[00134] It was investigated whether an IL-2/CD25 fusion protein amplifies the response of CD8^+ Pmel-1 against a tumor-related antigen, where the CD8^+ T cells are specific for a tumor-

related antigen. C57BL/6 mice adoptively transferred with Pmel-1 T cells were immunized with hgp100, LPS and mouse IL-2/CD25 fusion protein (mIL-2/mCD25) (**Fig. 1A**). Indeed, the mIL-2/mCD25 fusion protein was found to amplify the response of CD8⁺ Pmel-1 against a tumor-related antigen (**Figs. 1B and 1C**). The persistent cells were initially effector/memory cells but over time central memory cells predominated as shown by the increase in CD8⁺ T central memory cells (**Figs. 2A-2C**). It was also assessed whether a mIL-2/mCD25 fusion protein amplified CD4⁺ T cells using TCR transgenic class II-restricted TRP-1-specific CD4⁺ T cells. Again, a substantial expansion of TRP-1 T cells was observed (**Figs. 3A-3D**). However, unlike Pmel-1, the TRP-1 T cells rapidly contracted to low levels, without a readily detectable pool of memory cells. Collectively, these data indicate that a limited application of IL-2/CD25 very effectively expands CD4⁺ and CD8⁺ T effector cells, but long-lasting memory only occurs for CD8⁺ T cells.

Example 2: Assessing the anti-tumor activity of Pmel-1 and TRP-1 T cells and IL-2/CD25 fusion protein

[00135] The anti-tumor activity of Pmel-1 T cells were assessed. Initially it was tested whether persistent Pmel-1 memory T cells could reject a challenge with the B16.F10 melanoma, which expresses mouse gp100. Tumors grew rapidly in control untreated mice and mice that were immunized with only hgp100; in contrast, no tumor growth was evident over this time in the mice immunized with hgp100 and mIL-2/mCD25 (**Figs. 4A-4C**). Most mice remained tumor-free for approximately 100 days, but after this time tumors grew at variable rates. These tumors were likely antigen-loss variants as the tumor lost their dark pigment that depends on gp100 (not shown). When the tumors from these mice were examined, those immunized with gp100 and mIL-2/mCD25 had a very high number of Pmel-1 cells, which led to a high ratio of T effector:Treg cells (**Figs. 5A-5K**). Further, this led to an increase in the number of endogenous non-transgenic CD4⁺ Foxp3⁻ and CD8⁺ T and NK cells, but not B cells and myeloid lineage cells. In another experiment, mice that had a pre-existing tumor prior to immunization were best protected from tumor growth after immunization with hgp100 and mIL-2/mCD25 (**Figs. 6A-6E**). Assessment of the tumor microenvironment in this setting revealed a large increase in the number of Pmel-1 T cells that led to a high ratio of Pmel-1 to Treg cells (**Fig. 20B**). Trends were noted for increased prevalence of endogenous NK cells but decreased B cells. In an analogous manner, IL-2/CD25 promoted anti-tumor activity of TRP-1 T cells after immunization of tumor-

bearing mice with trp-1/LPS (**Figs. 21B-21C**). When the tumor microenvironment was assessed (**Fig. 22A**), trends were noted for increased prevalence of TRP-1 T cells, which led to an increased ratio of TRP-1 to Treg cells, as well as increased endogenous NK cells and macrophages (**Fig. 22B**). Collectively, these data indicate that limited application of IL-2/CD25 leads to amplification of a vaccine-induced anti-tumor response mediated by T effector and T memory cells. The single application of IL-2/CD25 is expected to substantially limit the severe toxicities and prolonged expansion of Tregs associated with high dose recombinant IL-2 therapy in cancer patients.

Example 3: Assessing the ability of IL-2/CD25 fusion protein to increase tumor immunity

[00136] An approach to test whether IL-2/CD25 boosts tumor immunity in polyclonal T cells in a normal mouse was developed, as this setting is analogous to that encountered in a cancer patient. Initial attempts focused on amplifying anti-tumor immunity induced by a cell-based vaccine (GVAX) or tumor-related peptides such as gp100. These approaches were unsuccessful, perhaps because it needed to break immune tolerance.

[00137] It was then assessed whether it was possible to amplify responses induced by vaccination with tumor neo-antigens. This approach is most analogous to the responses amplified by Pmel-1 and TRP-1 T cells. Some neo-antigens were already defined and tested for B16.F10 melanoma (15). Four of these neo-antigens were synthesized for testing (**Fig. 7**). Initial attempts directly evaluated the basic strategy used with Pmel-1 and TRP-1 T cells, i.e., priming tumor neo-antigens and then administering IL-2/CD25 one day later. However, unexpectedly, this approach did not lead to an increase in tumor neo-antigen-specific T cells, even when a prime/boost strategy was employed, where IL-2/CD25 was administered after each application of the tumor neo-antigen (**Figs. 8A-8E**). Only after several attempts, where the application of the IL-2/CD25 was varied, were conditions identified whereby the anti-tumor T cells response was substantially enhanced. The successful regimen depended upon, priming, boosting, and then applying mIL-2/mCD25, as this approach led to the highest responses to a mixture of neo-antigen peptides as measured by IFN γ ELISpots (**Fig. 8C**). This response was maintained when administering a second boost with the peptide/poly(I:C) and IL-2/CD25 (**Fig. 8C and 8E**). The neo-antigen specific T cells were largely CD4⁺ T cells (**Figs. 9A-9B**), with these neo-antigen peptides.

[00138] When polyclonal C57BL/6 mice were first immunized with these neo-antigens and then challenged with B16.F10, tumor protection was only noted when IL-2/CD25 was given after the boost with the neo-antigen peptides (**Figs. 10A-10C**). This prime/boost regimen followed by IL-2/CD25 also delayed the growth of pre-established B16.F10 (**Figs. 11A-11C**). Evaluation of lymphoid cells in the tumor microenvironment showed increases in T cells, NK cells, macrophages and myeloid lineage cells (**Figs. 12A-12R**). An increase in the Treg: CD4⁺ T cell ratio was noted, which might not be favorable for anti-tumor responses. However, these Tregs showed a lower fraction of activated CD44^{hi} effector Tregs, a phenotype associated with highly functional Tregs (**Figs. 13A-13C**). This finding suggests that IL-2/CD25 supports less functional Tregs in the tumor microenvironment. An IL-2/CD25-dependent change in the CD8⁺ and CD4⁺ T cell compartment within the tumor environment that would be favorable for an anti-tumor response was an increase in granzyme B, which is expected to enhance CTL activity (**Figs. 14A-14L**). Although minimally affected in CD4⁺ and CD8⁺ T conventional cells, expression of CTLA-4, PD-1, and Tim-3 was significantly altered on tumor-resident Tregs, but these changes seem unlikely to promote anti-tumor responses. Lastly, IL-2/CD25 promoted an increase in CD4⁺ and CD8⁺ T effector and memory cells in the tumor-microenvironment (**Figs. 15A-15G**). Presumably, the majority of the cells are tumor-antigen specific. In any case, these changes coincided with a favorable anti-tumor response that was supported by IL-2/CD25 and neo-antigen peptide vaccine. Thus, these findings indicate that application of neo-antigen peptides, adjuvant, here in the form of Poly (I:C), and IL-2/CD25 promoted an anti-tumor immune response.

[00139] The studies with B16.F10 melanoma primarily tested the capacity of neo-antigens/IL-2/CD25 combination to amplify CD4⁺ neo-antigen-specific T cells. To extend this approach to other neo-antigen peptides, five MHC class I-restricted neo-antigen peptides from the 4T1 mammary carcinoma (5, 15) (**Fig. 16**) were synthesized and used to induce CD8⁺ neo-antigen-specific T cell responses. The efficacy of different TLR adjuvants was also tested. Priming and boosting with 4T1 neo-antigens with Poly (I:C) or Gardiquimod, but not LPS, led to significantly increased numbers of peptide-specific T cells only when the IL-2/CD25 fusion protein was administered after the boost. (**Figs. 17A-17B**). In a second experiment, IL-2/CD25 increased the frequency of 4T1 neo-antigen-specific T cells when administered after the boost, but not after the priming injection only or after the priming and boost injections (**Fig. 23B**). These data indicate

that IL-2/CD25 amplifies both CD4⁺ and CD8⁺ polyclonal tumor-neo-antigen-specific T cells when used during boost injections with tumor neo-antigens.

Example 4: Amplification of neoantigen-specific T cells by IL-2/CD25 occurs with less frequent dosing at lower amounts when compared to recombinant IL-2.

[00140] When IL-2 is used in cancer immunotherapy, it is administered at a high dose over a long timeframe with accompanying toxicity that is clinically managed. The ability of IL-2/CD25 and IL-2 to amplify the expansion of Pmel-1 T cells was compared (the same amount of IL-2 and IL-2/CD25 was administered to compare the ability of each to amplify the expansion of Pmel-1 T cells) (**Fig. 18A**). A single injection of IL-2/CD25, but not IL-2, amplified the response of Pmel-1 T cells (**Fig. 18B**). Not only did the amount of IL-2 not amplify Pmel-1 T cells, an equivalent amount of IL-2 administered *five times* did not support amplification of the cells. In a second experiment (**Fig. 18C**), much higher doses of IL-2 (50 and 100 µg), which represent maximal tolerable amounts, were administered twice a day for 3 days. Under these conditions, IL-2 amplified Pmel-1 T cells. However, a single dose of IL-2/CD25, which is 10-20-fold less than the overall amount of IL-2 administered, was more effective in amplifying the expansion of Pmel-1 T cells (**Fig. 18D**). With respect to the response of polyclonal T cells to B16.F10 tumor-neo-antigens (**Fig. 19A**), a single dose of IL-2/CD25 amplified the tumor-specific T cells to a level nearly comparable to that supported by multiple injections of much higher doses of IL-2 (**Fig. 19B**). Thus, IL-2/CD25 readily enhances the frequency of tumor-reactive T cells when administered at lower doses and less frequently than IL-2, an approach that is expected to be accompanied by lower toxicity when compared to IL-2.

[00141] All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this disclosure that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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CLAIMS

WHAT IS CLAIMED:

1. A method of increasing neo-antigen-specific T cells in a mammalian subject, the method comprising administering to a subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.
2. A method of enhancing immunogenicity to a neo-antigen in a mammalian subject, the method comprising administering to the subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.
3. The method claim 2, wherein enhancing immunogenicity comprises enhancing cancer-specific T cell response in a mammalian subject.
4. A method of treating cancer, the method comprising administering to mammalian subject a therapeutically effective amount of an IL-2/CD25 fusion protein in combination with at least one neo-antigen.
5. The method of any one of claims 1-4, wherein administration of one or more doses of neo-antigen is followed by administration of one of more doses of the IL-2/CD25 fusion protein.
6. The method of any one of claims 1-4, wherein the IL-2/CD25 fusion protein is delivered within 7 days of administration of the neo-antigen peptide.
7. The method of any one of claims 1-4, wherein a second dose of the IL-2/CD25 fusion protein is delivered within 7 days after administration of the neo-antigen peptide.
8. The method of any one of claims 1-4, wherein one or more doses of neo-antigen peptide is administered no more than 10 days prior to administration with the IL-2/CD25 fusion protein.

9. The method of any one of claims 1-4, wherein the subject is administered a priming dose comprising at least one neo-antigen no more than 10 days prior to administration of the IL-2/CD25 fusion protein.

10. The method of claim 9, wherein the subject is administered one or more boosting doses comprising at least one neo-antigen 1-30 days after administration of the priming dose comprising at least one neo-antigen.

11. The method of claim 10, wherein the subject is administered a dose comprising the IL-2/CD25 fusion protein within 7 days of administration of the boosting dose comprising at least one neo-antigen.

12. The method of any one of claims 1-4, wherein the subject is administered a second dose comprising the IL-2/CD25 fusion protein within 7 days after administration of the initial dose comprising the IL-2/CD25 fusion protein.

13. The method of any one of claims 1-4, wherein the subject is administered a second dose comprising the IL-2/CD25 fusion protein within 10 days after administration of the initial dose comprising the IL-2/CD25 fusion protein.

14. The method of any one of claims 1-4 further comprising the steps of

- (a) administering a priming dose comprising at least one neo-antigen;
- (b) administering a first boosting dose comprising at least one of the neo-antigen(s) 1-30 days after step (a);
- (c) administering a first dose comprising the IL-2/CD25 fusion protein within 7 days after step (b);
- (d) administering a second or more boosting doses comprising at least one neo-antigen(s) within 30 days after step (c); and
- (e) administering a second dose comprising the IL-2/CD25 fusion protein within 7 days after step (d).

15. The method of any one of claims 1-4, wherein the IL-2/CD25 fusion protein comprises (a) a first polypeptide comprising Interleukin-2 (IL-2) or a functional variant or fragment thereof and (b) a second polypeptide, comprising CD25 or a functional variant or fragment thereof, fused in frame to said first polypeptide, wherein said fusion protein has IL-2 activity.
16. The method of any one of claims 1-4, wherein the administration step comprises administering to the subject a neo-antigen peptide.
17. The method of any one of claims 1-4, wherein the administration step comprises administering to the subject a nucleic acid molecule that encodes the neo-antigen peptide.
18. The method of any one of claims 1-4, wherein the administration step comprises administering to the subject a vector comprising a nucleic acid molecule that encodes the neo-antigen peptide.
19. The method of any one of claims 1-4, wherein the neo-antigen comprises a mutation specific to a tumor of the subject.
20. The method of any one of claims 1-4, wherein the neo-antigen is administered with an adjuvant.
21. The method of claim 20, wherein the adjuvant is Polyinosinic:polycytidylic acid (poly-IC), MPL, GLA, imiquimod, CpG ODN, LPS, Polyinosinic-Polycytidylic Acid stabilized with Polylysine and Carboxymethylcellulose (poly-IC LC) gardiquimod, aluminum, resiquimod, sodn-dsRNA, flagellin, or SMP-105.
22. The method of claim 20, wherein the adjuvant is STING agonists or liposomes.
23. The method of any one of claims 1-4, wherein administration of the IL-2/CD25 fusion protein increases the number of CD4⁺ and CD8⁺ T effector and memory cells.

24. The method of any one of claims 1-4, wherein the IL-2/CD25 fusion protein is administered in combination with one or more checkpoint inhibitors.
25. The method of claim 24, wherein the checkpoint inhibitor is selected from the group consisting of an anti-PD-1 antibody, an anti-PDL-1 antibody, an anti-CTLA-4 antibody, a PD-1 inhibitor, a PDL-1 inhibitor or a CTLA-4 inhibitor, anti-Tim-3 antibody, anti-LAG3 antibody, and anti-TIGIT antibody.
26. The method of any one of claims 1-4, wherein said mammalian subject is a human.
27. The method of any one of claims 1-3, wherein said subject is suffering from cancer.
28. The method of claim 4 or claim 27, wherein the cancer is melanoma, cutaneous melanoma, ocular melanoma, cervical cancer, follicular B cell non-Hodgkin's lymphoma, kidney cancer, prostate cancer, and multiple myeloma, breast cancer, lung cancer, colon cancer, ovarian cancer, bladder cancer, pancreatic cancer, endometrial cancer, liver cancer, thyroid cancer, or leukemia.

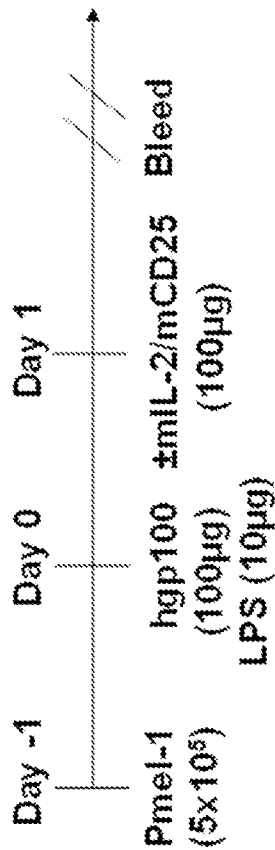
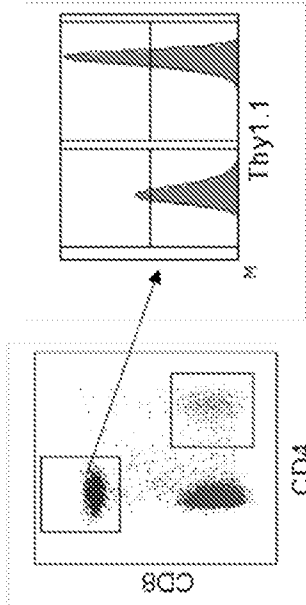


FIG. 1A



• Thy1.1 marks Pmel-1 cells

FIG. 1B

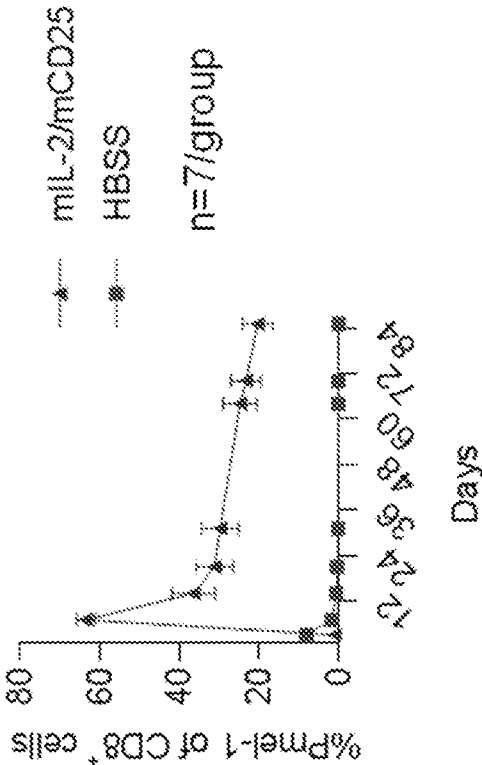
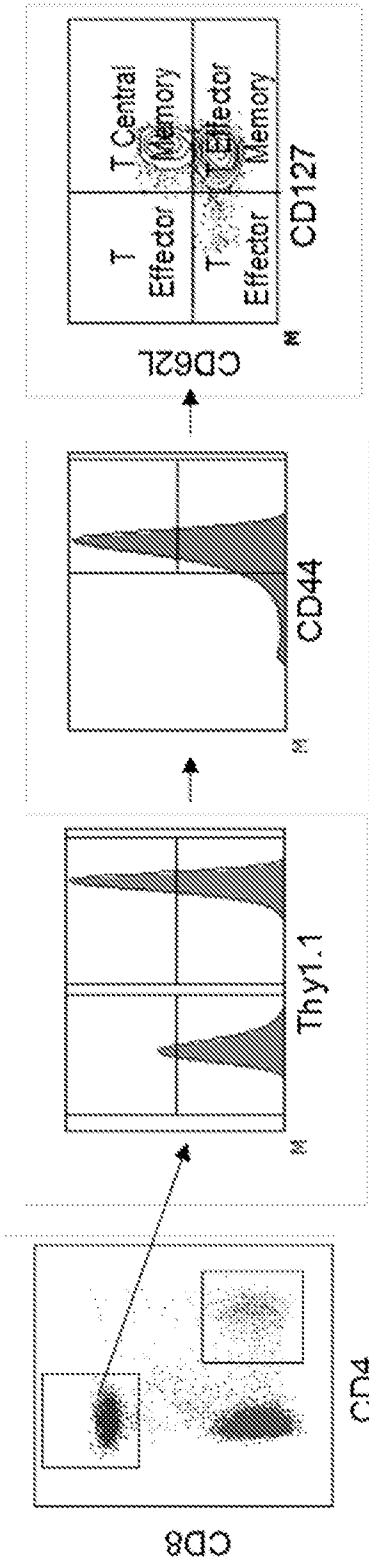


FIG. 1C



Effector Memory **FIG. 2A** Central Memory

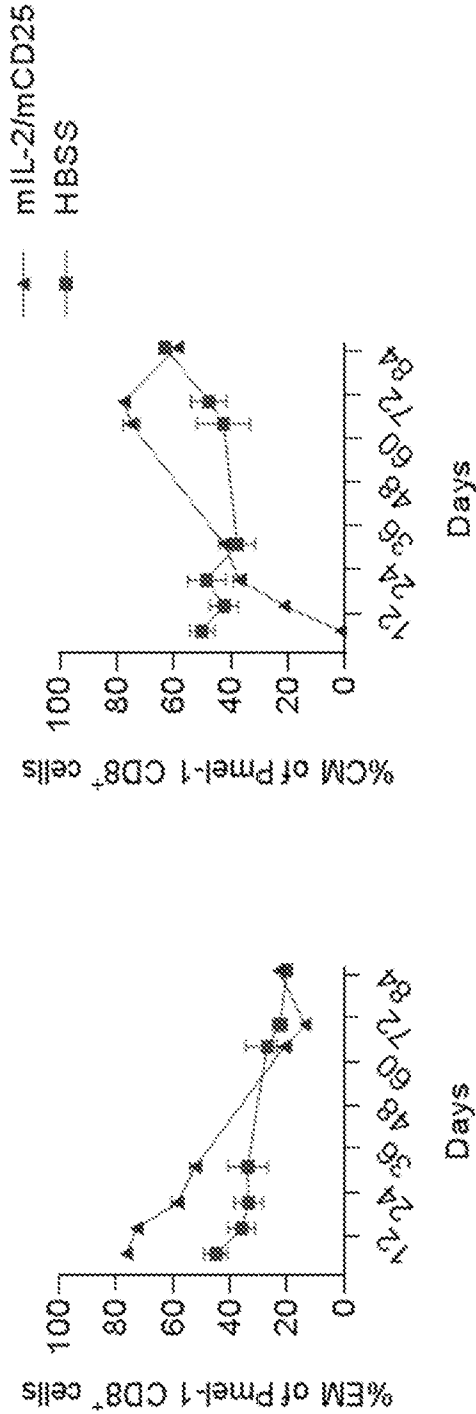


FIG. 2B **FIG. 2C**

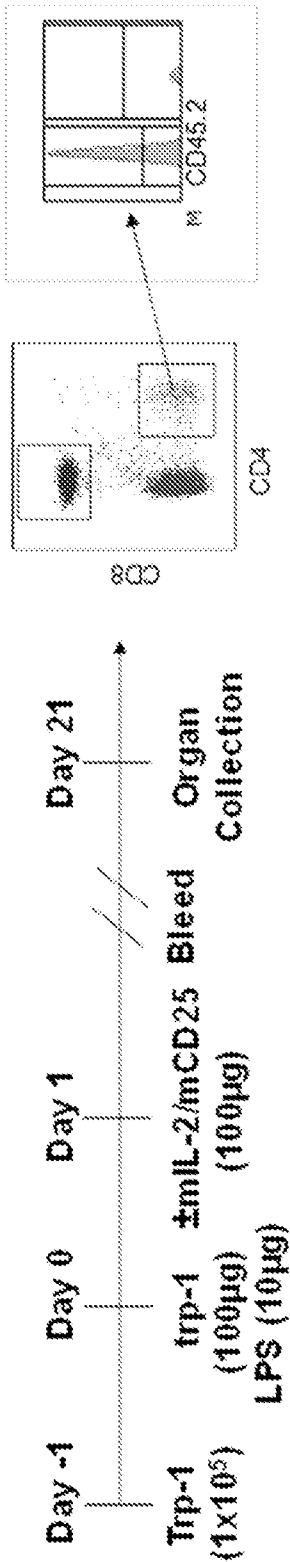


FIG. 3A

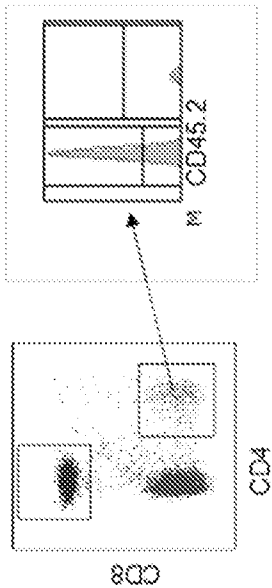


FIG. 3B

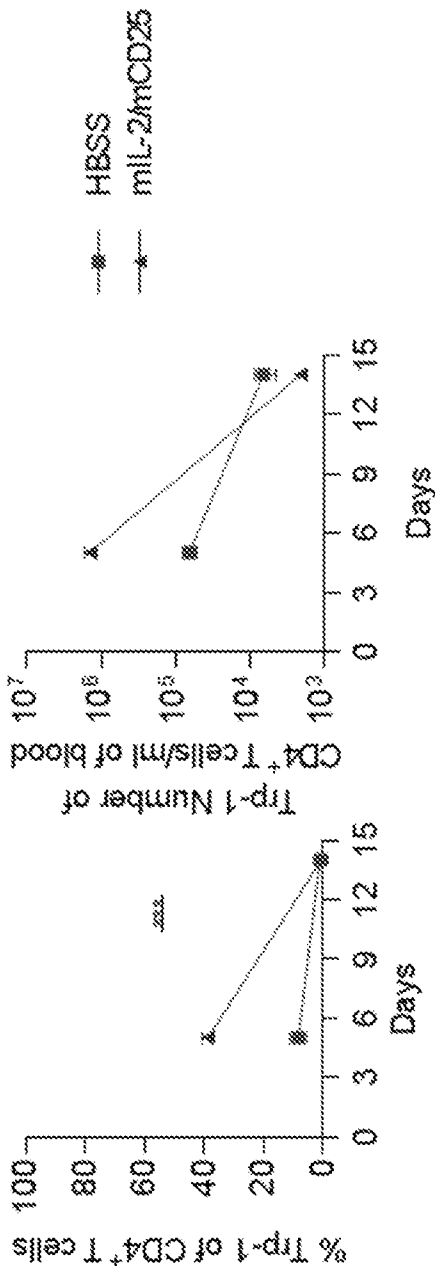


FIG. 3C

FIG. 3D

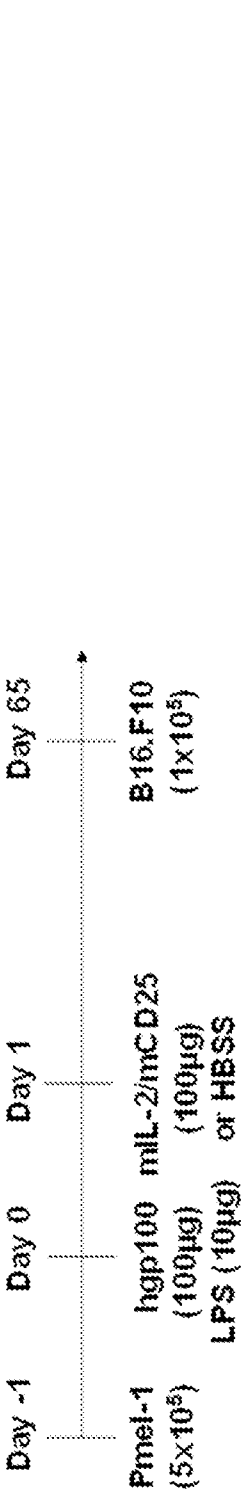


FIG. 4A

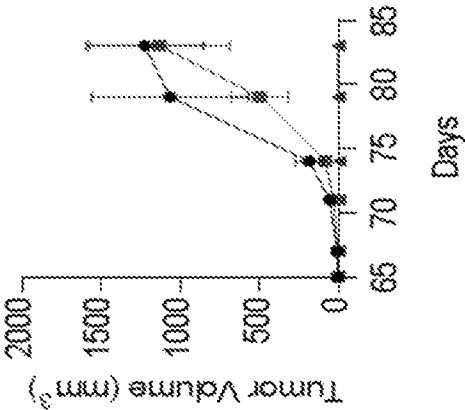


FIG. 4B

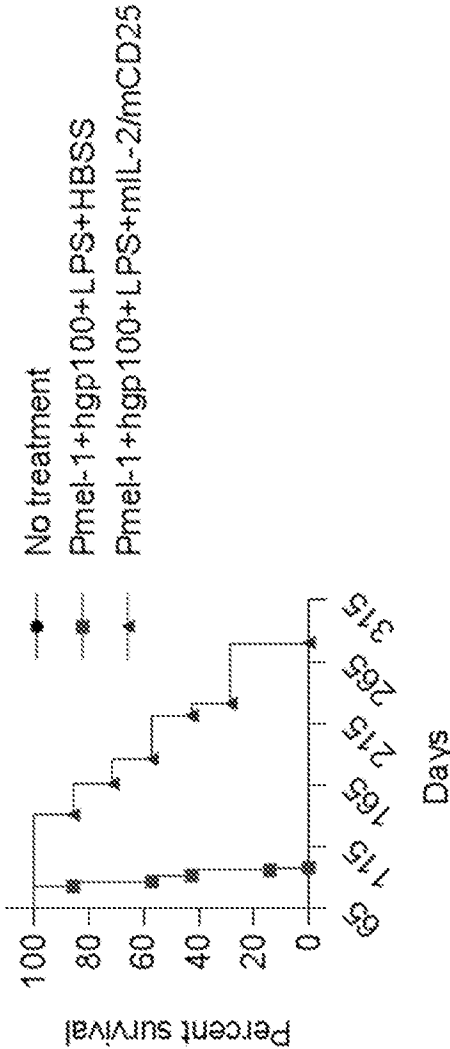


FIG. 4C

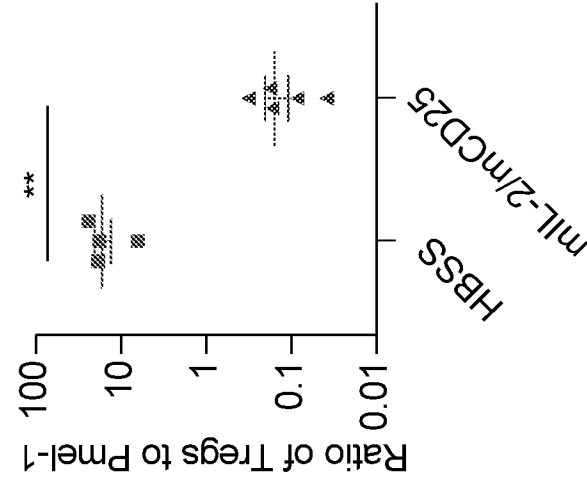


FIG. 5C

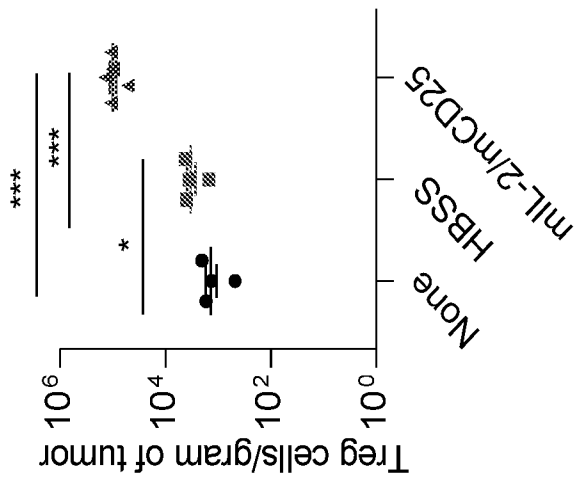


FIG. 5B

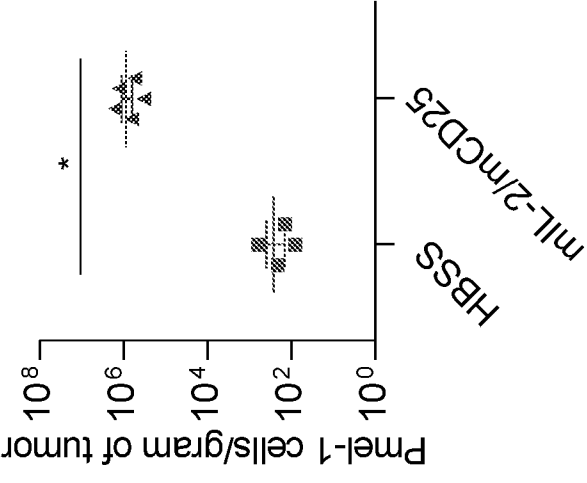


FIG. 5A

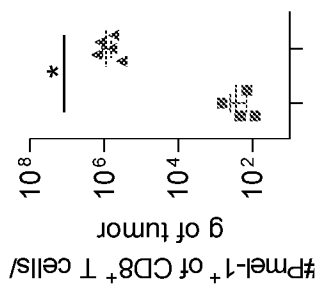


FIG. 5D

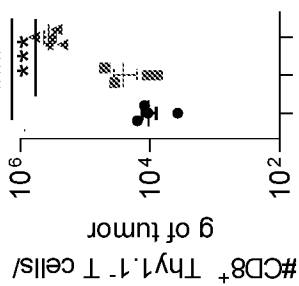


FIG. 5G

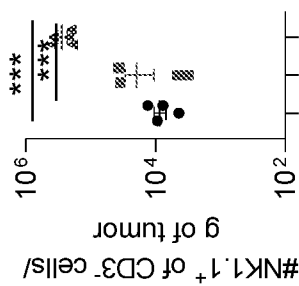


FIG. 5I

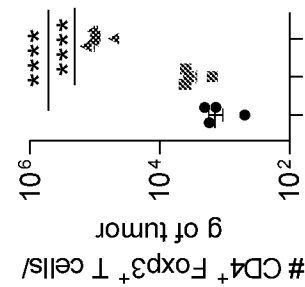


FIG. 5E

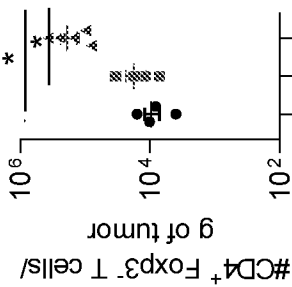


FIG. 5H

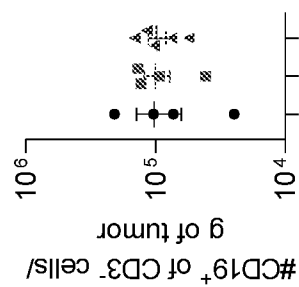


FIG. 5J

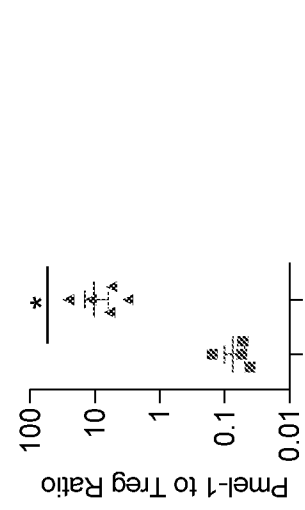


FIG. 5F

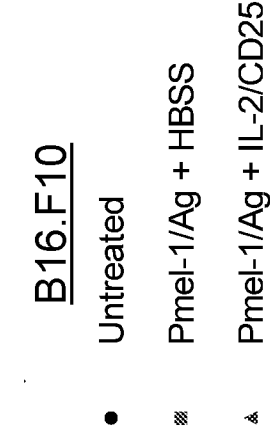


FIG. 5K

B16.F10

- Untreated
- Pmel-1/Ag + HBSS
- ▲ Pmel-1/Ag + IL-2/CD25

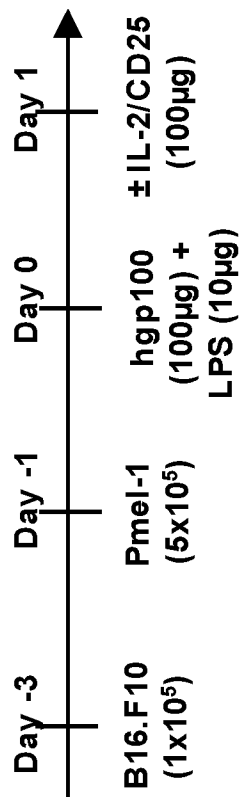


FIG. 6A

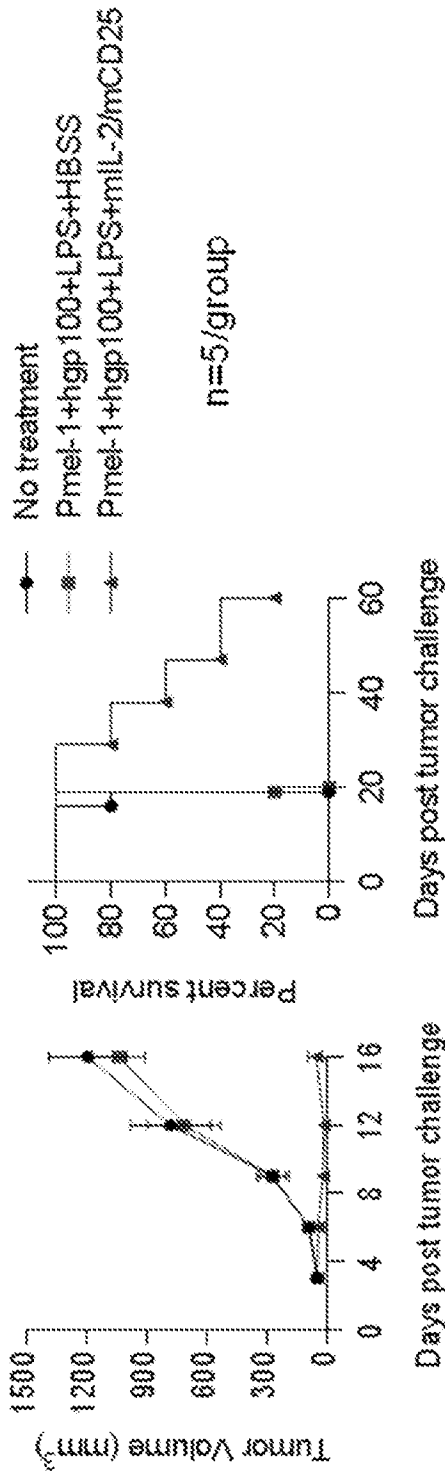


FIG. 6B

FIG. 6C

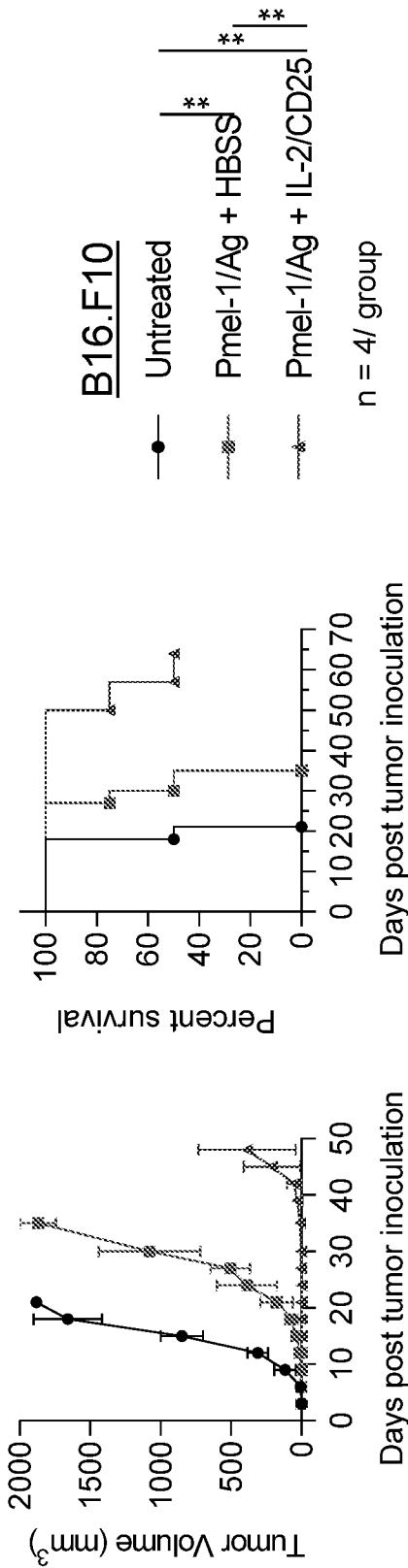


FIG. 6E

FIG. 6D

Mutated Peptides	Gene	Substitution	Reactive T cell subtype	Role
B16M27	Obsl1	T1764M	CD8	Cytoskeletal adaptor proteins
B16M30	Kif18b	K739N	CD4	Vesicle and Golgi to ER retrograde transport
B16M44	Cpsf3l	D314N	CD4	Transcription control
B16M48	Def8	R255G	CD4	Regulator of lysosome distribution

- 27 aa long synthetic peptides, not expressed in healthy mouse tissue
- MHC class I and II restricted with moderate binding capacity
- Response after vaccination obtained with either RNA or peptide sequence

FIG. 7

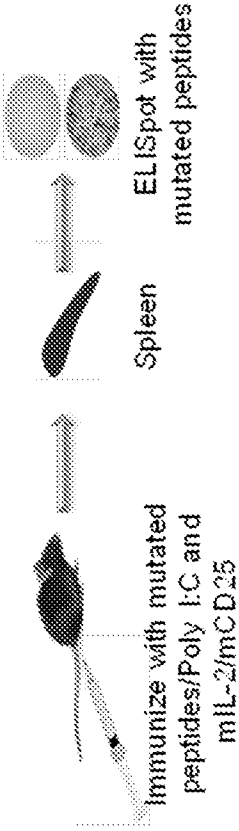
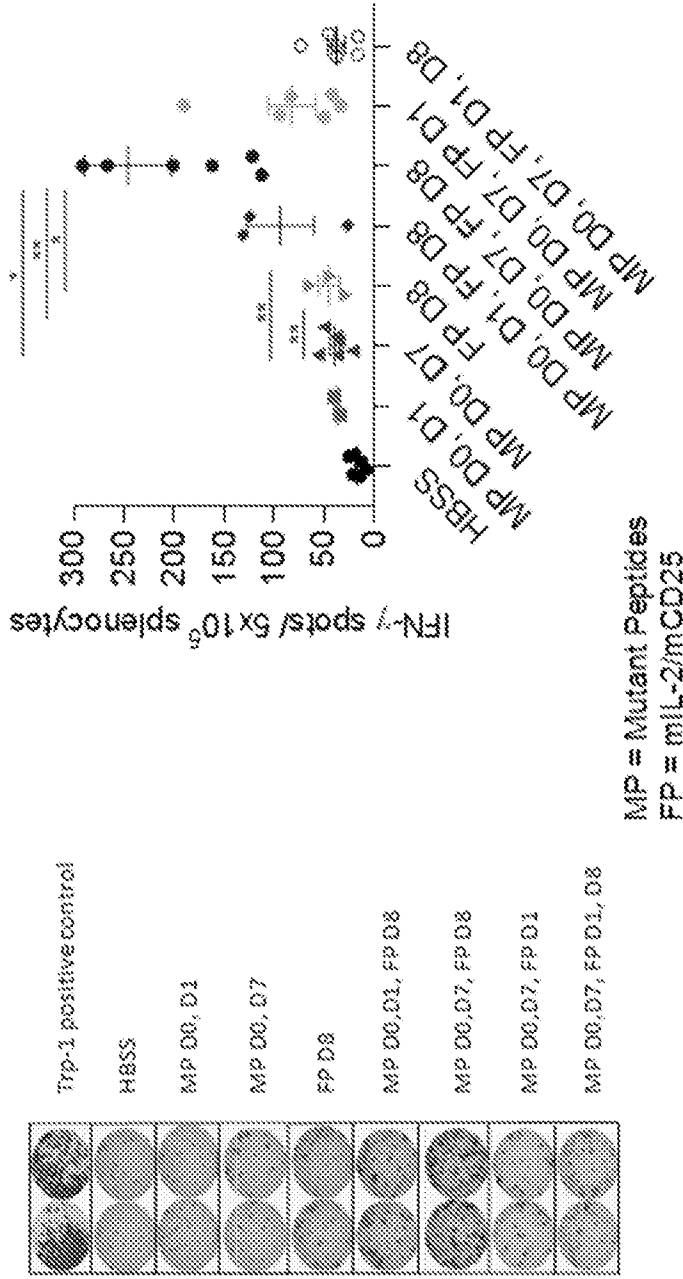


FIG. 8A



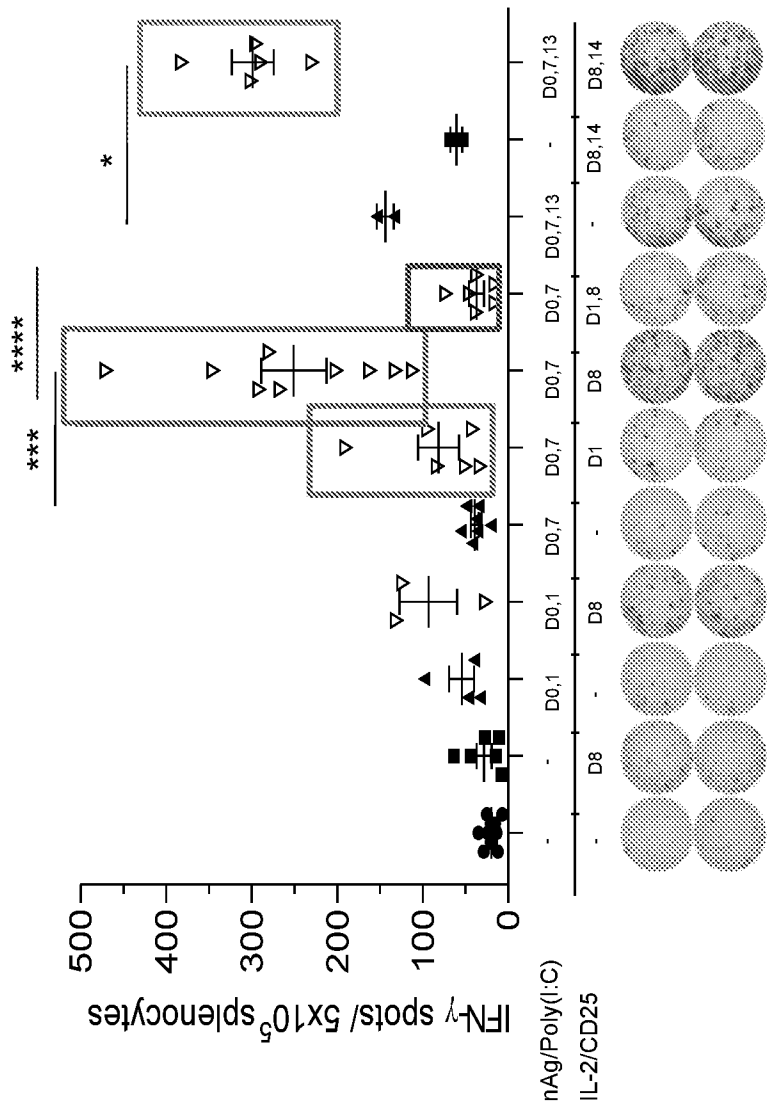
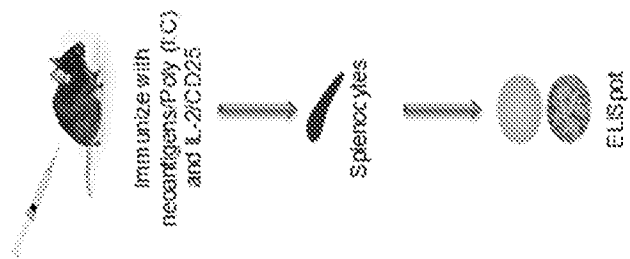


FIG. 8E



Dosing:
nAg/Poly(I:C) = 50 μ g/ 50 μ g/ dose
IL-2/CD25 = 50 μ g/ dose

FIG. 8D

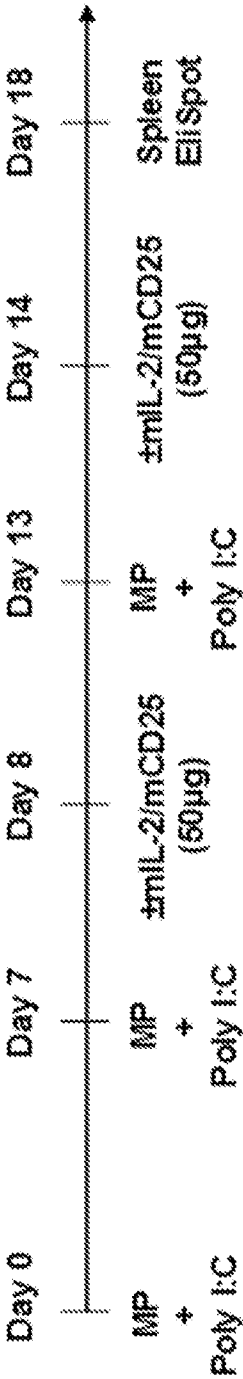


FIG. 9A

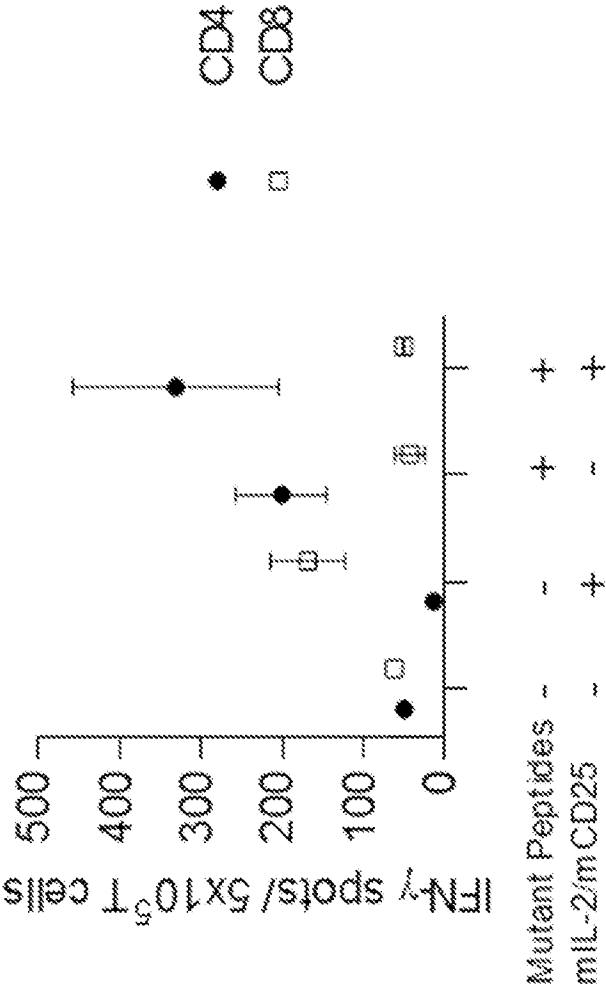


FIG. 9B

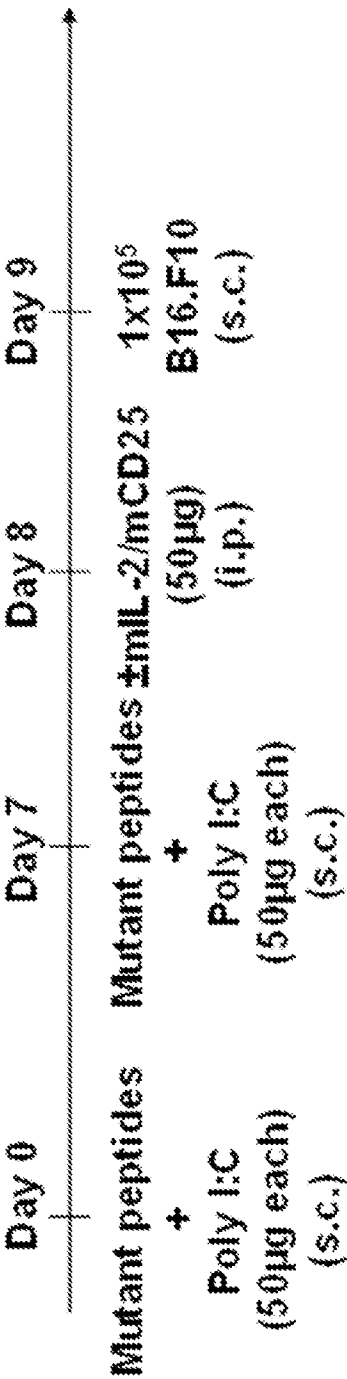


FIG. 10A

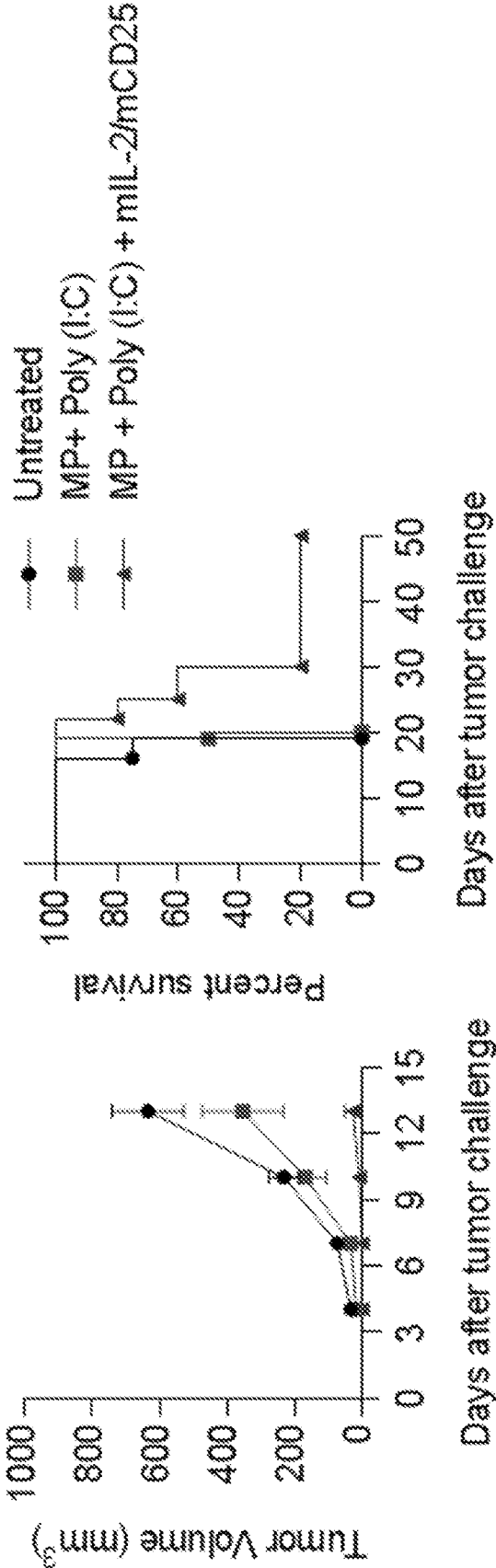


FIG. 10B

FIG. 10C

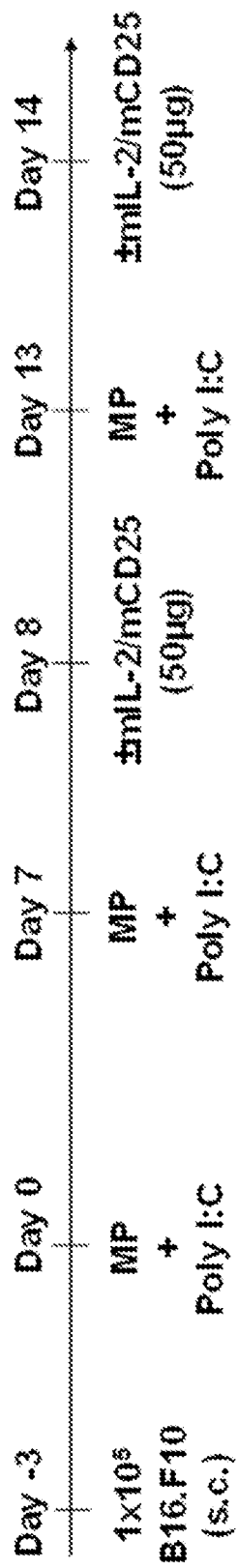


FIG. 11A

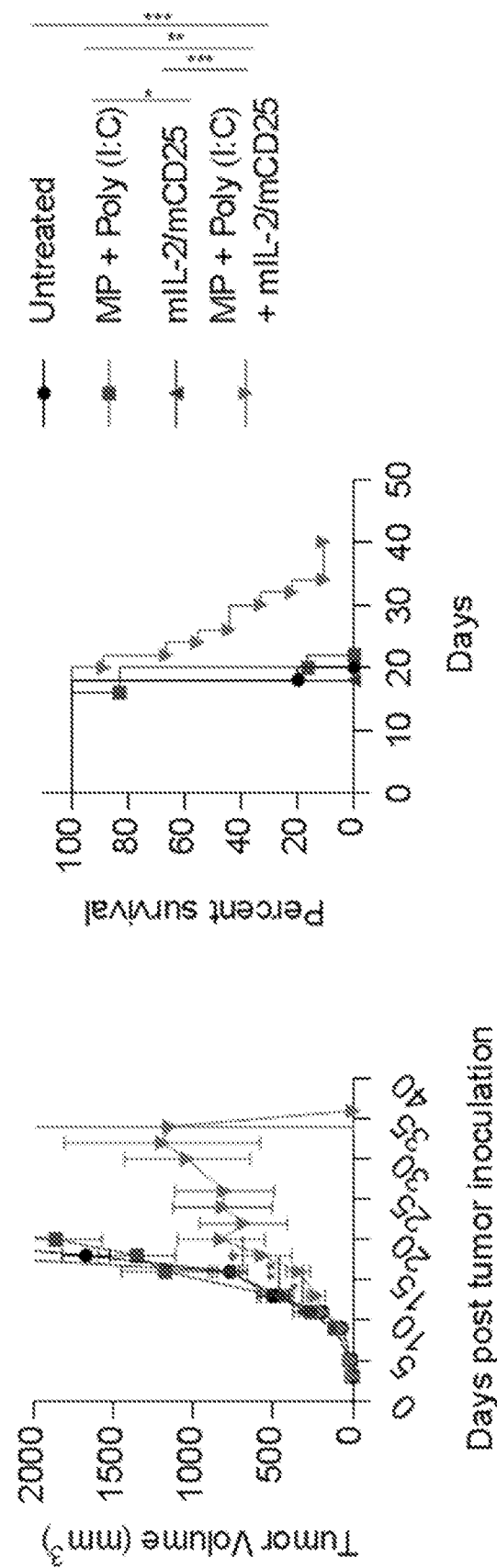


FIG. 11C

FIG. 11B

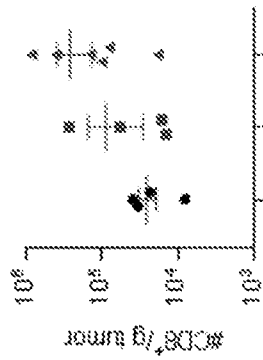


FIG. 12A

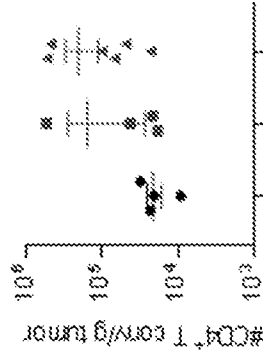


FIG. 12B

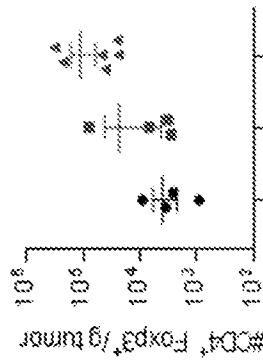


FIG. 12C

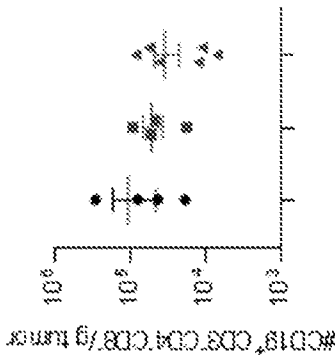


FIG. 12D

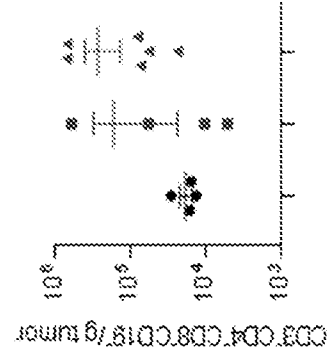


FIG. 12E

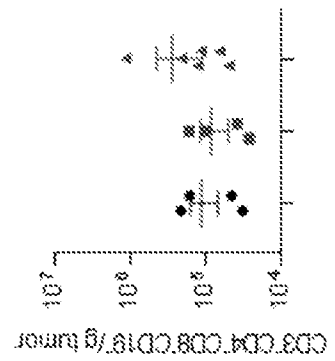


FIG. 12F

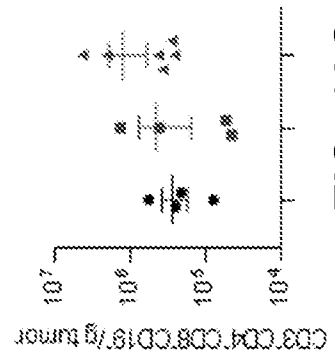


FIG. 12G

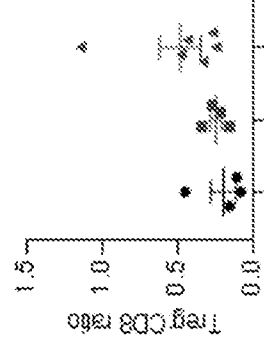


FIG. 12H

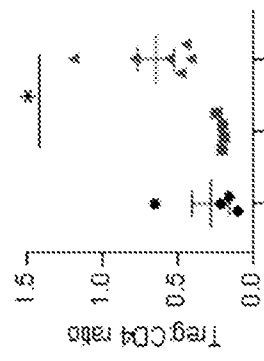


FIG. 12I

• Untreated
■ MP + Poly (I:C)
▲ MP + Poly (I:C)
+ mIL-2/mCD25

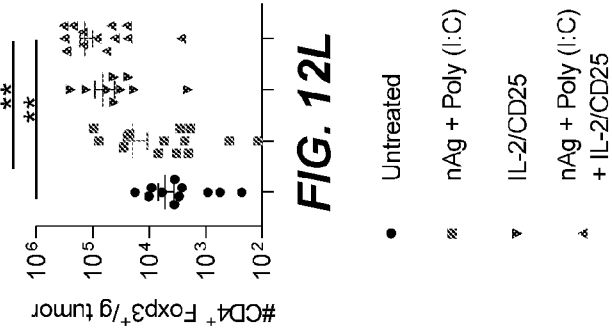


FIG. 12L

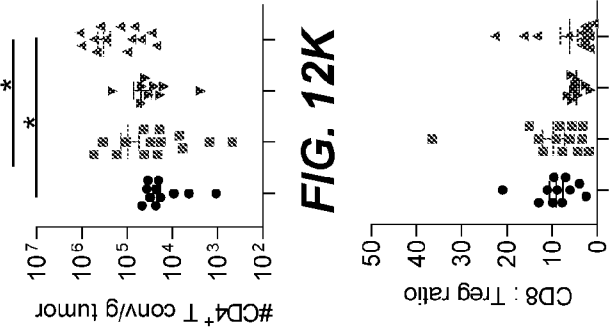


FIG. 12K

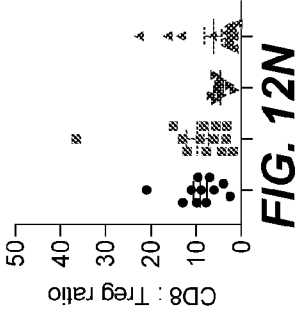


FIG. 12N

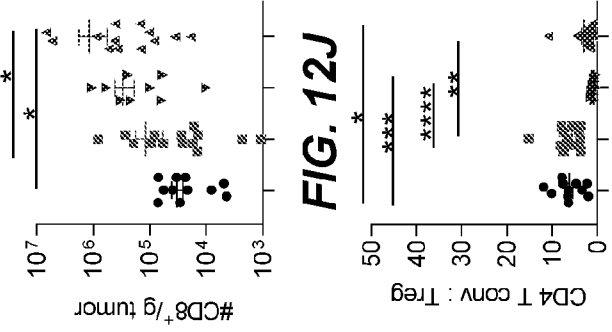


FIG. 12J

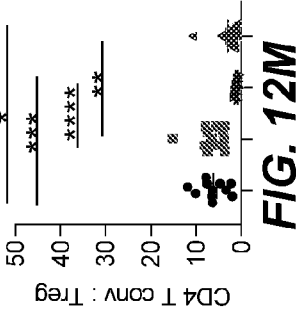


FIG. 12M

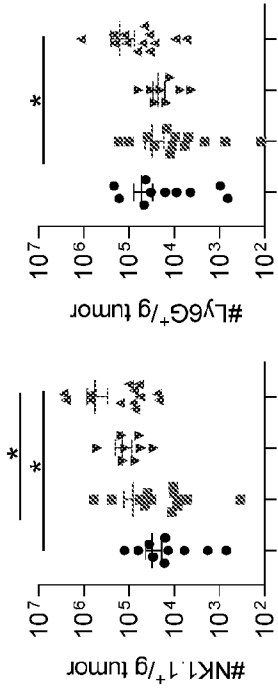


FIG. 12O

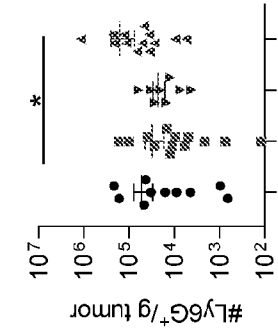


FIG. 12P



FIG. 12Q

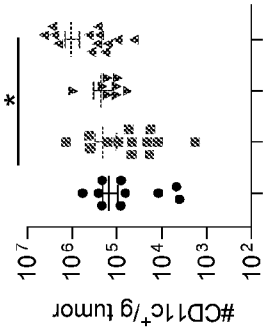


FIG. 12R

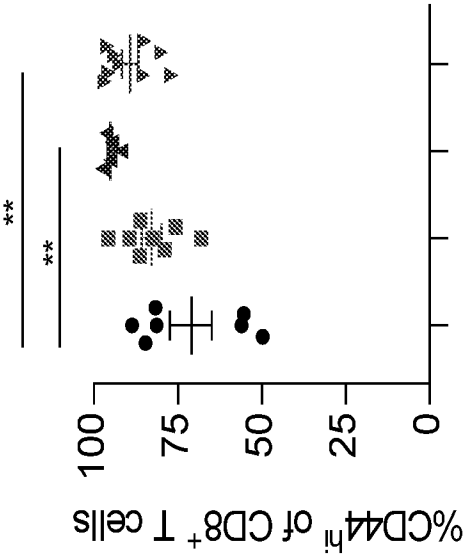


FIG. 13A

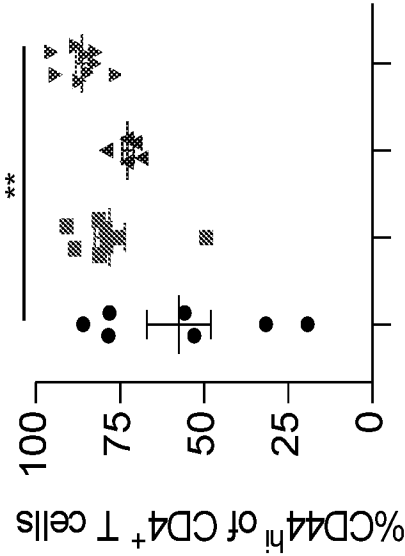


FIG. 13B

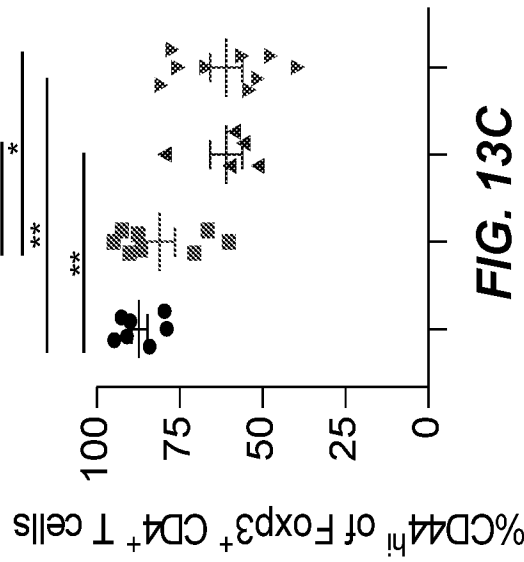
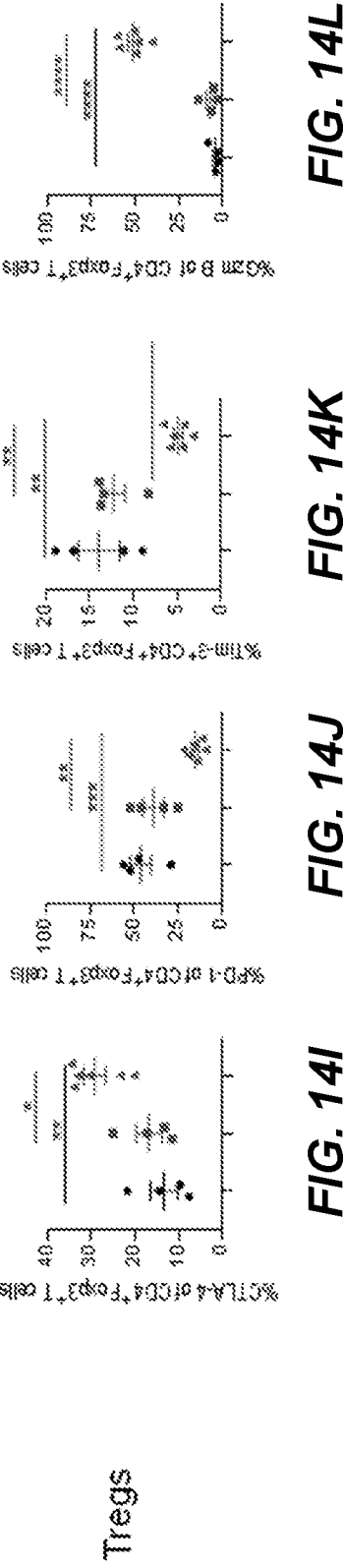
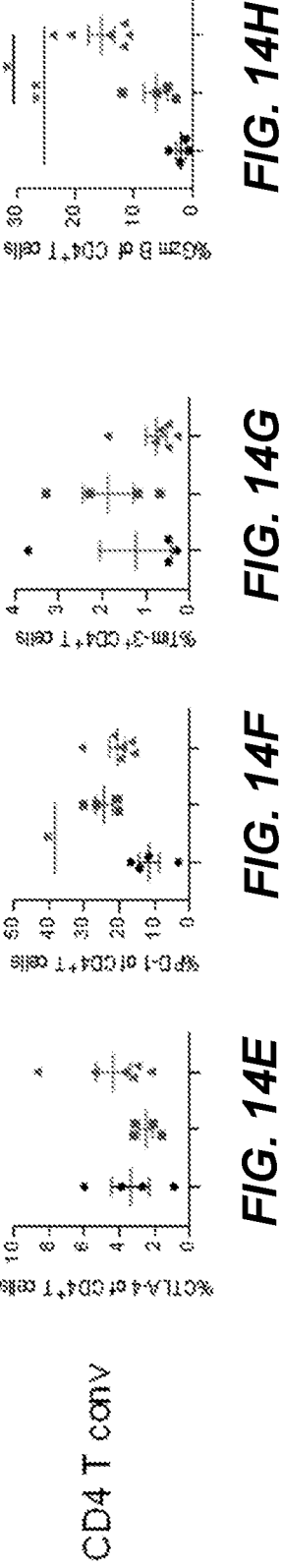
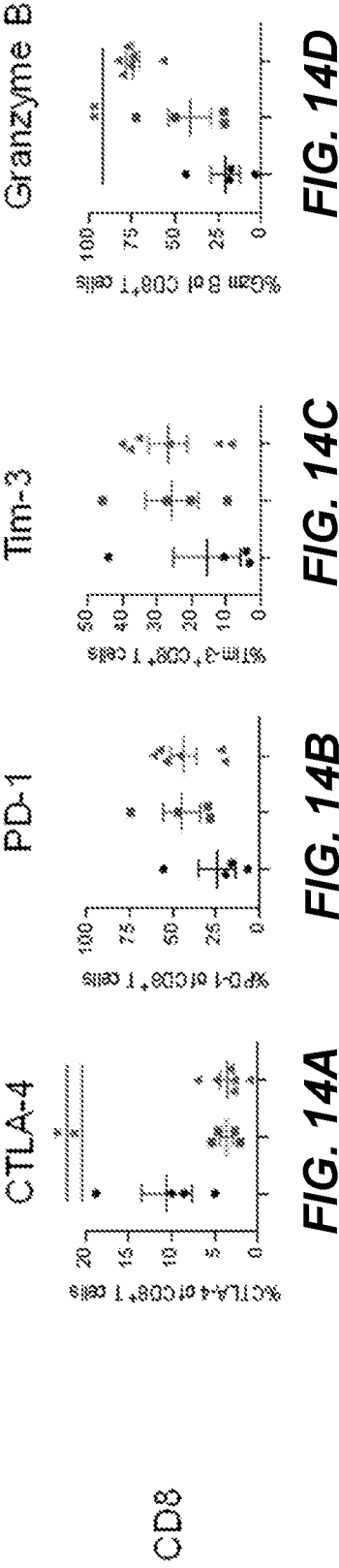
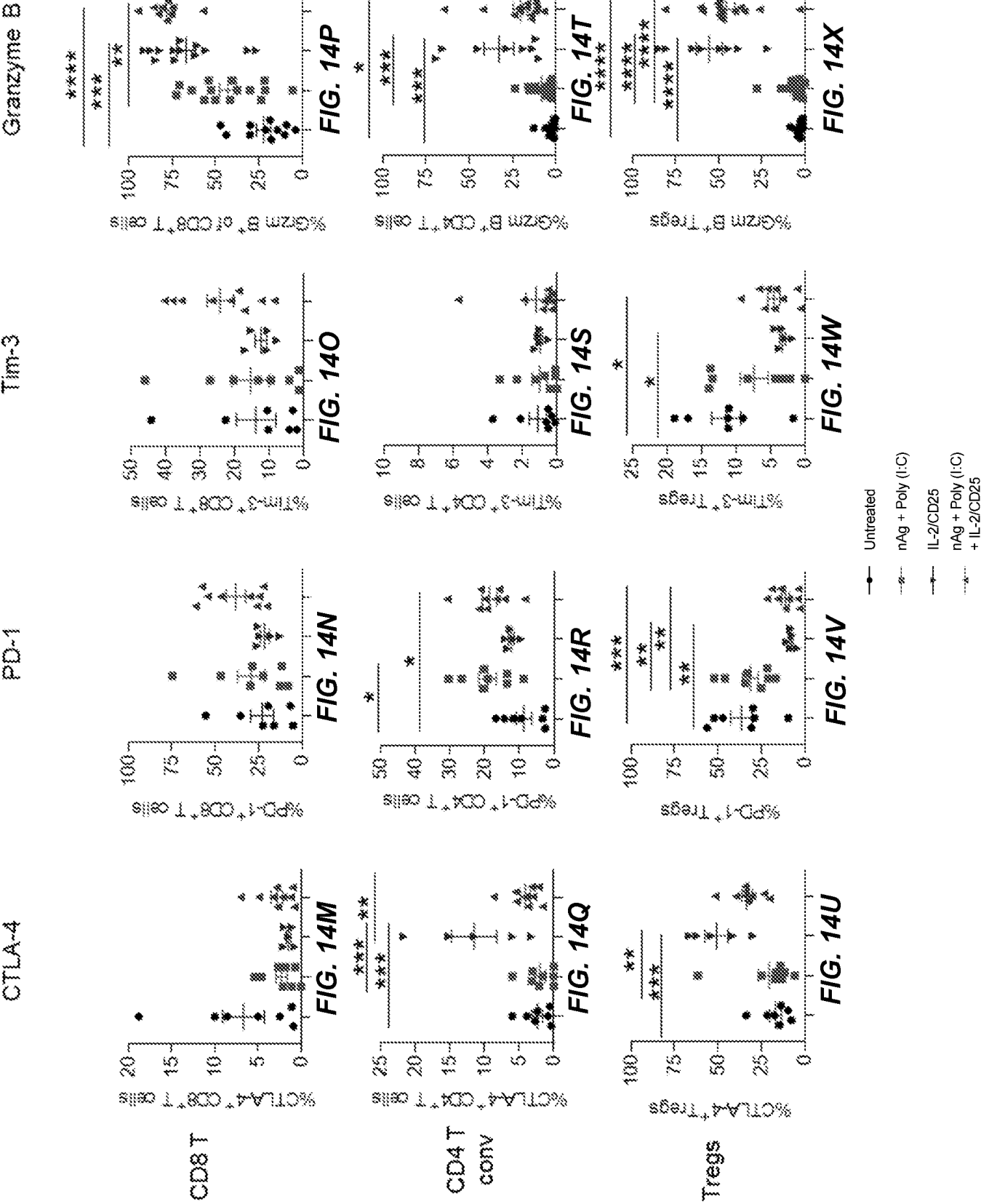


FIG. 13C





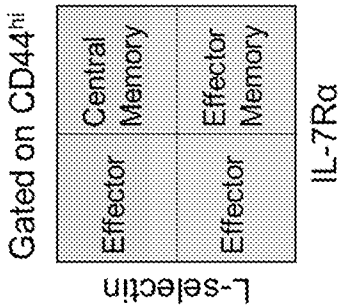
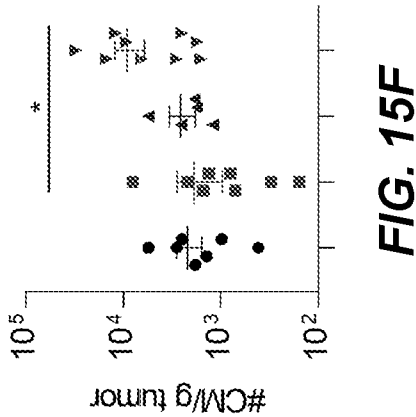
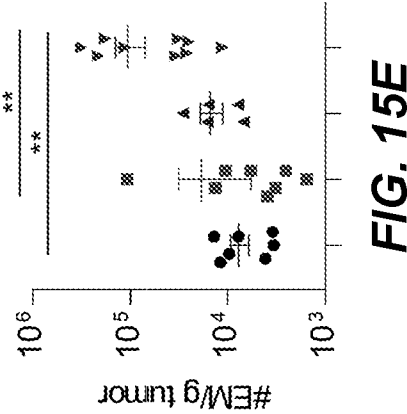
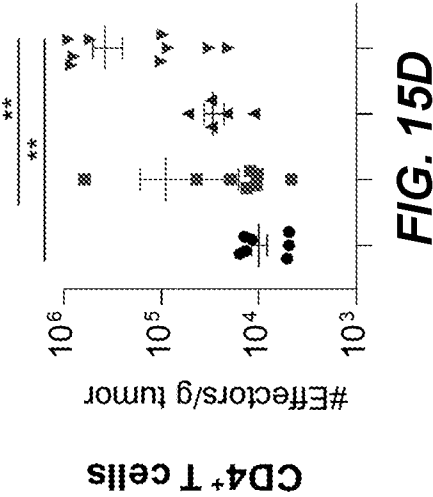
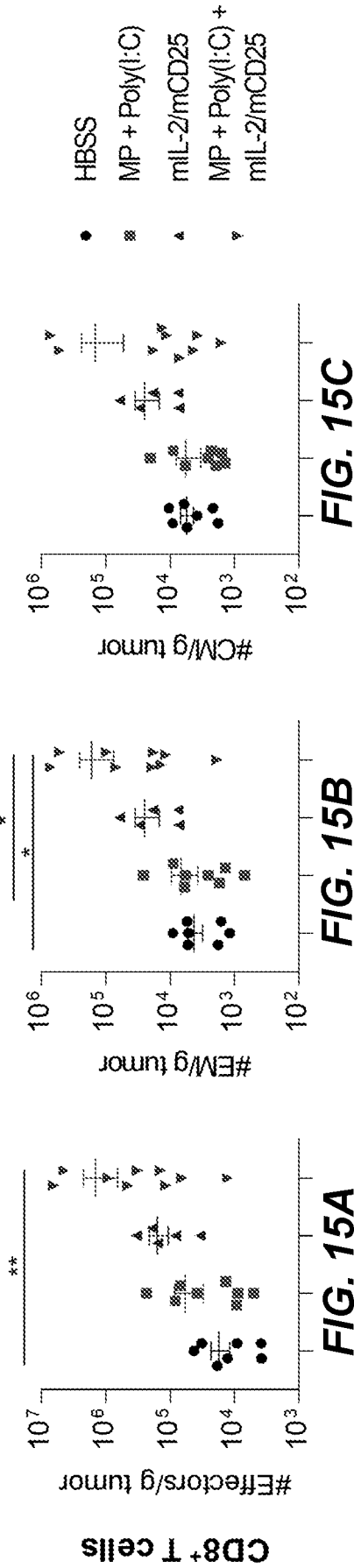


FIG. 15G

Mutated peptides	Gene	aa#	Reactive T cell subtype
4T1-M8	Tmtc2	27	CD8
4T1-M20	Wdr11	27	CD8
4T1-M26	Pzp	27	CD8
4T1-M27	Gprc5a	27	CD8
4T1-M35	Zzz3	27	CD8

• MHC class I restricted 27 aa long synthetic peptides, not expressed in healthy mouse tissue

FIG. 16

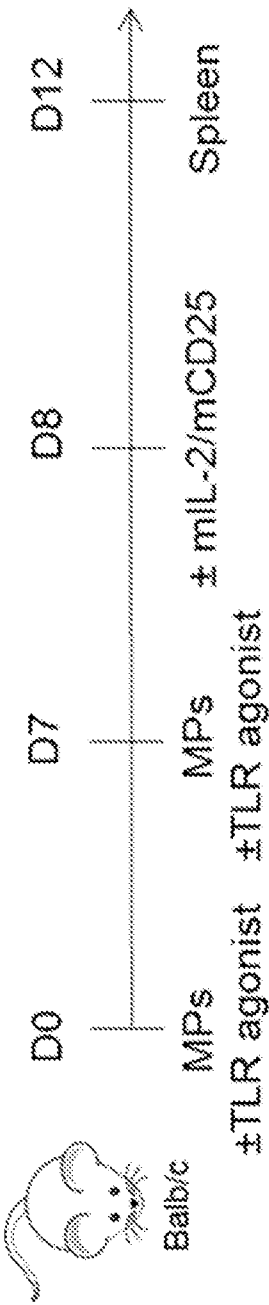


FIG. 17A

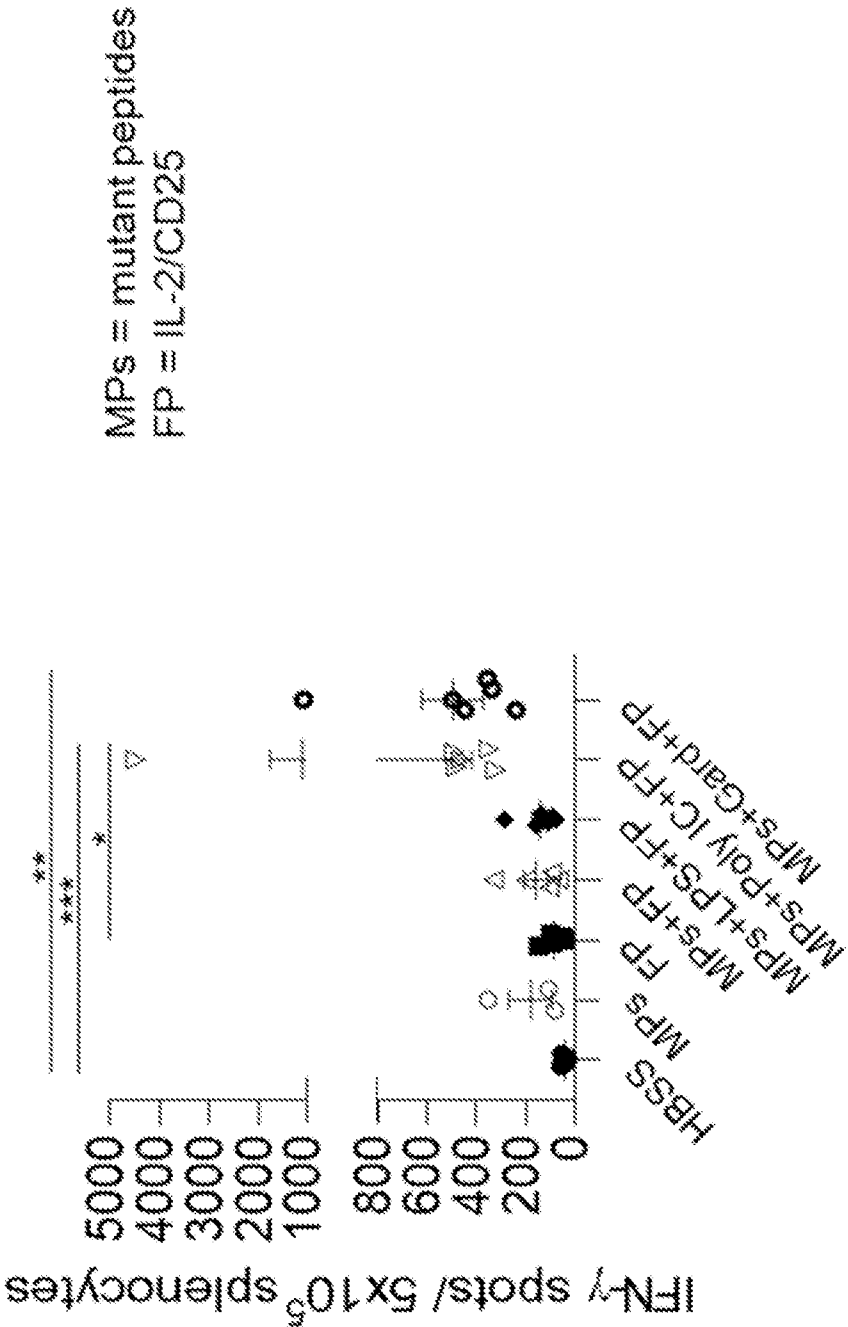


FIG. 17B

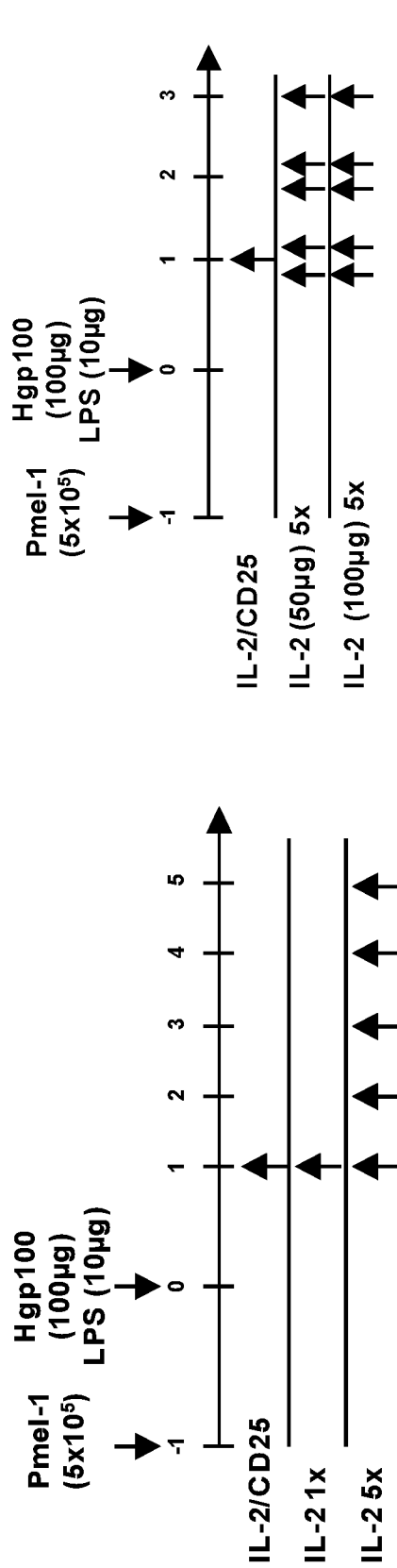


FIG. 18C

FIG. 18A

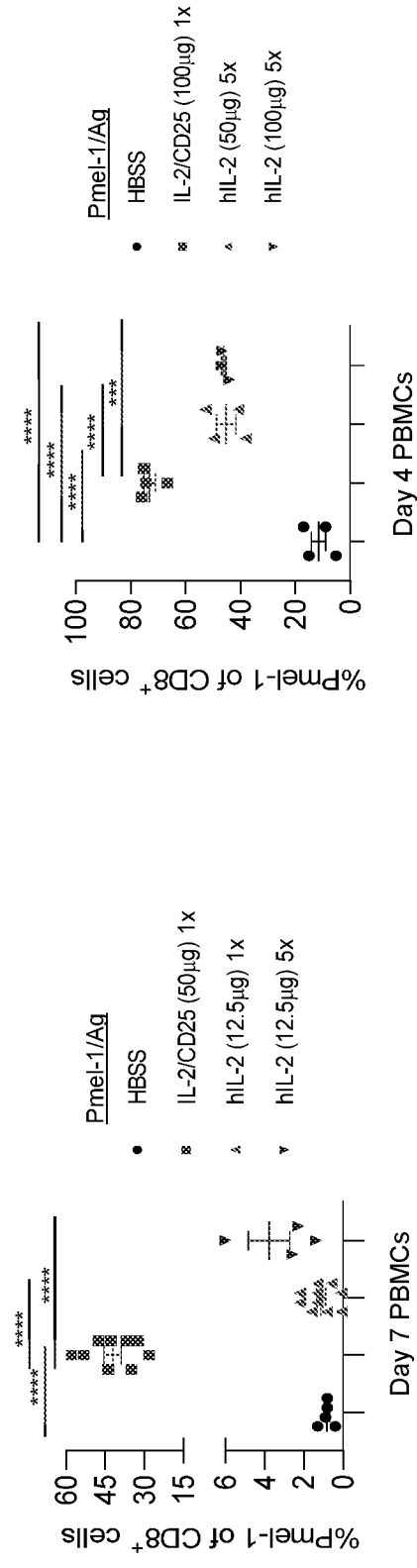


FIG. 18D

FIG. 18B

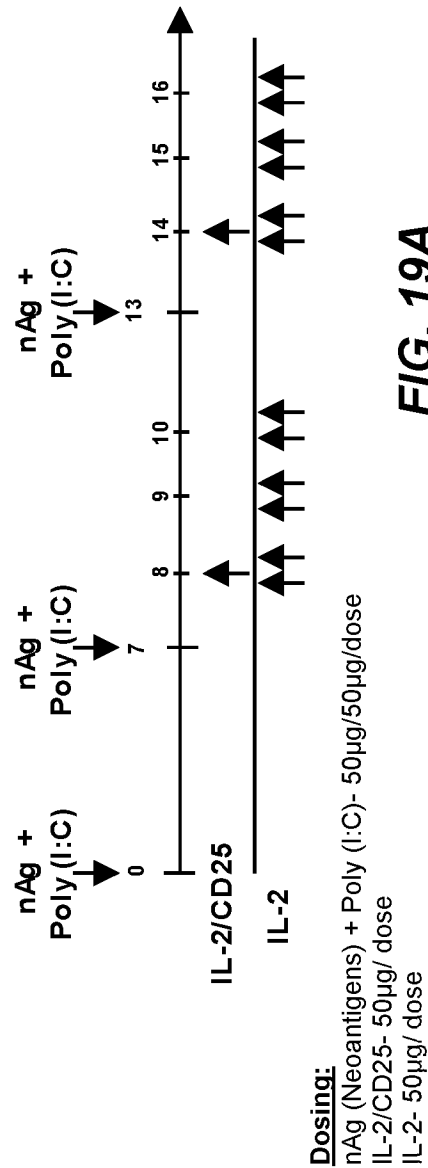


FIG. 19A

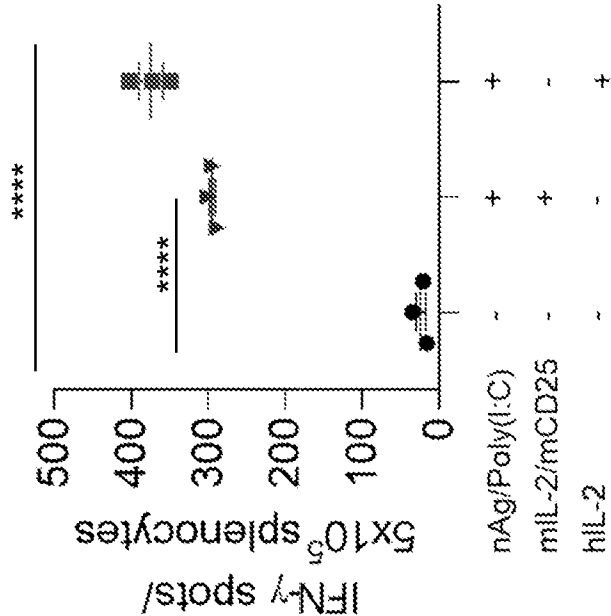


FIG. 19B

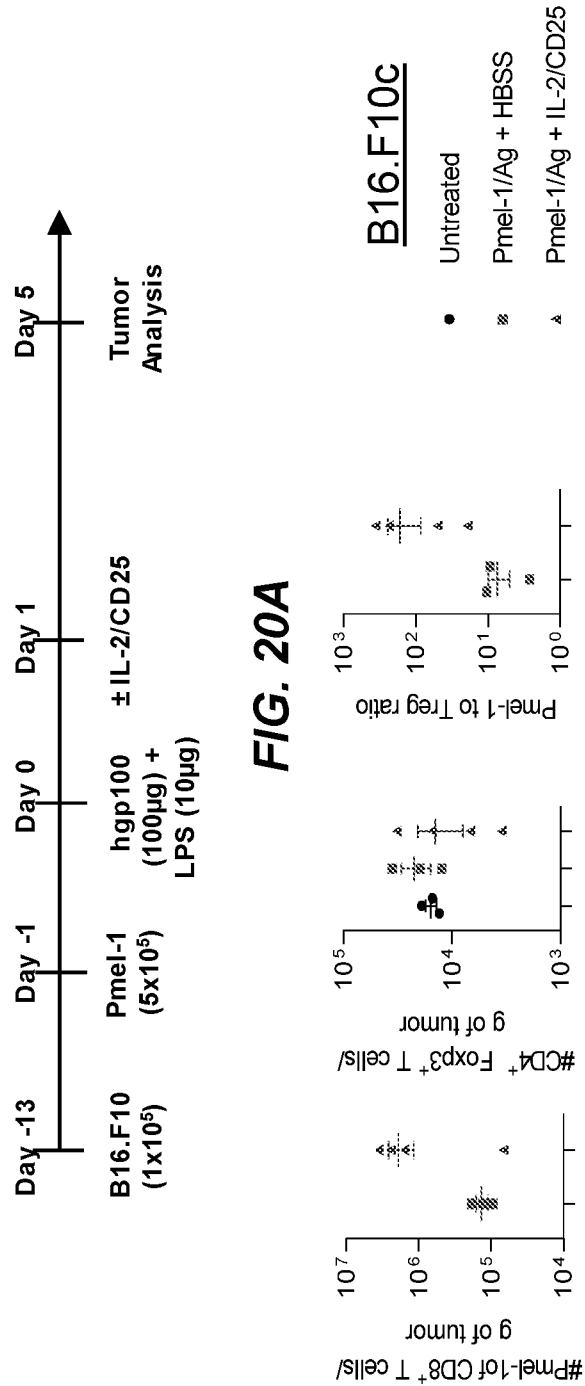


FIG. 20A

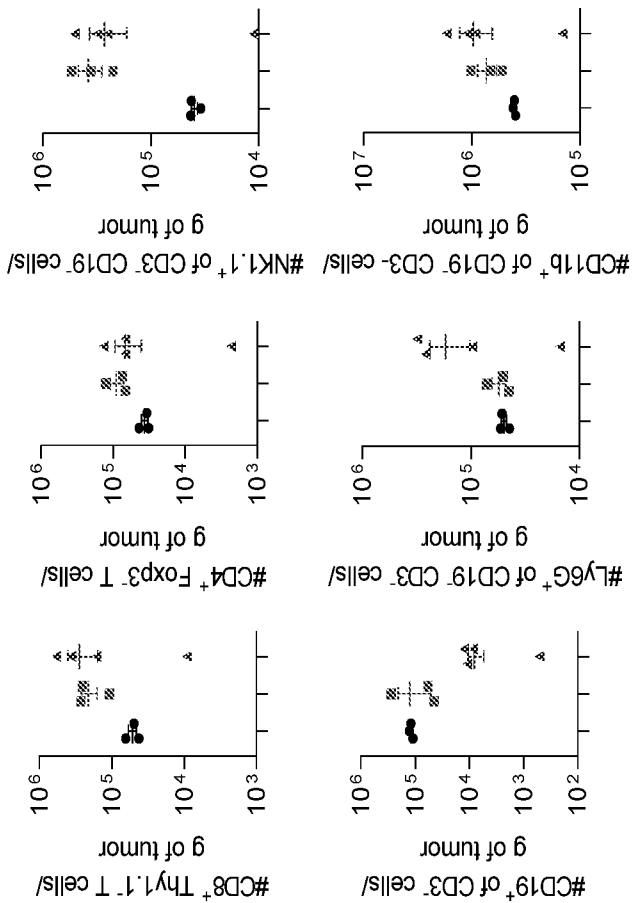


FIG. 20B

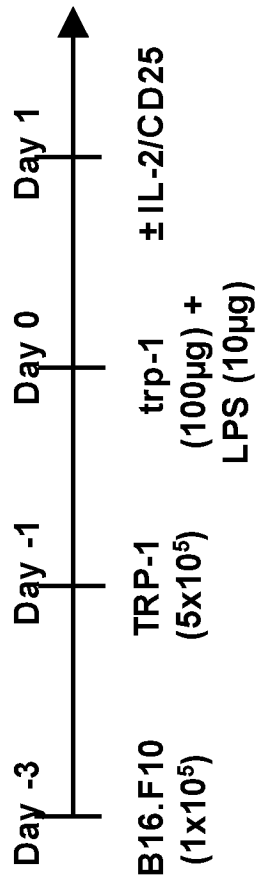


FIG. 21A

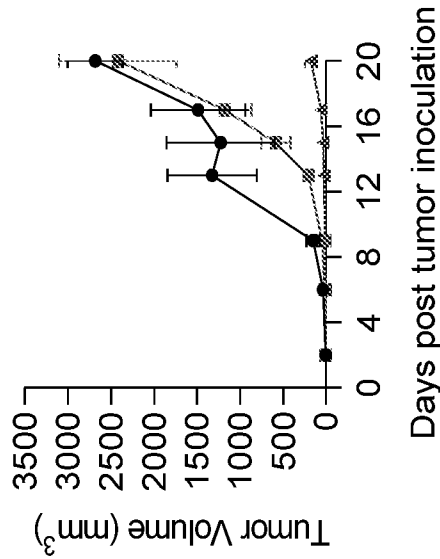


FIG. 21B

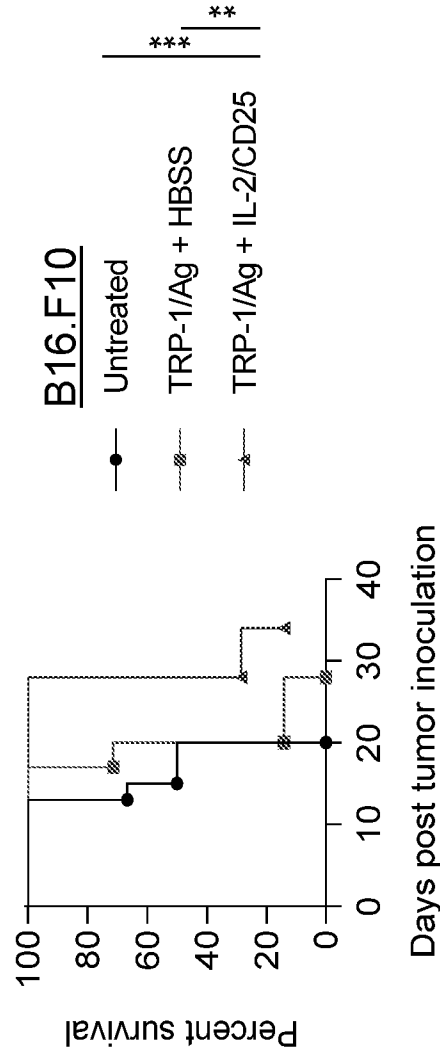


FIG. 21C

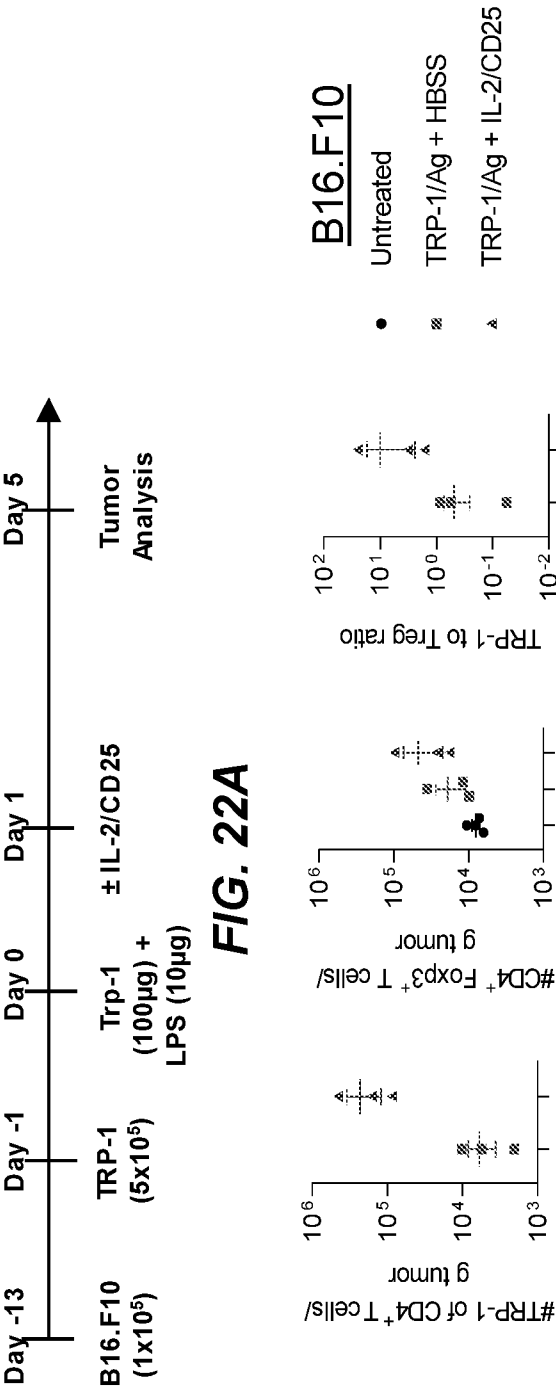


FIG. 22A

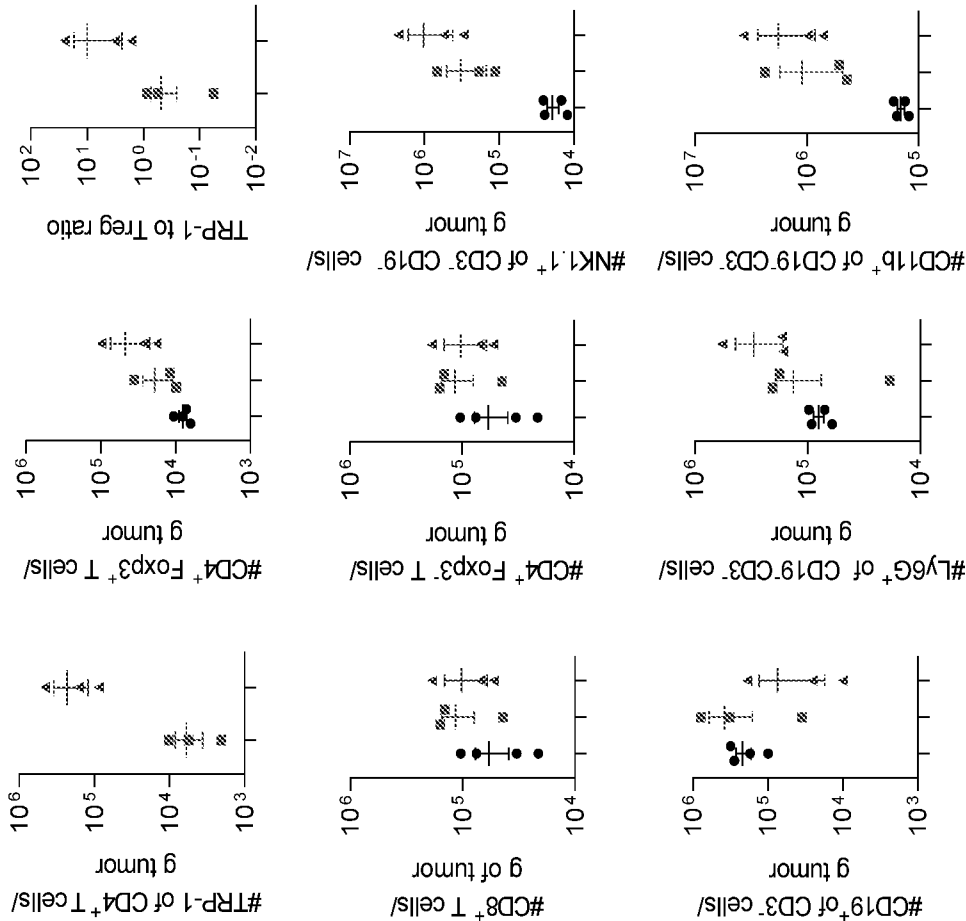
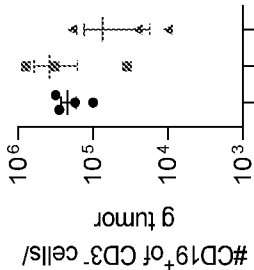
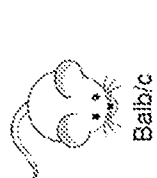


FIG. 22B





Immunize with
neofangens/Poly (I:C)
and IL-2/CD25

Splenocytes
12d post priming

106933

nAg/Poly (I:C) = (50µg each)
mIL-2/mCD25 = (50µg)

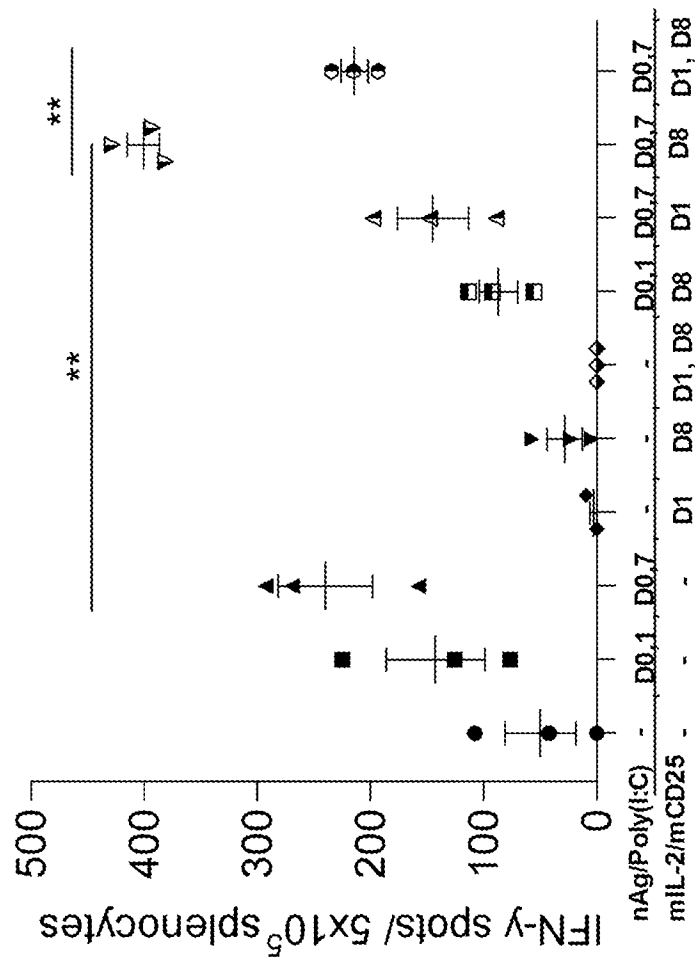


FIG. 23A

FIG. 23B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/13152

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/19, A61K 38/20, A61K 39/00, A61P 35/00, A61P 35/04, C07K 14/47 (2020.01)

CPC - A61K 39/0011, A61K 45/06, A61K 2039/884, A61K 2039/5158, A61K 2039/804, A61K 2039/82, C07K 14/705, C07K 14/47, C07K 14/55, A61P 35/00, A61K 38/00, A61K 2039/812, A61K 2039/892, A61K 2039/836, C07K 19/00, A61K 2039/86, C12N 9/00, A61K 2039/505, C07K 4/12, A61K 2039/876, A61K 2039/852, C07K 2319/70, C07K 2319/50, C07K 2319/21

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)

See Search History document

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

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PUSKAS, et al. Development of an attenuated interleukin-2 fusion protein that can be activated by tumour-expressed proteases. Immunology, June 2011, Vol 133, No 2, pp 206-20; Abstract, pg 207, col 2; pg 210, col 2; pg 216, Fig 6 and its legend	1-27
Y	WO 2018/102585 A1 (ADVAXIS INC., et al.) 07 June 2018 (07.06.2018) claims 1, 91; para [00346]-[00364], [00488], [00456]-[00489], [00511], [001000], [001293], [001321]	1-27

☐ 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

"D" document cited by the applicant in the international application

"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

19 March 2020

Date of mailing of the international search report

14 APR 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/13152

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 28
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 additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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.