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(54) **Title:** MONOVALENT ANTI-CD3 ADJUVANTS

(57) **Abstract:** This document provides methods and materials related to using anti-CD3 antibodies (e.g., monovalent anti-CD3 Fab fragments) and/or antibody mimetics having binding affinity for CD3 (e.g., anti-CD3 monobodies) as adjuvants to increase the immune response produced against an antigen (e.g., a tumor associated antigen). For example, vaccine compositions containing monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies in combination with tumor associated antigens (e.g., tumor associated antigens having little or no immunogenicity in the absence of monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies) alone or in combination with adjuvants for signals two and/or three required for full activation of T cell immune function, as well as methods and materials for using monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies to increase the immune response produced against an antigen (e.g., a tumor associated antigen) within a mammal (e.g., a human) are provided.



MONOVALENT ANTI-CD3 ADJUVANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Application
5 Serial No. 62/280,477, filed January 19, 2016. The disclosures of the prior applications are considered part of (and are incorporated by reference in) the disclosure of this application.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

10 This invention was made with government support under AI097187 awarded by National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

1. Technical Field

15 This document provides methods and materials related to using anti-CD3 antibodies (*e.g.*, monovalent anti-CD3 Fab fragments) and/or anti-CD3 antibody mimetics (*e.g.*, anti-CD3 monobodies) as adjuvants to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen). For example, this document provides monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and anti-CD3 $\gamma\epsilon/\delta\epsilon$
20 monobodies and vaccine compositions containing monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies in combination with tumor associated antigens (*e.g.*, tumor associated antigens having little or no immunogenicity in the absence of anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibodies and/or anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody mimetics) as well as methods and materials for using monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments to
25 increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen) within a mammal (*e.g.*, a human).

2. Background Information

Cancer is one of the leading causes of death in the world, responsible of about
30 13 percent of all human deaths. In spite of the significant advances achieved during recent decades, the efficiency of cancer treatments remains rather poor. Unfortunately, main treatments like radio- and chemotherapy do not specifically target cancer cells, damaging healthy tissues as well. As a result, these kinds of cancer therapies themselves cause significant morbidity and mortality. On the other

hand, these treatments frequently fail to eradicate cancer cells efficiently, leading to cancer recurrence.

Immunotherapy is an attractive alternative to treat cancer. The immune system has the capacity to identify cancer cells specifically, sparing healthy tissue from its attack. The main goal when stimulating the immune system against a tumor using vaccination strategies is to achieve an efficient anti-tumor T cell response that not only is specific for the cancer but also develops memory to control potential recurrence. T cell activation depends on T cells receiving three signals. Signal one consists of the recognition by the T cell receptor (TCR) of a foreign antigen in the shape of a peptide/MHC on the surface of professional antigen presenting cells (APCs). Co-stimulatory molecules on the APCs and their corresponding receptors in T cells provide signal two. Signal three is provided by soluble cytokines present in the T cell milieu.

Tumor associated antigens (TAA) come from mutated self-proteins, over/aberrantly expressed self-proteins, or unique foreign proteins from oncoviruses that are expressed by tumor cells. When mutated or from viral origin, tumor proteins might generate unique TAAs that can stimulate the TCR efficiently. However, in most cases, TAAs are closely related with self-proteins and are not very efficient in providing signal one. Some immunotherapies focus on the development of anti-tumor vaccines that incorporate the use of adjuvants that function to increase the efficiency of signals two and three such as toll like receptor ligands (*e.g.*, CpG and MLP), cytokines, (*e.g.*, IL-2, IL-12, and IFN α/β), and chemokines (*e.g.*, GM-CSF).

The option to improve the poorly immunogenic nature of most TAAs is reduced to a strategy of vaccinating mammals with altered peptide ligands (APLs). In an attempt to compensate for the weakness of signal one provided by TAAs, the sequence of the natural tumor peptide is modified to increase its affinity for the MHC and/or the TCR, with the expectation that the immunogenicity of the resulting APL will be higher than the natural TAA. This strategy, however, can be limited by the fact that once the natural tumor peptide is modified, (i) the TCRs stimulated by the APLs may not be specific to the natural TAAs from the tumor, (ii) the T cells may become anergized by the natural TAAs from the tumor, and/or (iii) the TCRs may be specific for other tissues, thereby displaying undesired side effects.

SUMMARY

This document provides methods and materials related to using monovalent anti-CD3 antibodies (*e.g.*, monovalent anti-CD3 Fab fragments) and/or anti-CD3 antibody mimetics (*e.g.*, anti-CD3 monobodies) as adjuvants to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen). For example, this document provides monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies as well as vaccine compositions containing monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies in combination with tumor associated antigens. In some cases, the tumor associated antigen can be an antigen having little or no immunogenicity in the absence of monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies. This document also provides methods and materials for using monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen) within a mammal (*e.g.*, a human).

As described herein, monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies can be used to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen). For example, monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies provided herein can be used to increase the immunogenicity of natural TAAs without having to change their peptide sequences. In some cases, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragment and/or an anti-CD3 monobody provided herein can be used to increase the immunogenicity of a natural TAA, an APL, a mixture of different TAAs, a mixture of different APLs, or a mixture of TAAs and APLs. In some cases, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragment and/or an anti-CD3 monobody provided herein can be used to increase the immunogenicity of tumor associated antigens in the form of a tumor cell lysate. For example, monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies provided herein can be combined with a tumor cell lysate to produce a mixture that is more immunogenic than the tumor cell lysate alone. In some cases, the monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 monobodies provided herein can be used in combination with tumor associated antigens and other particular adjuvants designed to increase signal two and/or signal three of T cell activation.

In general, one aspect of this document features a method for increasing an immune response against an antigen. The method comprises, or consists essentially of, administering a composition comprising an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody preparation

and the antigen or nucleic acid that expresses the antigen to a mammal, wherein the mammal produces an immune response against the antigen that is increased as compared to an immune response produced against the antigen when the antigen or the nucleic acid is administered to a comparable mammal in the absence of the anti-
5 CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody preparation. The mammal can be a human, and the anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody can include a human FN3 scaffold (*e.g.*, the tenth extracellular domain of human FN3). The antigen can be a tumor associated antigen. The tumor associated antigen can be a polypeptide. The antigen can be within an extract from a whole tumor cell lysate. The method can comprise administering the antigen to the
10 mammal. The method can comprise administering the nucleic acid to the mammal.

In another aspect, this document features a method for increasing an immune response against a cancer antigen. The method comprises, or consists essentially of, administering a composition comprising an anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody preparation to a mammal having cancer cells, wherein the mammal produces an immune response
15 against the antigen that is increased as compared to an immune response produced against the cancer antigen in a comparable mammal not administered the anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody preparation. The mammal can be a human, and the anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody can include a human FN3 scaffold (*e.g.*, the tenth extracellular domain of human FN3). The antigen can be a tumor associated antigen. The tumor
20 associated antigen can be a polypeptide. The antigen can be within an extract from a whole tumor cell lysate. The method can comprise administering the antigen to the mammal. The method can comprise administering nucleic acid encoding the antigen to the mammal. The method can comprise administering tumor specific cytotoxic T lymphocytes to the mammal. The method can comprise administering an IL-2
25 polypeptide to the mammal.

In another aspect, this document features a method for increasing an immune response against an antigen. The method comprises, or consists essentially of, administering a composition comprising an anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody preparation and tumor specific cytotoxic T lymphocytes, wherein the mammal produces an immune
30 response against the antigen that is increased as compared to an immune response produced against the antigen when the tumor specific cytotoxic T lymphocytes are administered to a comparable mammal in the absence of the anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody preparation. The mammal can be a human, and the anti-CD3 $\gamma\epsilon$ / $\delta\epsilon$ monoclonal antibody can include a human FN3 scaffold (*e.g.*, the tenth extracellular domain of human FN3).

The antigen can be a tumor associated antigen. The tumor associated antigen can be a polypeptide. The method can comprise administering the antigen to the mammal. The method can comprise administering a nucleic acid encoding the antigen to the mammal. The method can comprise administering an IL-2 polypeptide to the
5 mammal.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and
10 materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

15 The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

20 DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of a T cell receptor/CD3 complex of a T cell that is in the closed conformation.

Figure 2 is a diagram of a T cell receptor/CD3 complex of a T cell that is in an open conformation (CD3 Δ c), which can be induced by the binding of a peptide/MHC
25 complex.

Figure 3 is a diagram of a CD3 pull down (CD3-PB) assay using Nck-SH3.1 beads and a western blot with anti-CD3 ζ antibodies.

Figure 4 contains a diagram (left) of how an APA 1/1 antibody to the cytoplasmic tail of CD3 ϵ blocks the pull down of Nck SH3.1, and a diagram (right) of
30 how an 2C11 antibody to an extracellular domain of CD3 ϵ promotes the open conformation allowing detection by the pull down of Nck SH3.1.

Figure 5 is a diagram of a T cell receptor/CD3 complex in an open conformation (CD3 Δ c) along with a list of properties.

Figure 6 contains a diagram (left) of how poorly immunogenic or non-immunogenic antigens in combination with MHC molecules do not result in the CD3Δc conformation, and a diagram (right) of how immunogenic antigens in combination with MHC molecules do result in the CD3Δc conformation.

5 Figure 7 contains a diagram of how most TAA are poorly immunogenic or non-immunogenic antigens that when in combination with MHC molecules do not result in the CD3Δc conformation.

10 Figure 8 contains a diagram representing how a soluble agent can produce the open CD3Δc conformation even though a poorly immunogenic or non-immunogenic antigen in combination with an MHC molecule is engaging the T cell receptor/CD3 complex.

Figure 9 contains a diagram of a monovalent anti-CD3γ ϵ /δ ϵ Fab fragment (*e.g.*, Mono-7D6-Fab) bound to the extracellular domains of CD3γ ϵ /δ ϵ in a manner that triggers the open CD3Δc conformation.

15 Figure 10 contains a schematic diagram and graph showing the digestion of the 7D6 monoclonal antibody into Fab and Fc fragments.

Figure 11 contains graphs demonstrating that Mono-7D6-Fab binds to the T cell receptor and does not block the binding of peptide/MHC complexes (pMHC7) to T cell receptors. Ms IgG Fab is a non-specific Fab control for Mono-7D6-Fab.

20 Figure 12 is a western blot demonstrating that Mono-7D6-Fab induces the open CD3Δc conformation in mature T cells obtained from mice.

Figure 13 contains graphs demonstrating that Mono-7D6-Fab alone does not stimulate T cells.

25 Figure 14 is a graph listing various altered peptide ligands (APLs) derived from the OVA polypeptide (pOVA) and their levels of immunogenicity.

Figure 15 contains graphs plotting the percent of CD69⁺ or CD25⁺ OT-I T cells following exposure to T2-Kb antigen presenting cells (APCs) having H2-Kb MHC-I molecules loaded with the indicated OVA APLs plus either control Fab IgG from mouse (Ig Fab; 5 μg/mL) or Mono-7D6-Fab (5 μg/mL).

30 Figure 16 contains graphs plotting the percent OT-I T cells dividing (M3 %) following exposure to T2-Kb antigen presenting cells (APCs) having MHC-I molecules loaded with the indicated OVA APLs plus either control Fab IgG from mouse (Ig Fab; 5 μg/mL) or Mono-7D6-Fab (5 μg/mL).

Figure 17 is a bar graph plotting the percent of OT-I CTL specific killing of EL-4 tumor target cells when incubated in the presence of 2 μ M of the indicated OVA APLs with either control Fab IgG from mouse (Ig Fab; 5 μ g/mL) or Mono-7D6-Fab (5 μ g/mL). The OT-I T cell to target cell ratio was 10:1.

5 Figure 18 is a bar graph plotting the levels of indicated cytokines in blood serum from healthy mice at day 7 following intravenous injections of either control Ms Ig Fab; (10 μ g/mouse) or Mono-7D6-Fab (10 μ g/mouse) on days 1, 3, 5, and 7. Staphylococcal enterotoxin B (SEB) was used as a positive control for an autoimmune response that produces a specific cytokine profile tested in this experiment.

10 Figure 19 contains photographs of liver and kidney tissue from the mice tested in Fig 18 (3 mice injected with Mouse Ig Fab, 10 μ g/mouse; or Mono-7D6-Fab, 10 μ g/mL) demonstrating that Mono-7D6-Fab does not cause signs of an autoimmune response in the shape of tissue inflammation.

Figure 20 is a western blot of a CD3 pull down (CD3-PD) assay using Pmel
15 TCR transgenic CD8⁺ T cells that are specific for the natural/weak tumor antigen mouse gp100 (mg100). Pmel T cells were either treated with a non-specific Ig (Ig) or with the antigens mg100 or hgp100 (xenogeneic variant of mg100 from human melanocytes that functions as a strong antigen for Pmel TCR) for 1 hour at 37°C. Then, Pmel T cells were lysed, and the resulting samples were used to detect CD3
20 open conformation using the CD3-PD assay as described herein. The western blot revealed that while the strong/xenogeneic variant hgp100 induces CD3 Δ c (6 fold induction of open-CD3), the weak/natural melanoma antigen mgp100 fails to induce CD3 Δ c.

Figure 21 contains a set of photographs of lung tissue from mice injected with
25 B16.F10 tumor cells on day 0 and treated intravenously with mouse Ig Fab (10 μ g/mouse on day 0) or Mono-7D6-Fab (10 μ g/mouse on day 0). On day 21, the lungs were extracted and evaluated for the presence of metastatic melanoma tumor burden. Less melanoma burden was observed in mice treated with Mono-7D6-Fab. The two dot-plots present two different means of objective melanoma quantification in the
30 lungs of each group of mice. On the left plot, melanoma density was quantified using software that detects dark melanoma tissue in the lungs. On the right plot, lung weight was plotted to reflect the amount of melanoma burden. Both plots reveal a statistical difference in the melanoma burden between the mouse IgG Fab treated mice (higher burden) and the Mono-7D6-Fab treated mice (lower burden).

Figure 22 contains photographs of lung tissue from mice lacking T cells (CD3 ϵ ^{-/-} mice) injected with B16.F10 tumor cells on day 0 and treated intravenously with mouse Ig Fab; (10 μ g/mouse on day 0) or Mono-7D6-Fab (10 μ g/mouse on day 0). On day 21, the lungs were extracted and evaluated for the presence of metastatic melanoma tumor burden. Similar tumor burden was observed in both treatment groups, demonstrating that T cells are required for the response observed in Figure 21. On the left, there is an objective quantification of tumor burden in lung of mice of each group treatment using a dot plot that depicts melanoma density as specified in Figure 21. There is not a statistical difference in the melanoma burden between the mouse IgG Fab treated mice and the Mono-7D6-Fab treated mice.

Figure 23 contains a dot plot to quantify melanoma burden and photographs of lung tissue from mice with T cells (No Depl), without CD4 T cells (CD4 Depl; CD4 T cells were depleted by injecting anti-CD4 specific antibody), or without CD8 T cells (CD8 Depl; CD8 T cells were depleted by injecting anti-CD8 specific antibody) injected with B16.F10 tumor cells on day 0 and treated intravenously with either mouse IgG Fab; (10 μ g/mouse on day 0) or Mono-7D6-Fab (10 μ g/mouse on day 0). On day 21, the lungs were extracted and evaluated for the presence of metastatic melanoma tumor burden and quantified as described in Figures 21 and 22. The results demonstrate that both CD4 and CD8 T cells are required for the full anti-melanoma effect of Mono-7D6-Fab treatment observed when both CD4 and CD8 T cells are not depleted.

Figure 24 contains photographs of lung tissue from OT-I Rag2 KO mice. In these mice, the only kind of T cells present are OT-I CD8 T cells specific for an antigen from chicken ovalbumin. Therefore, these T cells are incapable of recognizing any antigens from the B16F10 melanoma tumor. All the OT-I mice were injected with B16.F10 tumor cells on day 0 and treated intravenously with mouse Ig Fab (10 μ g/mouse on day 0) or Mono-7D6-Fab (10 μ g/mouse on day 0). On day 21, the lungs were extracted and evaluated for the presence of metastatic melanoma tumor burden. Similar tumor burden was observed in both treatment groups, demonstrating that Mono-7D6-Fab treatment requires the presence of CD8 T cells specific for the tumor antigens to exert its anti-tumor effect. On the left, there is an objective quantification of tumor burden in lung of OT-I mice of each treatment group using a dot plot that depicts melanoma density, as specified in Figure 20. There is not a

statistical difference in the melanoma burden between the mouse IgG Fab treated mice and the Mono-7D6-Fab treated OT-I mice.

Figures 25A-E shows that mono-7D6-Fab promotes therapeutic anti-tumor T cell responses in the B16F10/B6 lung metastatic melanoma model when administered three days after tumor injection. Figure 25A is a schematic summary of the experimental procedure. Figure 25B contains photographs of the lungs that were evaluated for the presence of metastatic melanoma tumor burden. Figure 25C is a graph plotting melanoma density for mice treated with the indicated Fab (t test, *** $p < 0.0001$). Figure 25D is a graph plotting the percent of T cells positive for CD107a. Figure 25E is a graph plotting the percent of T cells positive for CD44 and CD62L.

Figures 26A-D show an anti-melanoma effect when Mono-7D6-Fab therapy is combined with adoptive transfer of melanoma specific cytotoxic lymphocytes. Figure 26A is a schematic summary of the experimental procedure. Figure 26B contains photographs of the lungs that were evaluated for the presence of metastatic melanoma tumor burden. Figure 26C is a graph plotting melanoma density for mice treated with the indicated Fab and CTLs. Figure 26D is a graph plotting the number of CD8 T cells from mediastinal lymph node or lung that stained with the Kd-tetramer gp100 to identify cells specific for the melanoma antigen gp100.

Figure 27 shows that mono-7D6-Fab increases survival of mice treated with tumor specific CTLs. ** $p < 0.005$, Mantel-Cox Log-Rank test.

Figures 28A-B show that mono-7D6-Fab synergizes with anti-melanoma immunotherapies that target co-inhibitory receptors on T cells. Figure 28A shows lung photographs from one independent experiment. Figure 28B shows quantification of melanoma found in lungs. Dots represent individual mice (mean $\pm$ SEM displayed; * $p < 0.05$, ** $p < 0.005$ *** $p < 0.0005$, two tailed, unpaired Student's t-test).

Figures 29A-D show properties of Mono-OKT3-Fab (anti-human CD3 $\gamma\epsilon/\delta\epsilon$) that make it suitable to increase anti-tumor responses of human T cells. Figure 29A shows that mono-OKT3-Fab binds to human CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ on T cells, as shown by positive staining of a Jurkat T cell line expressing the murine TCR OT-I and human CD3 complex (OT-I JRT3). Figure 29B shows that, when bound to T cells, mono-OKT3-Fab does not block TCR/antigen binding, as shown by unaffected flow cytometry staining of OT-I JRT3 cells with the fluorescent tetramer K^B/OVA-PE in

the presence of Mono-OKT3-Fab. Figure 29C shows that mono-OKT3-Fab is unable to stimulate T cells on its own, as shown by absent CD69 up-regulation and OT-I TCR internalization on OT-I JRT3 cells exposed to null peptide pFARL, nor blocks T cell cognate antigenic stimulation, as shown by equivalent CD69 up-regulation and OT-I TCR internalization on OT-I Jurkat T cells exposed to the agonist peptide pOVA in the presence of MsIgG Fab and Mono-OKT3-Fab. Figure 29D shows that mono-OKT3-Fab induces CD3 Δ c on hPBMCs, as detected using the CD3-PD assay.

Figure 30 shows that mono-OKT3-Fab increases the frequency of human CD8 T cells producing IFN γ *in vitro*. CD8, CD8 T cell positive PBMC populations; APC, CD8 T cell negative PBMC populations; PHA, phytohaemagglutinin. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, two-tailed, unpaired Student's t-test.

Figures 31A-C show that mono-OKT3-Fab does not interfere with the course of Graft versus Host Disease (GVHD) in NSG mice. Figures 31A-B show that mono-OKT3-Fab did not change the course of GvHD, monitored by weight loss (A) and percent survival (B). Figure 31C shows that mono-OKT3-Fab did not change the frequency of activated T cells (CD44^{Hi}) found in mice sacrificed due to GvHD (n.s. $p \geq 0.05$ two tailed, unpaired Student's t-test).

Figures 32A-E show that mono-OKT3-Fab reduces tumor burden of pre-established human melanoma in lungs. Figure 32A shows luciferase imaging of representative mice. Figure 32B shows average luciferase activity (10 mice/Fab condition, mean $\pm$ SEM displayed; * $p < 0.05$, ** $p < 0.005$, two tailed, unpaired Student's t-test). Figure 32C shows total live T cell counts found in the spleen of mice (average T cell counts in each group of mice $\pm$ SE; n.s. $p \geq 0.05$, two tailed, unpaired Student's t-test). Figure 32D-E show the %CD44⁺ T cells found in pooled peripheral (D) or mediastinal lymph nodes (E) (average % $\pm$ SD; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, two tailed, unpaired Student's t-test).

Figures 33A-B contains a schematic diagram and a sequence of a synthetic human FN3 sequence. Figure 33A contains a schematic drawing showing a comparison of the V_HH scaffold (left) and the FN3 scaffold (right) (Koide et al., *J. Mol. Biol.* 415:393-405 (2012)). β -strand domain sequences are designated A through G and loop regions designated AB loop, BC loop, CD loop, DE loop, EF loop, and FG loop connect the β -strand domain sequences. Figure 33B is an amino acid sequence (SEQ ID NO:4) and restriction sites of an exemplary synthetic human FN3

sequence. The BC loop corresponds to residues 21-31, the DE loop corresponds to residues 51-56, and the FG loop corresponds to residues 75-88.

Figures 34A and 34B are graphs showing the ability of anti-CD3 mono-Fabs to enhance T cell response to tumors. Figure 34A is a graph showing binding of mono-Fabs from mAbs Hit3a, SP34-2, SK7, and UCHT-I to CD3 ϵ γ / ϵ δ . Figure 34B is a graph showing the effect of mono-Fabs from mAbs Hit3a, SP34-2, SK7, and UCHT-I on peptide/MHC binding to TCR.

Figure 35 shows that mono-Fabs from OKT3, HIT3a, and SP34-2 neither blocked nor promoted a T cell response to cognate antigen stimulation.

Figure 36 shows that mono-Fabs from OKT3, HIT3a, and SP34-2 Abs induced CD3 Δ c on human CD3 ϵ .

Figure 37 shows that mono-Fabs from OKT3 and Hit3a increased the frequency of CD8 T cells reacting to autologous APCs.

DETAILED DESCRIPTION

This document provides methods and materials related to monovalent anti-CD3 γ ϵ / δ ϵ antibodies (*e.g.*, monovalent anti-CD3 Fab fragments) and/or anti-CD3 antibody mimetics (*e.g.*, anti-CD3 γ ϵ / δ ϵ monobodies). For example, this document provides monovalent anti-CD3 γ ϵ / δ ϵ antibody preparations, methods for making monovalent anti-CD3 γ ϵ / δ ϵ antibody preparations, and methods for using monovalent anti-CD3 γ ϵ / δ ϵ antibody preparations as adjuvants to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen). For example, this document provides monovalent anti-CD3 γ ϵ / δ ϵ Fab fragments as well as vaccine compositions containing monovalent anti-CD3 γ ϵ / δ ϵ Fab fragments in combination with tumor associated antigens. In some cases, the tumor associated antigen can be an antigen having little or no immunogenicity in the absence of monovalent anti-CD3 γ ϵ / δ ϵ Fab fragments. This document also provides methods and materials for using monovalent anti-CD3 γ ϵ / δ ϵ Fab fragments to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen) within a mammal (*e.g.*, a human).

In some cases, a monovalent anti-CD3 γ ϵ / δ ϵ antibody preparation or an anti-CD3 γ ϵ / δ ϵ monobody preparation provided herein can bind to a CD3 γ ϵ / δ ϵ dimer with little or no detectable binding to a CD3 ϵ polypeptide not in the form of a CD3 γ ϵ / δ ϵ dimer and with little or no detectable binding to a CD3 γ polypeptide not in the form

of a CD3 $\gamma\epsilon/\delta\epsilon$ dimer. For example, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody preparation or an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody preparation provided herein can bind to a human CD3 $\gamma\epsilon/\delta\epsilon$ dimer with little or no detectable binding to a human CD3 ϵ polypeptide not in the form of a CD3 $\gamma\epsilon/\delta\epsilon$ dimer and with little or no detectable binding to a human CD3 γ polypeptide or a human CD3 δ polypeptide not in the form of a CD3 $\gamma\epsilon$ or CD3 $\delta\epsilon$ dimer. An example of an antibody having the ability to bind to a CD3 $\gamma\epsilon/\delta\epsilon$ dimer with little or no detectable binding to a CD3 ϵ polypeptide not in the form of a CD3 $\gamma\epsilon$ or CD3 $\delta\epsilon$ dimer and with little or no detectable binding to a CD3 γ polypeptide or a CD3 δ polypeptide not in the form of a CD3 $\gamma\epsilon$ or CD3 $\delta\epsilon$ dimer includes, without limitation, the 7D6 antibody described elsewhere (Van Snick *et al.*, *Eu. J. Immunol.*, 21:1703-1709 (1991)), the OKT3 antibody described elsewhere (Kung *et al.*, *Science*, 206:347-9 (1979)), the Hit3a antibody described elsewhere (Schlossman *et al.* (Eds.) *Leucocyte Typing V*. Oxford University Press. New York (1995); Knapp, *Leucocyte Typing IV*. Oxford University Press. New York (1989); Barclay *et al.* *The Leucocyte Antigen Facts Book*. Academic Press Inc. San Diego (1997)), and the SP34-2 antibody described elsewhere (Pessano *et al.*, *The EMBO J.* 4(2):337-344 (1985)). Fab fragments from such an antibody can be used to obtain monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibodies (*e.g.*, monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments).

The term “antibody” as used herein refers to intact antibodies as well as antibody fragments that retain some ability to bind an epitope. Such fragments include, without limitation, Fab, F(ab')₂, and Fv antibody fragments.

The term “antibody mimetic” as used herein refers to artificial molecules that are structurally not related to antibodies, but can specifically bind an epitope. Antibody mimetics include, without limitation, monobodies, affibodies, alphabodies, and nanobodies.

The term “epitope” refers to an antigenic determinant on an antigen to which an antibody or antibody mimetic binds. Epitope determinants usually consist of chemically active surface groupings of molecules (*e.g.*, amino acid or sugar residues) and usually have specific three dimensional structural characteristics as well as specific charge characteristics.

The antibodies and/or antibody mimetics provided herein can be any antibody (*e.g.*, a monoclonal antibody), antibody fragment (*e.g.*, a monovalent Fab fragment), or antibody mimetic (*e.g.*, a monobody) having binding affinity (*e.g.*, specific binding affinity) for CD3 $\gamma\epsilon/\delta\epsilon$ with little or no detectable binding to a CD3 ϵ polypeptide or a

CD3 δ polypeptide not in the form of a dimer. For example, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody preparation can be a preparation of Fab fragments having the ability to bind to a CD3 $\gamma\epsilon$ /or a CD3 $\delta\epsilon$ dimer with little or no detectable binding to a CD3 ϵ polypeptide not in the form of a CD3 $\gamma\epsilon$ or a CD3 $\delta\epsilon$ dimer, with little or no detectable binding to a CD3 γ polypeptide not in the form of a CD3 $\gamma\epsilon$ dimer, or a CD3 δ polypeptide not in the form of a CD3 $\delta\epsilon$ dimer.

Any appropriate method can be used to produce Fab fragments from intact antibodies. For example, standard papain digestion methods can be used to make an Fab antibody preparation. In some cases, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody preparation provided herein can be a preparation of Fab fragments of humanized or fully-human anti-human CD3 $\gamma\epsilon/\delta\epsilon$ dimer antibodies. In some cases, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody preparation provided herein (*e.g.*, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody preparation containing Fab fragments of a humanized anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody) can have the ability to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen).

Antibodies provided herein can be prepared using any appropriate method. For example, a sample containing a CD3 $\gamma\epsilon/\delta\epsilon$ dimer (*e.g.*, a human CD3 $\gamma\epsilon/\delta\epsilon$ dimer or a chimeric mouse/human CD3 $\gamma\epsilon/\delta\epsilon$ dimer) can be used as an immunogen to elicit an immune response in an animal such that specific antibodies are produced. The immunogen used to immunize an animal can be chemically synthesized or derived from translated cDNA. In some cases, cells (*e.g.*, mouse T cells) transfected to express a CD3 $\gamma\epsilon/\delta\epsilon$ dimer (*e.g.*, a human CD3 $\gamma\epsilon/\delta\epsilon$ dimer or a chimeric mouse/human CD3 $\gamma\epsilon/\delta\epsilon$ dimer) can be used as an immunogen. In some cases, the immunogen can be conjugated to a carrier polypeptide, if desired. Commonly used carriers that are chemically coupled to an immunizing polypeptide include, without limitation, keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid.

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, *e.g.*, Green *et al.*, Production of Polyclonal Antisera, in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992) and Coligan *et al.*, Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in *Current Protocols In Immunology*, section 2.4.1 (1992). In addition, those of skill in the art will know of various techniques common in the immunology arts for purification and

concentration of polyclonal antibodies, as well as monoclonal antibodies (Coligan *et al.*, Unit 9, Current Protocols in Immunology, Wiley Interscience, 1994).

The preparation of monoclonal antibodies also is well-known to those skilled in the art. See, *e.g.*, Kohler & Milstein, *Nature* 256:495 (1975); Coligan *et al.*, sections 2.5.1 2.6.7; and Harlow *et al.*, *Antibodies: A Laboratory Manual*, page 726 (Cold Spring Harbor Pub. 1988). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by analyzing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well established techniques. Such isolation techniques include affinity chromatography with Protein A Sepharose, size exclusion chromatography, and ion exchange chromatography. See, *e.g.*, Coligan *et al.*, sections 2.7.1 2.7.12 and sections 2.9.1 2.9.3; Barnes *et al.*, Purification of Immunoglobulin G (IgG), in *Methods In Molecular Biology*, Vol. 10, pages 79 104 (Humana Press 1992).

In addition, methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies are well known to those skilled in the art. Multiplication *in vitro* can be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by mammalian serum such as fetal calf serum, or trace elements and growth sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, and bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* may be carried out by injecting cell clones into mammals histocompatible with the parent cells (*e.g.*, osyngeneic mice) to cause growth of antibody producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

In some cases, the antibodies provided herein can be made using non-human primates. General techniques for raising therapeutically useful antibodies in baboons

can be found, for example, in Goldenberg *et al.*, International Patent Publication WO 91/11465 (1991) and Losman *et al.*, *Int. J. Cancer*, 46:310 (1990).

In some cases, the antibodies can be humanized monoclonal antibodies.

Humanized monoclonal antibodies can be produced by transferring mouse

- 5 complementarity determining regions (CDRs) from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions
- 10 when treating humans. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l. Acad. Sci. USA* 86:3833 (1989). Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321:522 (1986); Riechmann *et al.*, *Nature* 332:323 (1988); Verhoeyen *et al.*, *Science* 239:1534 (1988); Carter *et al.*,
15 *Proc. Nat'l. Acad. Sci. USA* 89:4285 (1992); and Sandhu, *Crit. Rev. Biotech.* 12:437 (1992); Singer *et al.*, *J. Immunol.* 150:2844 (1993). In some cases, humanization such as super humanization can be used as described elsewhere (Hwang *et al.*, *Methods*, 36:35-42 (2005)). In some cases, SDR grafting (Kashmiri *et al.*, *Methods*, 36:25-34 (2005)), human string content optimization (Lazar *et al.*, *Mol. Immunol.*, 44:1986-
20 1998 (2007)), framework shuffling (Dall'Acqua *et al.*, *Methods*, 36:43-60 (2005); and Damschroder *et al.*, *Mol. Immunol.*, 44:3049-3060 (2007)), and phage display approaches (Rosok *et al.*, *J. Biol. Chem.*, 271:22611-22618 (1996); Radar *et al.*, *Proc. Natl Acad. Sci. USA*, 95:8910-8915 (1998); and Huse *et al.*, *Science*, 246:1275-1281 (1989)) can be used to obtain anti-CD3 $\gamma\epsilon/\delta\epsilon$ antibody preparations. In some cases,
25 fully human antibodies can be generated from recombinant human antibody library screening techniques as described elsewhere (Griffiths *et al.*, *EMBO J.*, 13:3245-3260 (1994); and Knappik *et al.*, *J. Mol. Biol.*, 296:57-86 (2000)).

Antibodies provided herein can be derived from human antibody fragments

isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et*

- 30 *al.*, *Methods: A Companion To Methods In Enzymology*, Vol. 2, page 119 (1991) and Winter *et al.*, *Ann. Rev. Immunol.* 12: 433 (1994). Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

In addition, antibodies provided herein can be derived from a human monoclonal antibody. Such antibodies can be obtained from transgenic mice that have been “engineered” to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens and can be used to produce human antibody secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.* (*Nature Genet.*, 7:13 (1994)), Lonberg *et al.* (*Nature*, 368:856 (1994)), and Taylor *et al.* (*Int. Immunol.*, 6:579 (1994)).

Antibody fragments can be prepared by proteolytic hydrolysis of an intact antibody or by the expression of a nucleic acid encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of intact antibodies by conventional methods. For example, Fab fragments can be produced by enzymatic cleavage of antibodies with papain. In some cases, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. In some cases, an enzymatic cleavage using pepsin can be used to produce two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg (U.S. Patent Nos. 4,036,945 and 4,331,647). See also Nisonhoff *et al.*, *Arch. Biochem. Biophys.* 89:230 (1960); Porter, *Biochem. J.* 73:119 (1959); Edelman *et al.*, *Methods In Enzymology*, Vol. 1, page 422 (Academic Press 1967); and Coligan *et al.* at sections 2.8.1 2.8.10 and 2.10.1 2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used provided the fragments retain some ability to bind (*e.g.*, selectively bind) its epitope.

Any appropriate method can be used to produce antibody mimetics. For example, a fibronectin type III domain (FN3) can be used as a molecular scaffold to make a monobody. In some cases, an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody preparation provided herein can be produced using human FN3 as a scaffold. In some cases, an anti-

CD3 $\gamma\epsilon/\delta\epsilon$ monobody preparation provided herein can have the ability to increase the immune response produced against an antigen (*e.g.*, a tumor associated antigen).

Regions of FN3 (*e.g.*, the tenth extracellular domain) are structurally similar to antibody variable domains, including exposed loops which correspond to
 5 complementarity determining regions. In some cases, monobodies can be engineered by screening combinatorial libraries of the tenth extracellular domain FN3. For example, monobodies can be produced from combinatorial libraries in which one or more loop portions of the FN3 scaffold contain diversified amino acids. In some cases, the length of the loop portions can be varied. For example, one or more loop
 10 portions can be extended by about 10 to about 13 amino acid residues. In some cases, monobodies can be produced from combinatorial libraries in which other portions of the FN3 scaffold (*e.g.*, the β -sheet and surface loop that together form a concave surface) contain diversified amino acids. Amino acids can be diversified using any suitable mutagenesis technique. For example, methods useful for creating diversified
 15 FN3 regions include, without limitation, directed evolution, Kunkel mutagenesis, site-directed mutagenesis, chemical mutagenesis, and error prone PCR techniques.

Libraries can be screened for monobodies which bind to specific ligands (*e.g.*, CD3) using any suitable assay for detecting specific binding. For example, assays useful for detecting monobody-CD3 binding include, without limitation, phage
 20 display, yeast surface display, mRNA display, two-hybrid screening (*e.g.*, a yeast two-hybrid), protein array, bimolecular fluorescence complementation, and tandem affinity purification techniques. For example, anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies can be produced by creating a library having diverse amino acid residues in the BC, DE, and FG loops of FN3, and using phage-display with a CD3 target to screen the library for
 25 suitable monobodies.

In some cases, the methods of making monobodies described elsewhere, for example, by Koide (U.S. 6,462,189; U.S. 6,673,901; U.S. 7,119,171; U.S. 7,598,352; *J. Mol. Biol.*, 284:1141-1151 (1998); *Proc. Natl. Acad. Sci.*, 99:1253-1258 (2002); and *J. Mol. Biol.*, 415:393-405 (2012)) can be used. See, also, Man *et al.*, *Chem Biol.*,
 30 8:608-616 (2013); Hackel *et al.*, *J. Mol. Biol.*, 401:84-96 (2010); Wojcik *et al.*, *Nat. Struct. Mol Biol.*, 17:519-527 (2010); Bloom *et al.*, *Drug Disc. Today*, 14:949-955 (2009); Koide *et al.*, *Protein Engineering Protocols: Methods in Mol. Biol.*, Vol. 355, pages 95-109 (Humana Press 2007); and Koide *et al.*, *Protein Engineering and Design*, Chapter 5. Design and Engineering of Synthetic Binding Proteins Using

Nonantibody Scaffolds (CRC Press 2009).

The antibodies (*e.g.*, anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments) and/or antibody mimetics (*e.g.*, anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies) provided herein can be substantially pure. The term “substantially pure” as used herein with reference to an antibody or antibody mimetic means the antibody or antibody mimetic is substantially free of other polypeptides, lipids, carbohydrates, and nucleic acid with which it is naturally associated. Thus, a substantially pure antibody is any antibody that is removed from its natural environment and is at least 60 percent pure. A substantially pure antibody or antibody mimetic can be at least about 65, 70, 75, 80, 85, 90, 95, or 99 percent pure.

As described herein, the monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments and/or anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies provided herein can be used to increase the immune response produced against an antigen. Examples of such antigens include, without limitation, germ cell-cancer associated tumor antigens, tumor antigens derived from genetic mutations and atypical gene products, tumor differentiation antigens, and tumor polypeptide ligands. Antigens (*e.g.*, tumor associated antigens) can be administered as, for example, polypeptides (*e.g.*, short or truncated polypeptides or full length polypeptides), DNA encoding such polypeptides, viral particles designed to express such polypeptides, extracts from whole tumor cell lysates, or dendritic cells loaded with such polypeptides or tumor cell lysates. Examples of tumor polypeptide ligands include, without limitation, altered peptide ligands (APLs), xenogeneic tumor peptides, and heteroclitic tumor peptides.

In some cases, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragment or an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody provided herein can be combined with one or more antigens to produce a vaccine composition. For example, a monovalent anti-human CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragment preparation can be combined with a tumor associated antigen such as whole tumor protein, whole tumor cell lysate, or an altered, xenogeneic, or heteroclitic tumor peptides derived from a whole tumor protein to produce a vaccine composition capable of producing an immune response against tumor cells that is increased in comparison to a comparable vaccine composition lacking monovalent anti-human CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments.

In some cases, a monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragment or an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody provided herein can be used to increase the immunogenicity of tumor associated antigens provided in the form of a tumor cell lysate. For example, monovalent anti-CD3 $\gamma\epsilon/\delta\epsilon$ Fab fragments or anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobodies provided

herein can be combined with a tumor cell lysate to produce a mixture or vaccine composition that is more immunogenic than the tumor cell lysate alone.

In some cases, a vaccine composition can include additional components such as adjuvants designed to increase signal two and/or signal three of T cell activation.

5 Examples of adjuvants designed to increase signal two of T cell activation include, without limitation, Freund's adjuvants and Toll like receptor ligands (like LPS, CpG, and PolyI:C). Examples of adjuvants designed to increase signal three of T cell activation include, without limitation, cytokines (IL-2, IL-12, IFN α , or IFN β), and chemokines (GM-CSF).

10 In some cases, a human monovalent CD3 $\epsilon\gamma$ -Fab preparation or an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monoclonal body preparation specific for human T cells can be used as an adjuvant to increase the immunogenicity of natural TAAs. Such a human reagent can allow the development of tumor vaccines using natural TAA avoiding the problems related with anti-tumor vaccines: limited repertoire of TAAs immunogenic enough to
15 be considered for the development the vaccine, very intricate design of APLs derived from the analogue natural TAAs to achieve higher affinity for MHC and/or TCR molecules, high risk of stimulating a T cell repertoire not specific for the natural TAAs when using such APLs, together with high risk of promoting adverse effects of stimulated T cell repertoire by the vaccine, and the need to personalize the selection
20 of APLs used to immunize. Additionally, using a human monovalent CD3 $\epsilon\gamma$ -Fab preparation or an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monoclonal body preparation to boost natural TAA immunogenicity can be compatibility with: (i) vaccines using single TAAs, TAAs mixtures or tumor lysates as a source of tumor antigenic specificity; or (ii) any existing or new adjuvants designed to increase signal two and/or three to stimulate T
25 cells.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

30 Example 1 – Using Monovalent Anti-CD3 $\gamma\epsilon/\delta\epsilon$ Dimer Fab Fragments as Adjuvants to Increase Immunogenicity

Mono-7D6-Fab specific for mouse T cells was capable of increasing the immunogenicity of antigens that are weak and poorly immunogenic (Figures 8-9).

Mono-7D6-Fab is a monovalent Fab fragment specific for the CD3 $\epsilon\gamma$ dimer of the CD3 complex (Figures 1, 9, and 10). The following was performed to confirm the ability of Mono-7D6-Fab to increase the immunogenicity of antigens in the context of cancer disease using a mouse model of lung metastatic melanoma (Figures 20-24).

5 Poorly immunogenic antigens failed to stimulate T cells due to their incompetence to induce a conformational change in the CD3 complex (CD3 Δ c) (Figures 6-7). See, also, Gil *et al.*, *J. Immunol.*, 180(6):3900-9 (2008)). The CD3 complex is a group of trans-membrane proteins associated to the T cell antigen receptor (TCR) that are in charge of starting the process of T cell stimulation once the
10 TCR interacts with a given antigen (Figure 1). The TCR distinguishes the quality of different antigens in order to instruct the activation of immune function. The most upstream marker currently known to be uniformly indicative of T cell stimulatory antigen recognition is CD3 Δ c (Figure 2). CD3 Δ c has the following attributes (Figures 2, 5, and 6): (i) it uncovers a cryptic proline rich sequence (PRS) in CD3 ϵ
15 that is a binding site for specific SH3 domains of several cytoplasmic proteins, including Nck; (ii) it is induced by either anti-TCR/CD3 antibodies, anti-CD3 Fab fragments, or antigenic peptide-MHC ligands; (iii) it occurs earlier than (and is independent of) TCR/CD3 crosslinking and src-kinase activity; (iv) when tested as an isolated variable, CD3 Δ c is required for optimal T cell signaling and immune
20 function. Considering these observations together, CD3 Δ c marks an initial communication to CD3 that a signaling-relevant peptide/MHC ligand has been bound by TCR. In mature T cells, weak peptide/MHC ligands fail to induce CD3 Δ c (Figure 6). See, also, Gil *et al.*, *J. Immunol.*, 180(6):3900-9 (2008)). Most tumor-associated antigens (TAA) are poorly immunogenic and do not stimulated anti-tumor T cell
25 function. It was hypothesized that most natural TAA fail to induce a T cell immune response due to their failure to induce CD3 Δ c (Figure 7). Poorly immunogenic TAA from melanoma like mouse gp100, indeed failed to induce CD3 Δ c (Figure 20). Mono-7D6-Fab was tested as a means to provide CD3 Δ c *in trans* during weak TCR/antigens interactions to increase T cell function triggered by weak/poorly
30 immunogenic antigens. The results provided herein demonstrate that Mono-7D6-Fab is precisely monovalent (Figure 10), it binds to mouse T cells but does not block peptide/MHC:TCR interactions (Figure 11), it induces CD3 Δ c on its own (Figure 12) (CD3 Δ c measured by the CD3-PD assay (Figures 3-4)), and it is functionally inert to

non-antigen engaged T cells both *in vitro* (Figures 13-17) and *in vivo* (Figures 13, 18, and 19). Moreover, the administration of Mono-7D6-Fab enhanced T cell signaling induced by weak peptide/MHC antigens *in vitro*, as shown in experiments using T cells from the OT-I TCR transgenic mouse model (Figures 14-17).

5 Mono-7D6-Fab's capacity to increase T cell responses to weak antigens was tested *in vivo* using a mouse model for lung metastatic melanoma. The B16.F10 cell line is a transplantable melanoma in B6 mice that colonizes lungs when injected intravenously (i.v.). This B16.F10 melanoma line is very aggressive and fast growing in B6 mice, and it is considered poorly immunogenic. In the absence of any specific
10 treatment, T cells from B6 mice fail to mount productive immune responses against the natural TAAs of B16.F10 cells. A single low-dose of Mono-7D6-Fab significantly reduced melanoma burden in the lungs of B6 mice when compared with mouse IgG Fab control treated mice in six different experiments (Figure 21). Mono-7D6-Fab anti-B16.F10 effects required the presence of T cells in B6 mice (Figure 22),
15 and both CD4 and CD8 T cells contribute to such effect (Figure 23). In addition, antigenic specificity of T cells mediating Mono-7D6-Fab is required for Mono-7D6-Fab anti-tumor effects, since in mice lacking T cells specific for B16.F10 Mono-7D6-Fab failed to reduce melanoma burden (Figure 24). These results demonstrate that Mono-7D6-Fab converted poorly stimulatory mouse TAAs of B16.F10 into efficient
20 stimuli for B16.F10 specific CD4 and CD8 T cells among the natural B6 cell repertoire.

Example 2 – Mono-7D6-Fab promotes therapeutic anti-tumor T cell responses in the B16F10/B6 lung metastatic melanoma model when administered three days after tumor injection

25 Mice were injected intravenously with a melanoma cell line (B16F10). Three days later, half of the mice were injected intravenously with a control mouse Fab fragment (Ms IgG Fab) or Mono-7D6-Fab (10 µg/mouse). 21 days after melanoma injection, all mice were sacrificed, and lungs and peripheral lymphoid organs were
30 collected (Figure 25A). Pictures of the lungs were evaluated for the presence of metastatic melanoma tumor burden. Less melanoma burden was observed in mice treated with Mono-7D6-Fab (Figure 25B). Lung pictures of each mouse in the experiment were analyzed by a software to quantify melanoma presence. A graph was prepared showing the results of melanoma density in lungs of mice treated with

either Ms IgG Fab or Mono-7D6-Fab (Figure 25C). Melanoma density in each group was statistically different (t test; *** $p < 0.0001$), showing a reduced lung metastasis in mice that received Mono-7D6-Fab (Figure 25C). In addition, T cells were isolated from the lung draining lymph nodes of the mice and stained for different activation markers: CD107a, CD44, and CD62L. Bar graphs were prepared, revealing the % of positive CD4 and CD8 T cells for these markers (Figures 25D and 25E). The results demonstrate an increase in the percentage of CD4 and CD8 T cells being activated in mice receiving Mono-7D6-Fab (Figures 25D and 25E).

10 Example 3 – Anti-melanoma effect when Mono-7D6-Fab therapy is combined with adoptive transfer of melanoma specific cytotoxic lymphocytes

Mice were injected intravenously with the melanoma cell line (B16F10). Three days later, half of the mice were injected intravenously with a control mouse Fab fragment (Ms IgG Fab) or Mono-7D6-Fab (10 $\mu\text{g}/\text{mouse}$). In addition, mice were injected at the same time with cytotoxic T lymphocytes (CTLs) and IL-2. IL-2 administration was repeated on days 4 and 5. The CTLs were either non-tumor specific (OT-I CTLs) or tumor specific (Pmel-1 CTLs). 28 days after melanoma injection, all mice were sacrificed, and lungs and peripheral lymphoid organs were collected (Figure 26A). Pictures of the lungs were evaluated for the presence of metastatic melanoma tumor burden (Figure 26B). In addition, lung pictures of each mouse in the experiment were analyzed by software to quantify melanoma presence (Figure 26C). The results of melanoma density in lungs of mice in the four experimental groups generated in the experiment (Ms IgG Fab/OT-I CTLs, Mono-7D6-Fab/OT-I CTLs, Ms IgG Fab/Pmel-1 CTLs, and Mono-7D6-Fab/Pmel-1 CTLs) were graphed (Figure 26D). Statistical analysis of melanoma density indicates that there was a synergistic effect against melanoma when Mono-7D6-Fab was combined with the Pmel-1 CTLs specific for the tumor but not when combined with the non-tumor specific OT-I CTLs (t test, * $p < 0.005$ ** $p < 0.001$ *** $p < 0.0001$). CD8 T cells present in the mediastinal lymph node (Figure 26D) or the lungs (Figure 26E) of the mice engaged in this experiment were stained with the Kd-tetramer gp100 to identify cells specific for the melanoma antigen gp100. The bar graphs revealed the increase in CD8 T cell counts specific for the melanoma in the mice receiving the Mono-7D6-Fab (Figure 26D).

Example 4 – Mono-7D6-Fab increases survival of mice treated with tumor specific CTLs

B6 mice were injected with B16-F10. Three days later, Pmel-1 CTLs were adoptively transferred into the B6 mice, together with Ms IgG Fab or Mono-7D6-Fab treatments (as described in Example 3). Survival was monitored for 100 days. (n=10, ** p < 0.005, Mantel-Cox Log-Rank test). As shown in Figure 27, mono-7D6-Fab increases survival of mice treated with tumor specific CTLs.

Example 5 – Mono-7D6-Fab synergizes with anti-melanoma immunotherapies that target co-inhibitory receptors on T cells

B6 mice were i.v. injected with B16-F10 melanoma. Three days later, mice were i.p injected with anti-CTLA-4 and anti-PD-1, or control antibodies. Additionally on day 3, mice were i.v. injected with either Mono-7D6-Fab or control Ms IgG Fab. Subsequently on days 5, 7, and 9, mice were re-treated with anti-CTLA-4 and anti-PD-1 (or control) antibodies. After 24 days, lungs were collected. (Figure 28A) Lung photographs from one independent experiment (Figure 28A). Quantification of melanoma found in lungs. Dots represent individual mice (mean +/- SEM displayed; * p < 0.05, ** p < 0.005 *** p < 0.0005, two tailed, unpaired Student's t-test) (Figure 28B). As shown in Figure 28, mono-7D6-Fab synergizes with anti-melanoma immunotherapies that target co-inhibitory receptors on T cells.

Example 6 – Using an anti-human CD3 $\gamma\epsilon$ / $\delta\epsilon$ CD3 monovalent Fab (Mono-OKT3-Fab) to increase immune responses of human T cells

Mono-OKT3-Fab binds to human CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ on T cells, as shown by positive staining of a Jurkat T cell line expressing the murine TCR OT-I and human CD3 complex (OT-I JRT3) (Figure 29A). When bound to T cells, Mono-OKT3-Fab does not block TCR/antigen binding, as shown by unaffected flow cytometry staining of OT-I JRT3 cells with the fluorescent tetramer K^B/OVA-PE in the presence of Mono-OKT3-Fab (Figure 29B). Mono-OKT3-Fab is unable to stimulate T cells on its own, as shown by absent CD69 up-regulation and OT-I TCR internalization on OT-I JRT3 cells exposed to null peptide pFARL, nor blocks T cell cognate antigenic stimulation, as shown by equivalent CD69 up-regulation and OT-I TCR internalization on OT-I Jurkat T cells exposed to the agonist peptide pOVA in the

presence of Ms IgG Fab and Mono-OKT3-Fab (Figure 29C). Mono-OKT3-Fab induces CD3Δc on hPBMCs, as detected using the CD3-PD assay (Figure 29D).

Example 7 – Mono-OKT3-Fab increases de frequency of human CD8 T cells producing IFN γ in vitro

Human PBMCs (hPBMCs) were isolated from 3 different healthy donors and were fractionated into CD8 T cell positive (CD8) and CD8 T cell negative (APC) PBMC populations. Irradiated APCs and CD8 T cells were cultured for 72 hours separately or mixed at a 1:1 ratio in the presence of Ms IgG Fab or Mono-OKT3-Fab and after culture IFN γ production was detected from triplicate samples by ELISPOT. A positive control for CD8 T cell stimulation was generated by culture in the presence of phytohaemagglutinin (PHA) * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, two-tailed, unpaired Student's t-test. As shown in Figure 30, mono-OKT3-Fab increases de frequency of human CD8 T cells producing IFN γ *in vitro*.

Example 8 – Mono-OKT3-Fab does not interfere with the course of Graft versus Host Disease (GVHD) in NSG mice

18×10^6 hu PBMCs were adoptively transferred into immunodeficient NSG recipient mice via intra-peritoneal injection. On the same day, mice were injected with 10 μ g of control Ms IgG Fab or Mono-OKT3-Fab (5 mice/ Fab condition). Mice were monitored for external signs of GvHD. Time of euthanasia was established when mice presented 15% weight loss, hunched posture, ruffled fur, reduced mobility, and hair loss. Mono-OKT3-Fab did not change the course of GvHD, monitored by weight loss (Figure 31A) and percent survival (Figure 31B). Mono-OKT3-Fab did not change the frequency of activated T cells (CD44^{Hi}) found in mice sacrificed due to GvHD (Figure 31C). As shown in Figure 31, mono-OKT3-Fab does not interfere with the course of Graft versus Host Disease (GVHD) in NSG mice.

Example 9 – Mono-OKT3-Fab reduces tumor burden of pre-established human melanoma in lungs in a humanized mouse model

Immunocompromised NSG mice were i.v. injected with the human metastatic melanoma cell line A375RC-Luc. Live mice were imaged to monitor tumor growth at the indicated time points by measuring luciferase activity. On day 10, tumor burden

in lungs was considered established. At that time point mice were adoptively transferred with 10×10^6 hPBMCs from a random healthy donor. After that mice were i.v. injected of 10 μ g of control Ms IgG Fab or Mono-OKT3-Fab. The same dose of Fabs was injected on days 13 and 17. Luciferase imaging of representative mice is shown in Figure 32A. Average luciferase activity is shown in Figure 32B. 10 mice were analyzed for each Fab condition. The mean $\pm$ SEM is displayed; * $p < 0.05$, ** $p < 0.005$, two tailed, unpaired Student's t-test.

Mice were sacrificed at day 27 and cell suspension from spleens, peripheral lymph nodes, and mediastinal lymph nodes were prepared for staining of T cell markers. Samples were subjected to flow cytometry analysis. Figure 32C shows the total live T cell counts found in the spleen of mice. Figure 32D shows the total live T cell counts found in the peripheral lymph nodes of mice. Figure 32E shows the total live T cell counts found in the mediastinal lymph nodes of mice. Total live T cell counts shown are average T cell counts ($\pm$ SE) in each group of mice. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, two tailed, unpaired Student's t-test.

Example 10 – Anti-CD3 Monobodies

A synthetic sequence for the tenth extracellular domain of human FN3 (Gribskov *et al.*, *Nuc. Acids. Res.*, 12:539-549 (1984)) is used, as shown in Figure 33.

Monobody libraries are constructed by diversifying residues in several loop regions of the human FN3. Libraries are prepared by diversifying residues in the BC loop and randomizing residues in the FG loop. Additional libraries are prepared by inserting seven diversified residues between Pro-15 and Thr-16 in the AB loop, and by randomizing residues and inserting an additional eight randomized residues in the FG loop. In each instance, the above-noted residues are randomized using the NNK codon (N denotes a mixture of A, T, G, C; K denotes a mixture of G and T) or NNS codon (S denotes a mixture of G and C) by Kunkel mutagenesis (Kunkel *et al.*, *Meth. Enzymol.*, 154:367-382 (1987)).

Phage display libraries are constructed by inserting randomized FN3 sequences into a phage coat protein gene, causing the phage to “display” the protein on its outside. Phages are produced and purified according to standard methods (Sambrook *et al.*, (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory).

Yeast display libraries are constructed by cloning the randomized FN3

sequences into a yeast plasmid so that expressed randomized FN3 protein is fused to the Aga2p protein on the surface of yeast as described elsewhere (Boder *et al.*, *Nat. Biotech.*, 15:553-557 (1997)).

Library screening using CD3 target molecules is carried out in order to select
5 displayed anti-CD3 monobodies.

An exemplary extracellular domain of human CD3 ϵ can be NCBI accession no. NP_000724 (Version: NP_000724.1; GI: 4502671):

dgneemgg itqtpykvsi sgttviltcp qypgseilwq hndkniggde
ddknigsded hlslkefsel eqsgyyvcyp rgskpedanf ylylrarvce ncmemd

10 (SEQ ID NO:1).

An exemplary extracellular domain of human CD3 γ can be NCBI accession no. NP_000064 (Version: NP_000064.1; GI: 4557429):

aiillqgt laqsikgnhl vkvydyqedg svlltcdaea knitwfkdgk
migfltedkk kwnlgsnakd prgmyqckgs qnkskplqvy yrmcqniciel naatis

15 (SEQ ID NO:2).

An exemplary extracellular domain of human CD3 δ can be NCBI accession no. NP_000723 (Version: NP_000723.1; GI: 4502669):

latllsqvs pkipieele drvfvncnts itwvegtvgt llsditrldl
gkrildprgi yrcngtdiyk dkestvqvh y rmcqscveld patva (SEQ ID NO:3).

20 FN3 mutants that bind to CD3 are recovered and sequenced. Sequences identified as encoding anti-CD3 monobodies are cloned into expression vectors. Anti-CD3 monobodies are expressed and purified.

Example 11 - Mono-Hit3a-Fab and Mono-SP34-2-Fab improve performance of 25 human T cells

Mono-OKT3-Fab bound to human CD3 $\gamma\epsilon$ and CD3 $\delta\epsilon$ on T cells, as shown by positive staining of a Jurkat T cell line expressing the murine TCR OT-I and human CD3 complex (OT-I JRT3) (Figure 29A). When bound to T cells, mono-OKT3-Fab did not block TCR/antigen binding, as shown by unaffected flow cytometry staining
30 of OT-I JRT3 cells with the fluorescent tetramer K^B/OVA-PE in the presence of Mono-OKT3-Fab (Figure 29B). Mono-OKT3-Fab was unable to stimulate T cells on its own, as shown by absent CD69 up-regulation and OT-I TCR internalization on OT-I JRT3 cells exposed to null peptide pFARL, nor block T cell cognate antigenic stimulation, as shown by equivalent CD69 up-regulation and OT-I TCR

internalization on OT-I Jurkat T cells exposed to the agonist peptide pOVA in the presence of Ms IgG Fab and Mono-OKT3-Fab (Figure 29C).

Anti-human CD3 $\epsilon\gamma/\epsilon\delta$ mono-Fabs from mAbs Hit3a, SP34-2, SK7, and UCHT-I bound to human CD3, as shown by positive staining of a Jurkat T cell line expressing the murine TCR OT-I and human CD3 complex (OT-I JRT3) (Figure 34A). HIT3a and SP34-2 mono-Fabs did not block TCR/Ag interaction, while SK7 and UCHT1 mono-Fabs blocked TCR/Ag interaction, as shown by flow cytometry staining of OT-I JRT3 cells with the fluorescent tetramer K^B/OVA-PE in the presence of these Fabs (Figure 34B).

Mono-Fabs from OKT3, HIT3a, and SP34-2 neither blocked nor promoted a T cell response to cognate antigen stimulation (Figure 35). The mono-Fabs were unable to stimulate T cells alone, as shown by absent CD69 up-regulation and OT-I TCR internalization on OT-I JRT3 cells exposed to null peptide pFARL, nor block T cell cognate antigenic stimulation, as shown by equivalent CD69 up-regulation and OT-I TCR internalization on OT-I Jurkat T cells exposed to the agonist peptide pOVA in the presence of Ms IgG Fab and mono- Fabs OKT3, Hit3a and SP34-2.

Mono-Fabs from OKT3, HIT3a, and SP34-2 Abs induced CD3 Δc on human CD3 ϵ (Figure 36). The CD3 Δc was measured by the CD3-PD assay (Figures 3 and 4). T cells expressing human CD3 ϵ were lysed and post-nuclear fractions were used to capture TCR/CD3 complex with CD3 Δc using the CD3-PD assay as described in Figures 3 and 4. Samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane for detection of captured TCR/CD3 complex by Western blot against subunits of the CD3 complex like CD3 γ in the upper panel, or CD3 ζ , lower panel.

Mono-Fabs from OKT3 and Hit3a increased the frequency of CD8 T cells reacting to autologous APCs (Figure 37).

Human PBMCs (hPBMCs) were isolated from a healthy donor and were fractionated into CD8 T cell positive (CD8) and CD8 T cell negative (APC) PBMC populations. Irradiated APCs and CD8 T cells were cultured for 72 hours separately or mixed at a 1:1 ratio in the presence of Ms IgG Fab, Mono-OKT3-Fab or Mono-Hit3a-Fab, and after culture IFN γ production was detected from triplicate samples by ELISPOT. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, two-tailed, unpaired Student's t-test. As shown in Figure 30 for mono-OKT3-Fab, both Mono-OKT3-Fab and Mono-Hit3a-Fab increased the frequency of human CD8 T cells producing IFN γ *in vitro*.

These results demonstrated that mono-Fabs from mAbs OKTs, Hit3a, and SP34-2 can enhance T cell response to tumors.

OTHER EMBODIMENTS

- 5 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

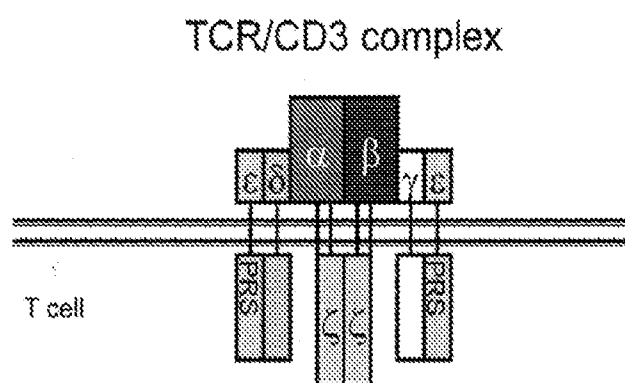
1. A method for increasing an immune response against an antigen, wherein said method comprises administering a composition comprising an anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody preparation and said antigen or nucleic acid that expresses said antigen to a mammal, wherein said mammal produces an immune response against said antigen that is increased as compared to an immune response produced against said antigen when said antigen or said nucleic acid is administered to a comparable mammal in the absence of said anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody preparation.
2. The method of claim 1, wherein said mammal is a human, and said anti-CD3 $\gamma\epsilon/\delta\epsilon$ monobody comprises a human FN3 scaffold.
3. The method of claim 2, wherein said human FN3 scaffold is a tenth extracellular domain of human FN3.
4. The method of claim 1, wherein said antigen is a tumor associated antigen.
5. The method of claim 4, wherein said tumor associated antigen is a polypeptide.
6. The method of claim 1, wherein said antigen is within an extract from a whole tumor cell lysate.
7. The method of claim 1, wherein said method comprises administering said antigen to said mammal.
8. The method of claim 1, wherein said method comprises administering said nucleic acid to said mammal.
9. A monovalent OKT3-Fab, monovalent Hit3a-Fab, or a monovalent SP34-2-Fab.
10. A method for increasing an immune response against an antigen, wherein said method comprises administering a composition comprising (a) a monovalent OKT3-

Fab, a monovalent Hit3a-Fab, or a monovalent SP34-2-Fab and (b) said antigen or nucleic acid that expresses said antigen, to a mammal, wherein said mammal produces an immune response against said antigen that is increased as compared to an immune response produced against said antigen when said antigen or said nucleic acid is administered to a comparable mammal in the absence of said monovalent OKT3-Fab, said monovalent Hit3a-Fab, and said monovalent SP34-2-Fab.

11. The method of claim 10, wherein said mammal is a human.
12. The method of claim 10, wherein said antigen is a tumor associated antigen.
13. The method of claim 12, wherein said tumor associated antigen is a polypeptide.
14. The method of claim 10, wherein said antigen is within an extract from a whole tumor cell lysate.
15. The method of claim 10, wherein said method comprises administering said antigen to said mammal.
16. The method of claim 10, wherein said method comprises administering said nucleic acid to said mammal.

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FIG. 1



In the absence of stimulus → CD3 Closed conformation

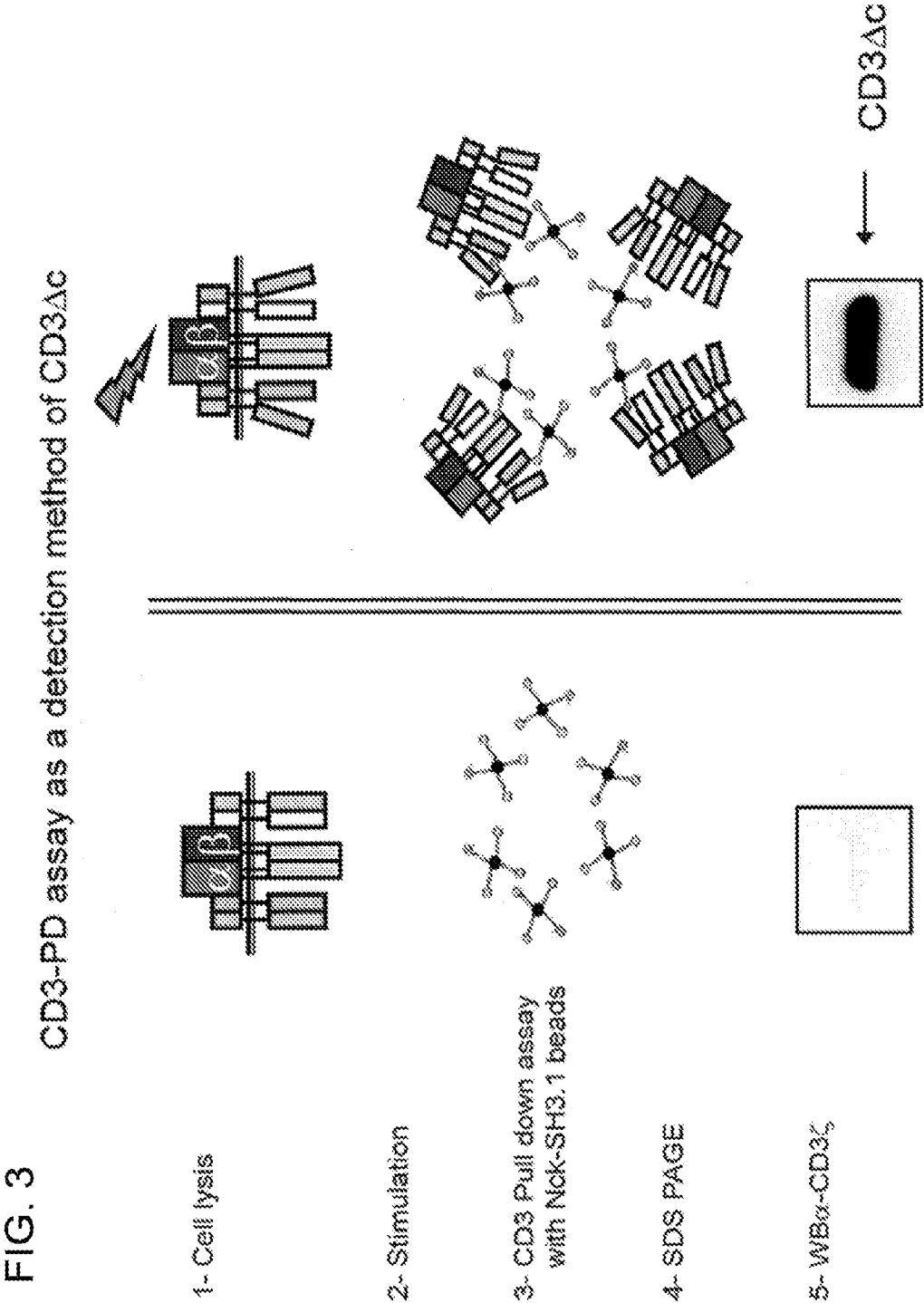


FIG. 4

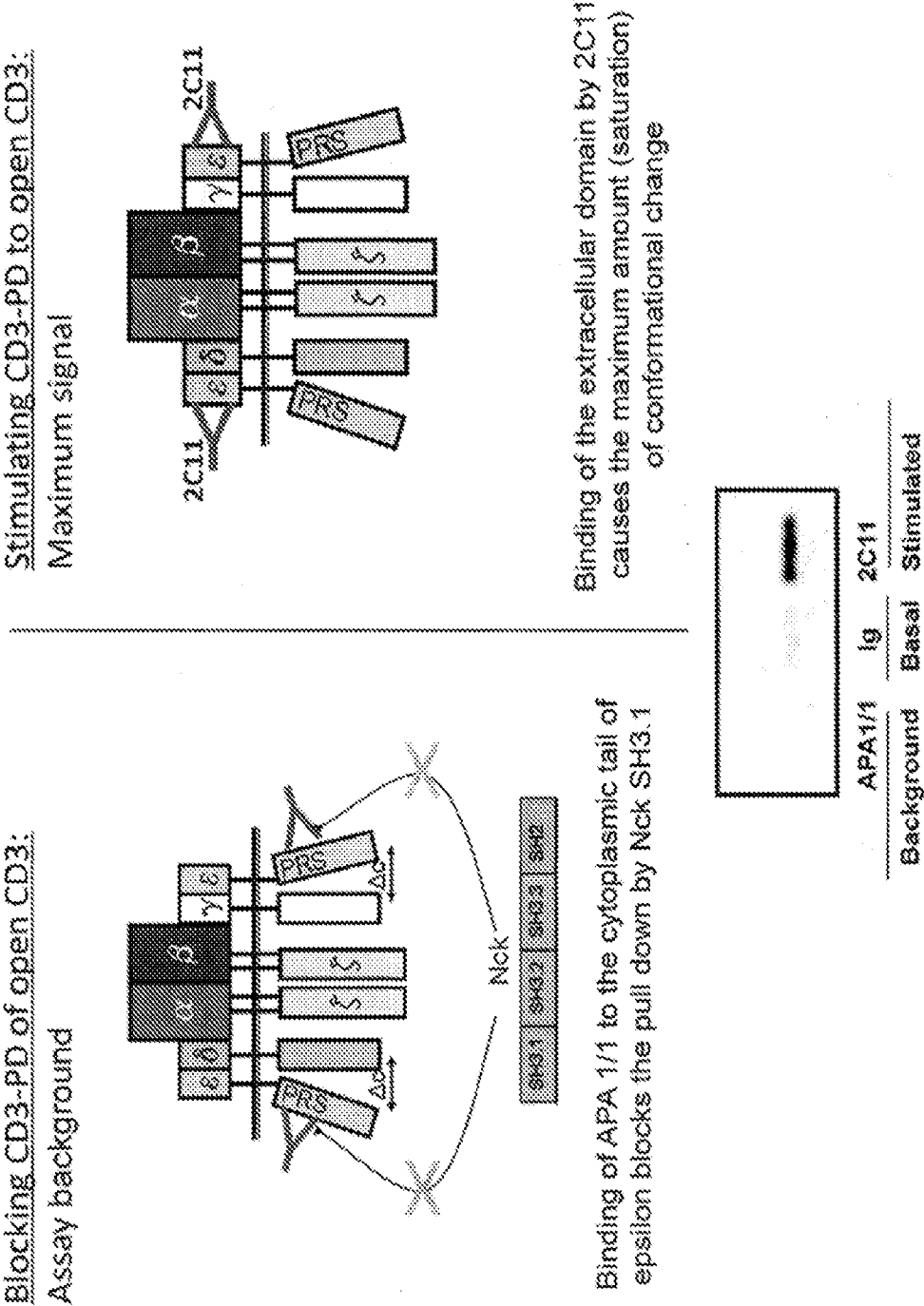
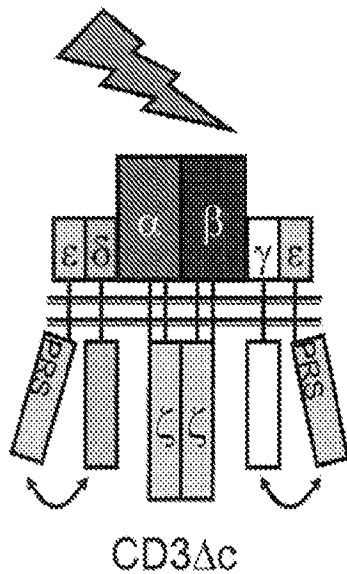


FIG. 5



CD3Δc Physical Properties

- Independent of Src kinase and other enzymatic activity
- Dependent on proteins within TCR/CD3
- Dependent on TCR/pMHC binding or anti-CD3mAb engagement

Only strong/immunogenic antigens induce CD3Δc in Mature T cells

CD3Δc Functional Properties

- Required for full TCR/CD3 signaling
- Possible role in thymic selection
- Possible role in T cell proliferation

CD3Δc PRS Functional Properties

- Role in TCR/CD3 expression, ubiquitination, and degradation
- Role in efficiency of thymocyte development
- Role in synapse formation

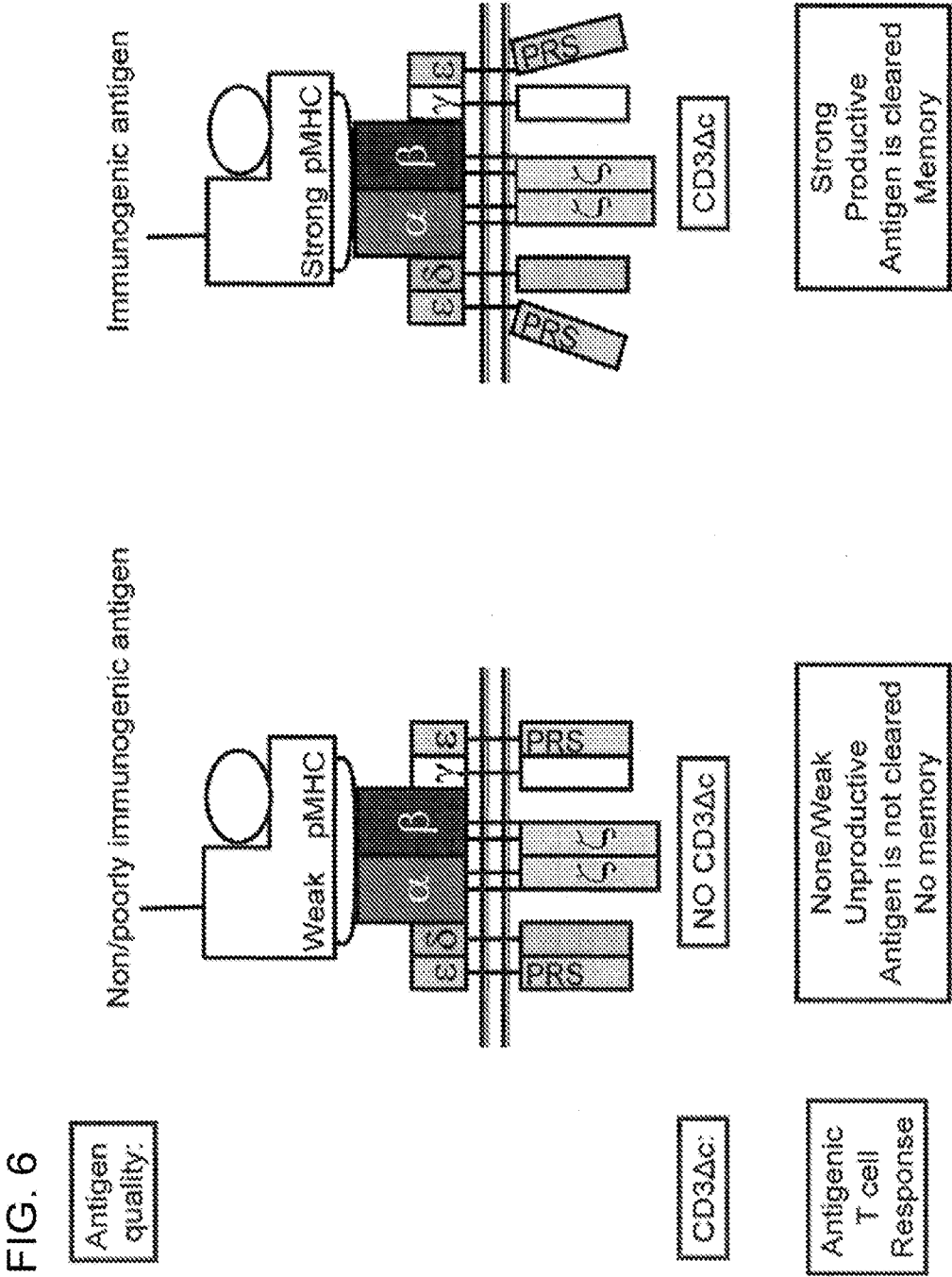


FIG. 7

Tumor associated antigens (TAA)
 Most are poorly immunogenic because they are generated from self proteins
 or altered self proteins (mutated, overexpressed, incorrectly expressed self proteins)

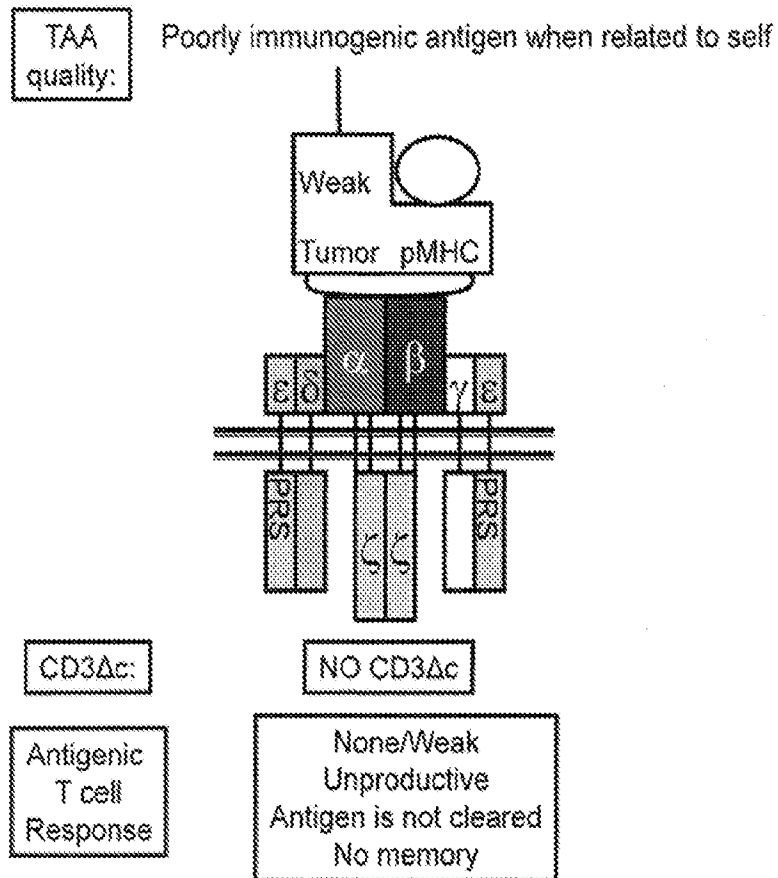


FIG. 8
Complementation of Weak antigens to achieve productive T cell response

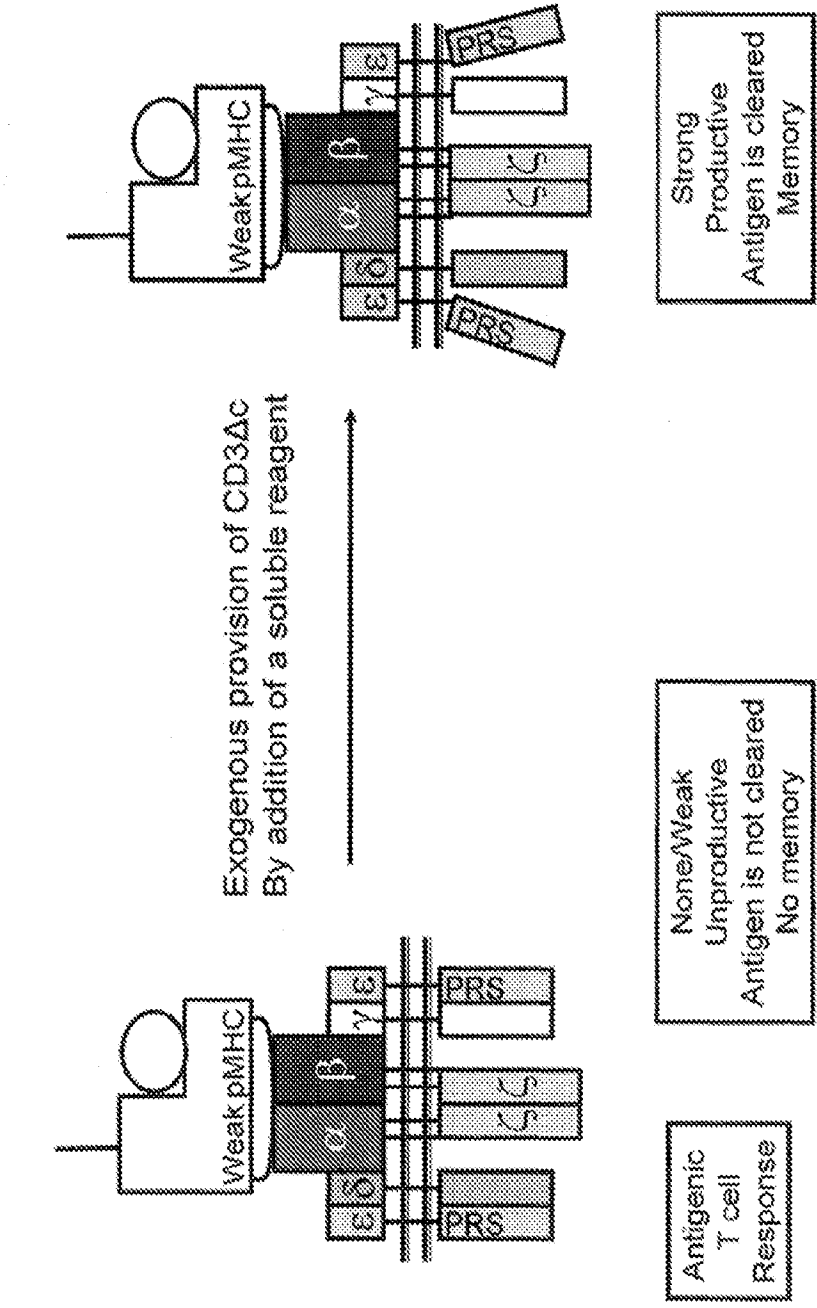
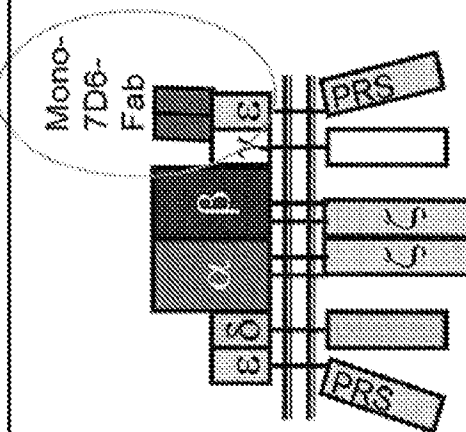


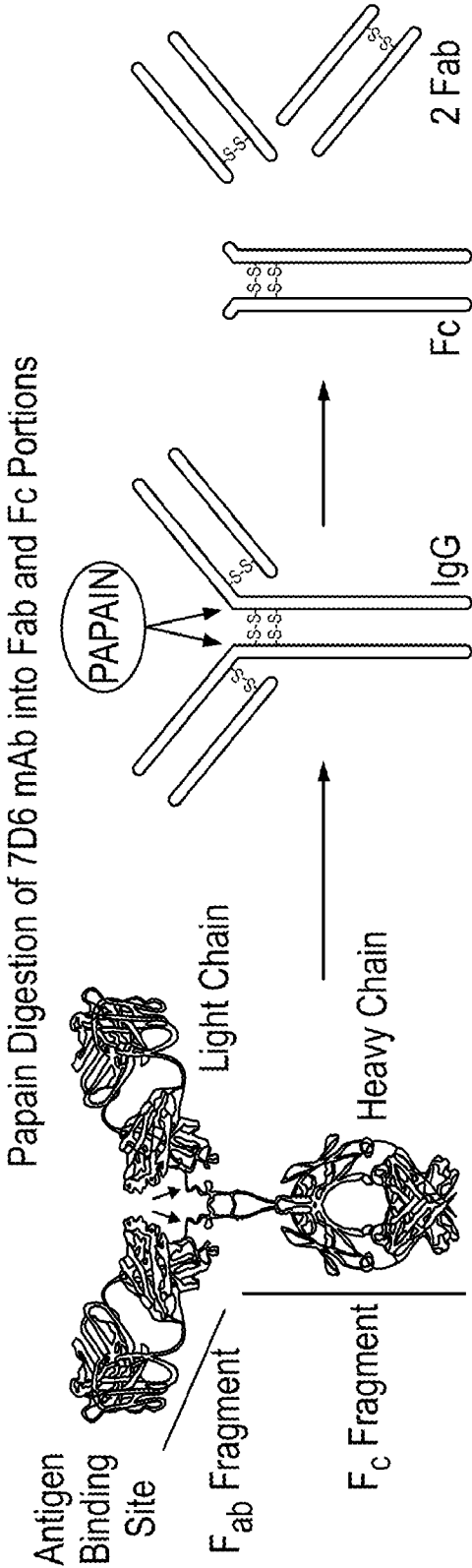
FIG. 9

Requirements of reagent to achieve complementation of weak antigens :

- Binds specifically to T cells
- Induces CD3Δc
- Fails to stimulate T cell responses in the absence of TCR/Antigen binding
- Does not interfere with TCR/Antigen interaction

Mono-7D6-Fab fulfills all these requirement





FPLC Purification of Mono-7F6-Fab in 2M Proline

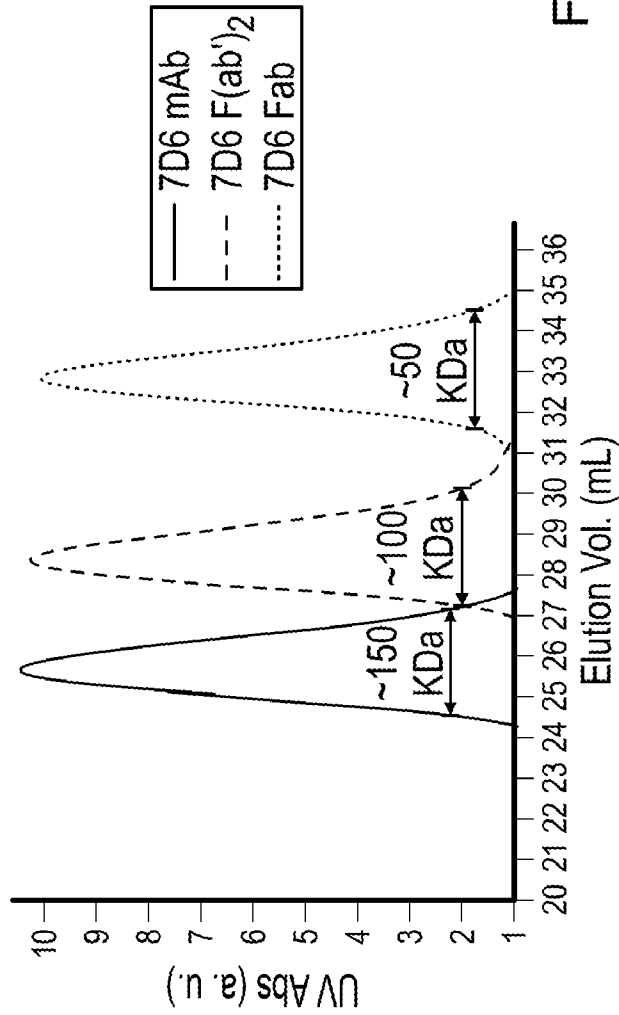
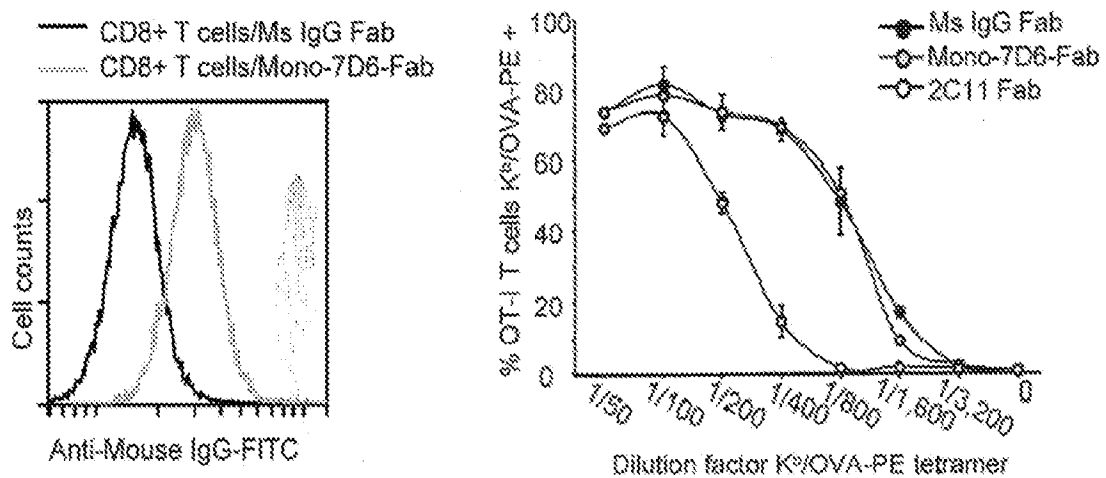


FIG. 10

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FIG. 11

Mono-7D6-Fab binds the TCR and does not block pMHC7TCR binding



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FIG. 12

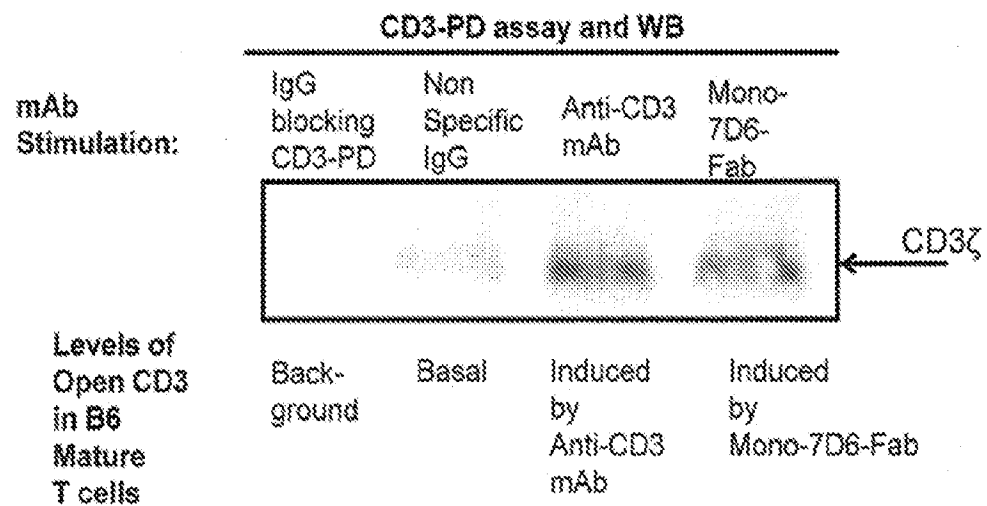
Mono-7D6-Fab induces CD3 Δ c in Mature T cells from mice

FIG. 13 Mono-7D6-Fab does not stimulate T cells on its own

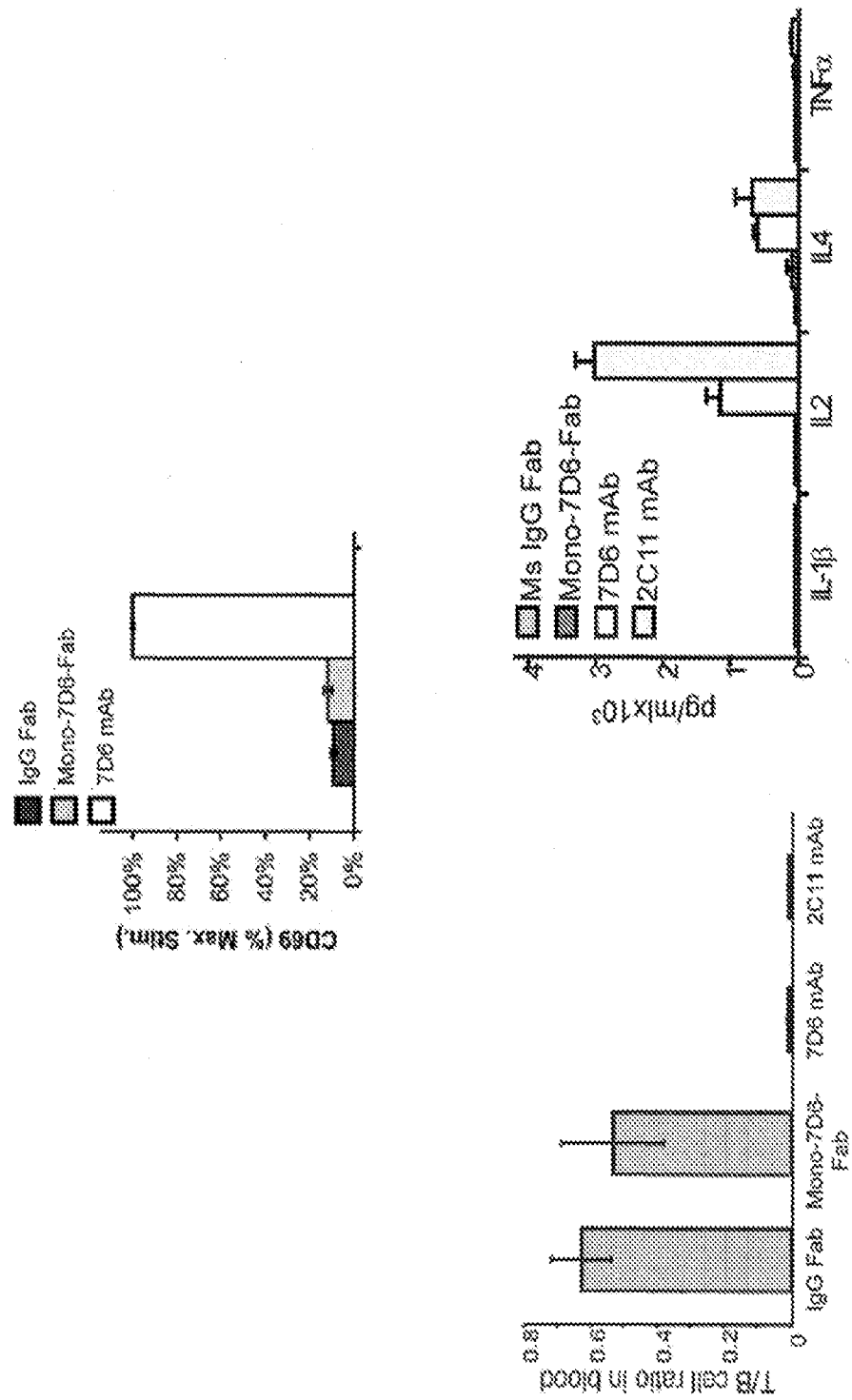


FIG. 14

Use altered peptide ligands (APLs) for the OT-I transgenic TCR to test reactivity against weak antigens

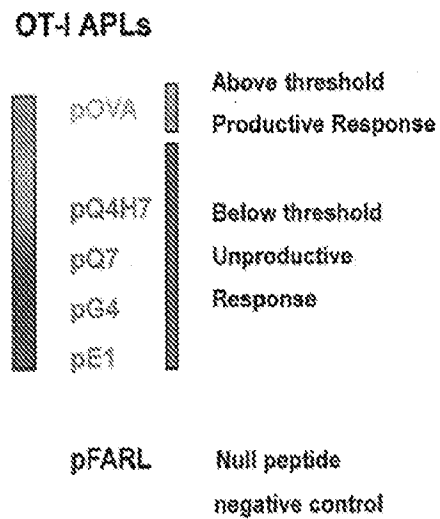
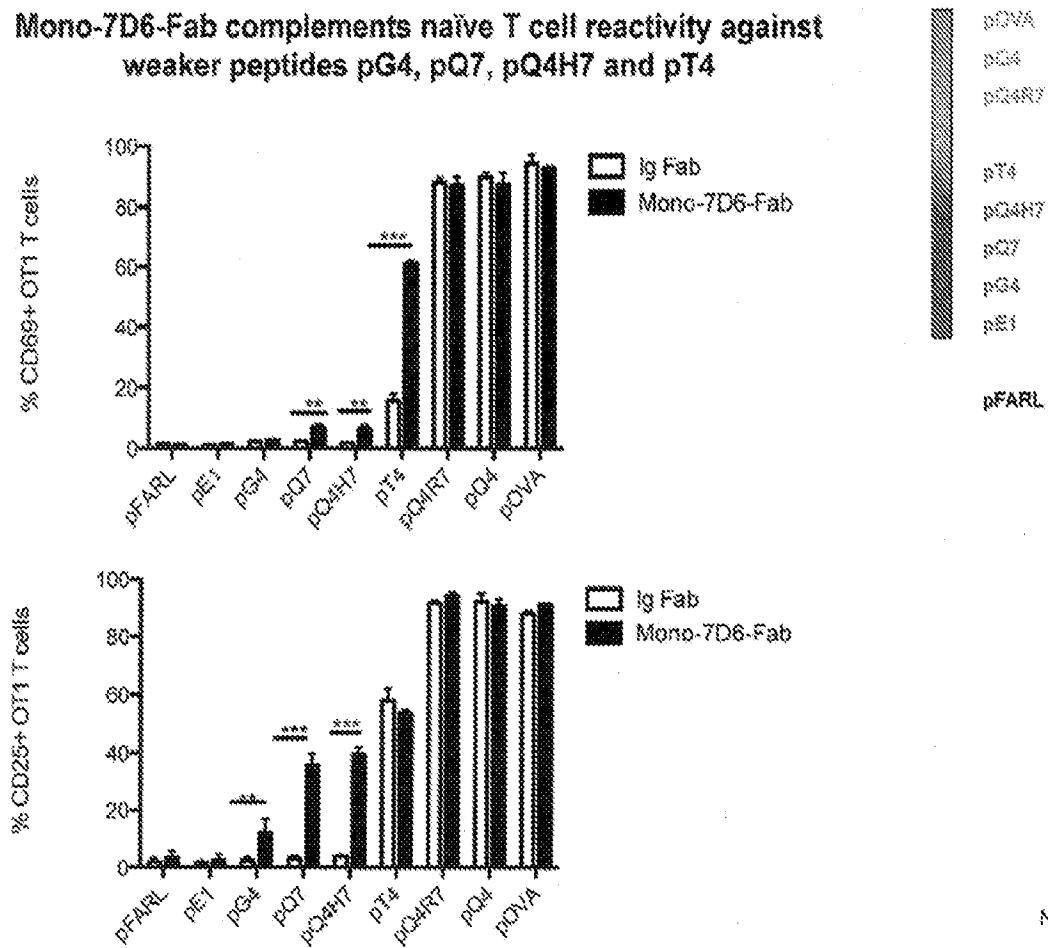


FIG. 15

Mono-7D6-Fab complements naïve T cell reactivity against weaker peptides pG4, pQ7, pQ4H7 and pT4



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FIG. 16

Addition of Mono-7D6-Fab allows for increased proliferation with weak peptides pQ7, pQ4H7

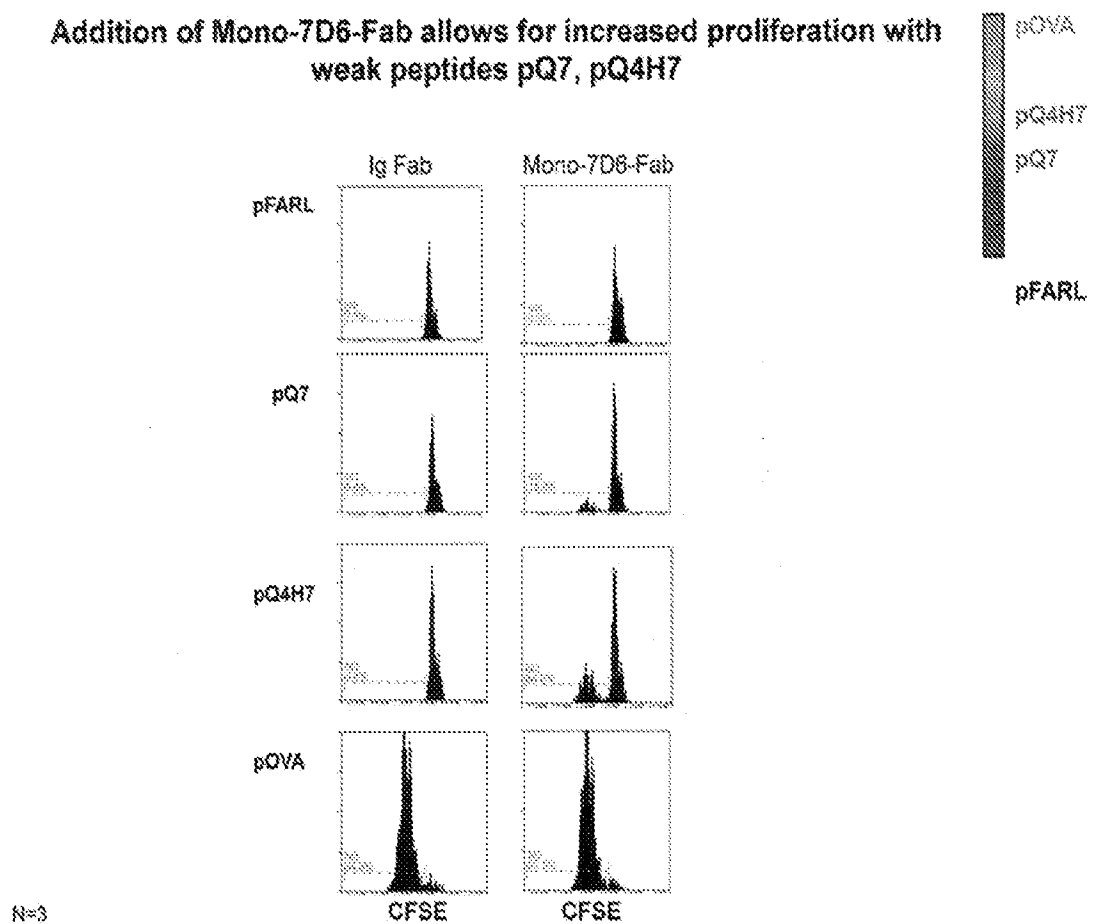


FIG. 17

Addition of Mono-7D6-Fab allows for increased OT-1 CTL killing
against weak peptides pE1 and pG4

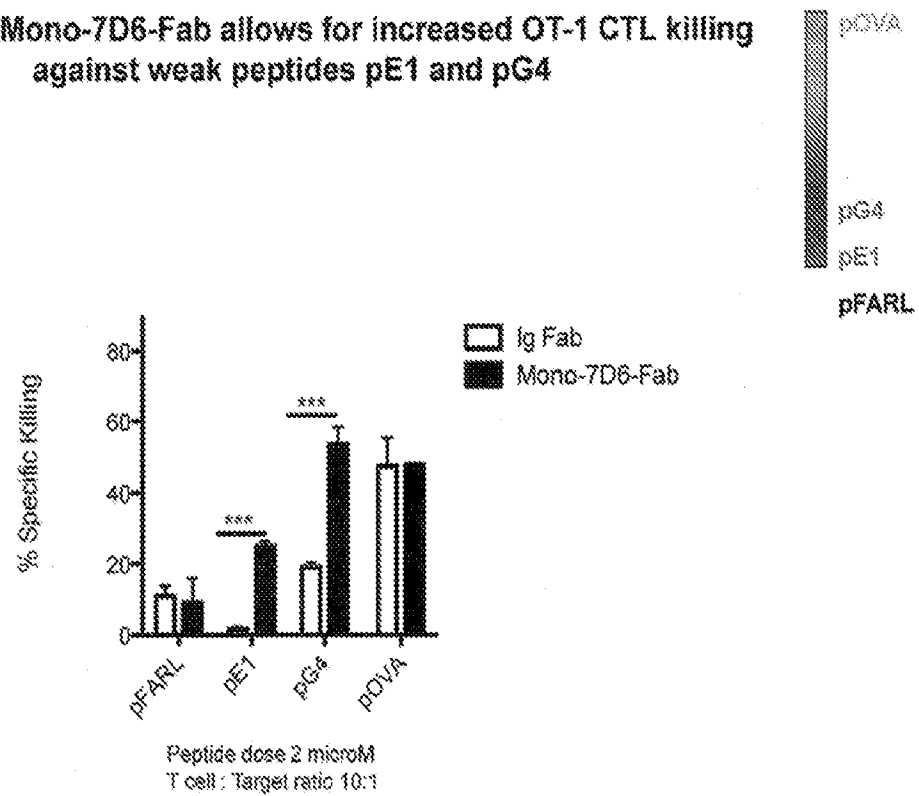


FIG. 18
Mono-7D6-Fab does not cause autoimmune responses when injected in healthy mice (I)

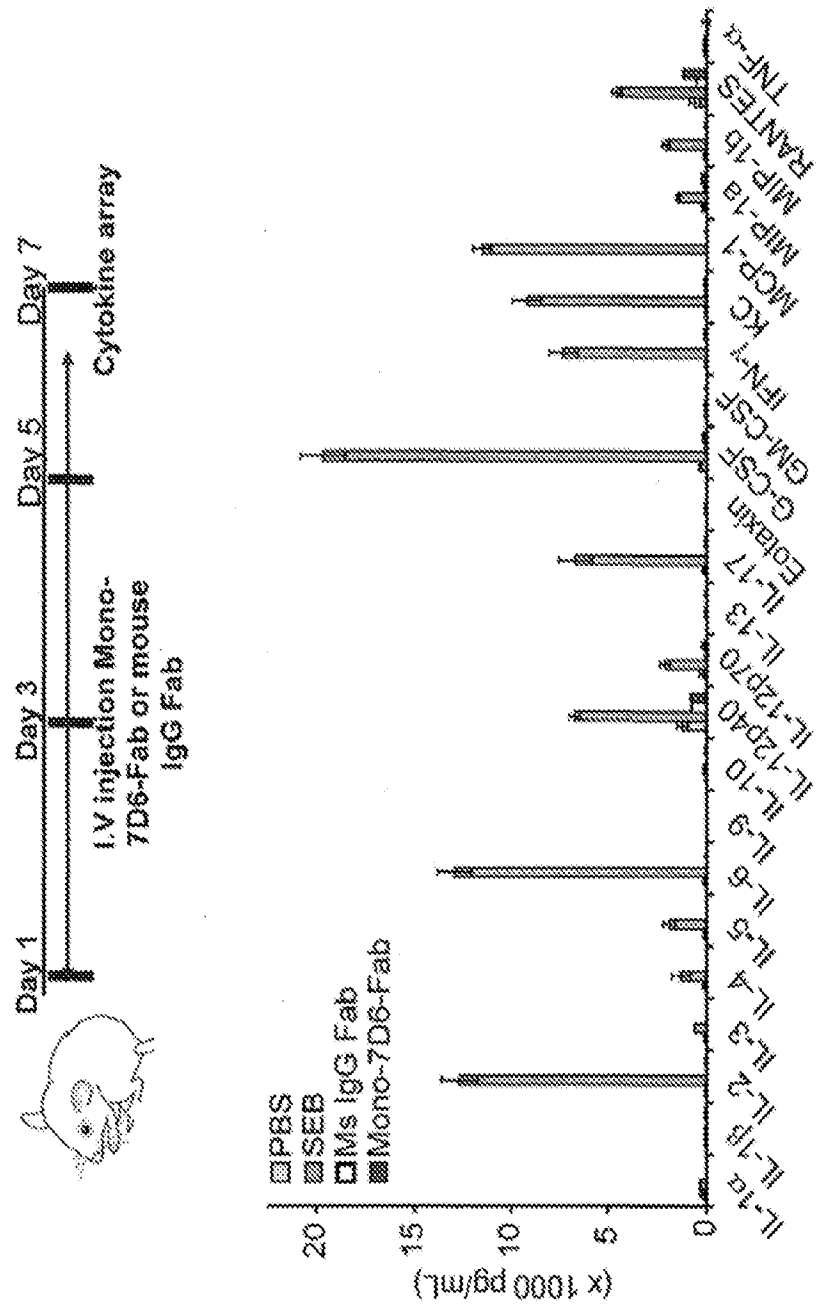
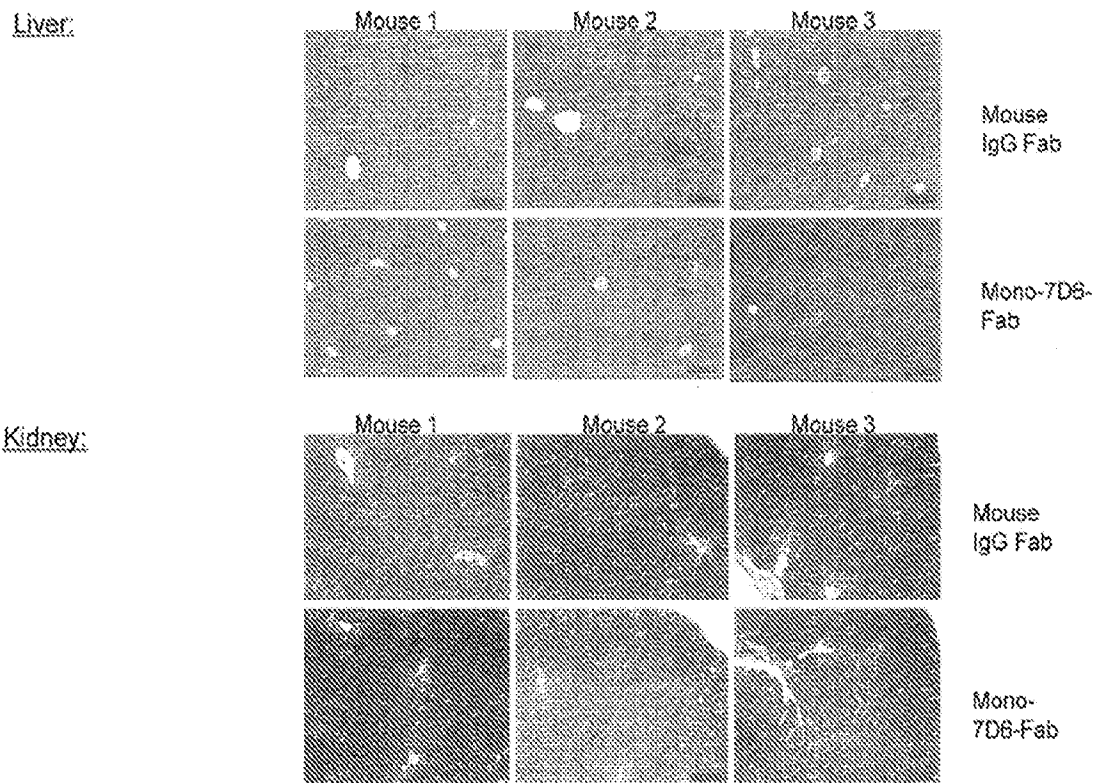


FIG. 19

Mono-7D6-Fab does not cause autoimmune responses when injected in healthy mice (II)



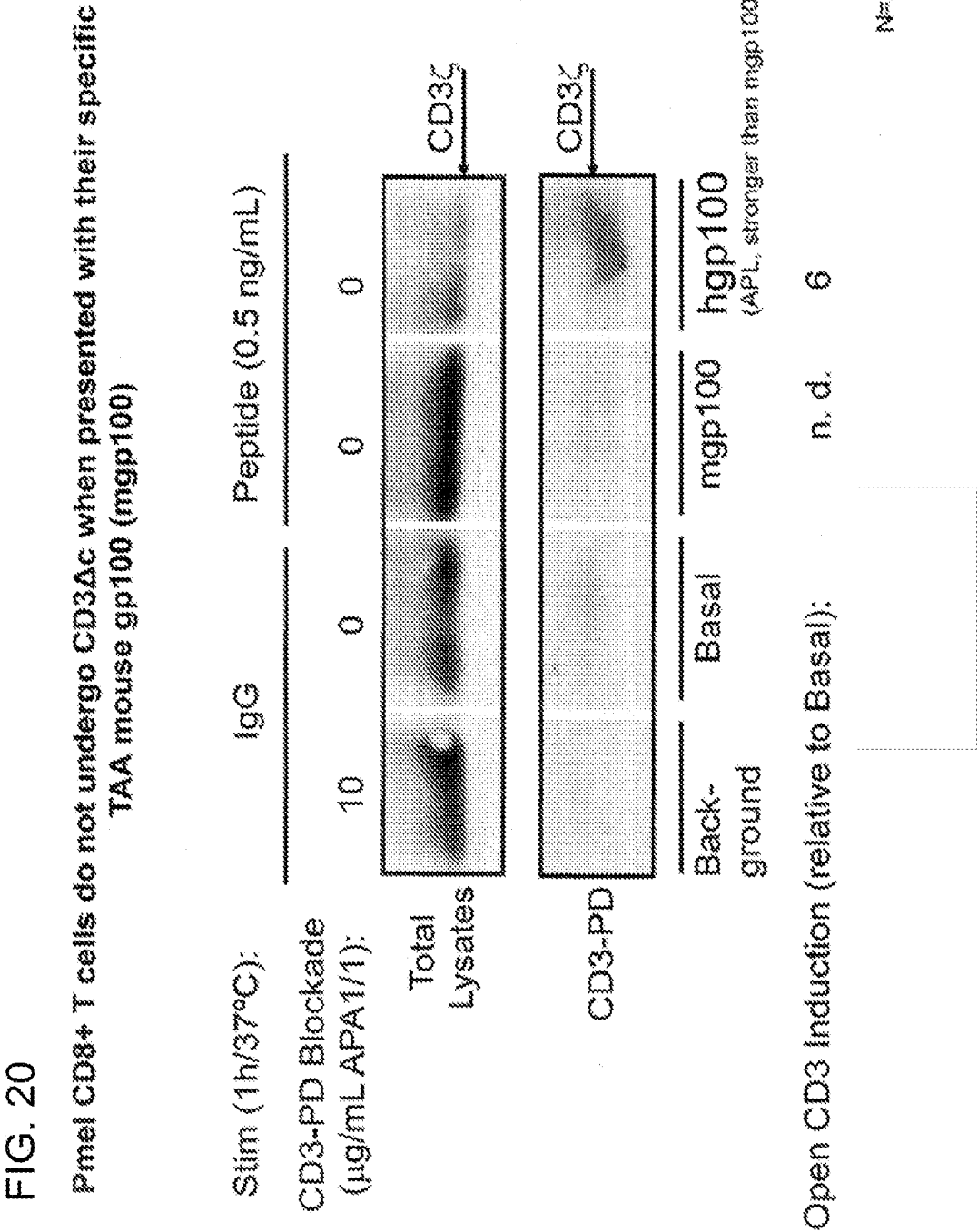


FIG. 21

Injection of Mono-7D6-Fab results in decreased B16F10 metastatic melanoma tumor burden in B6 mice

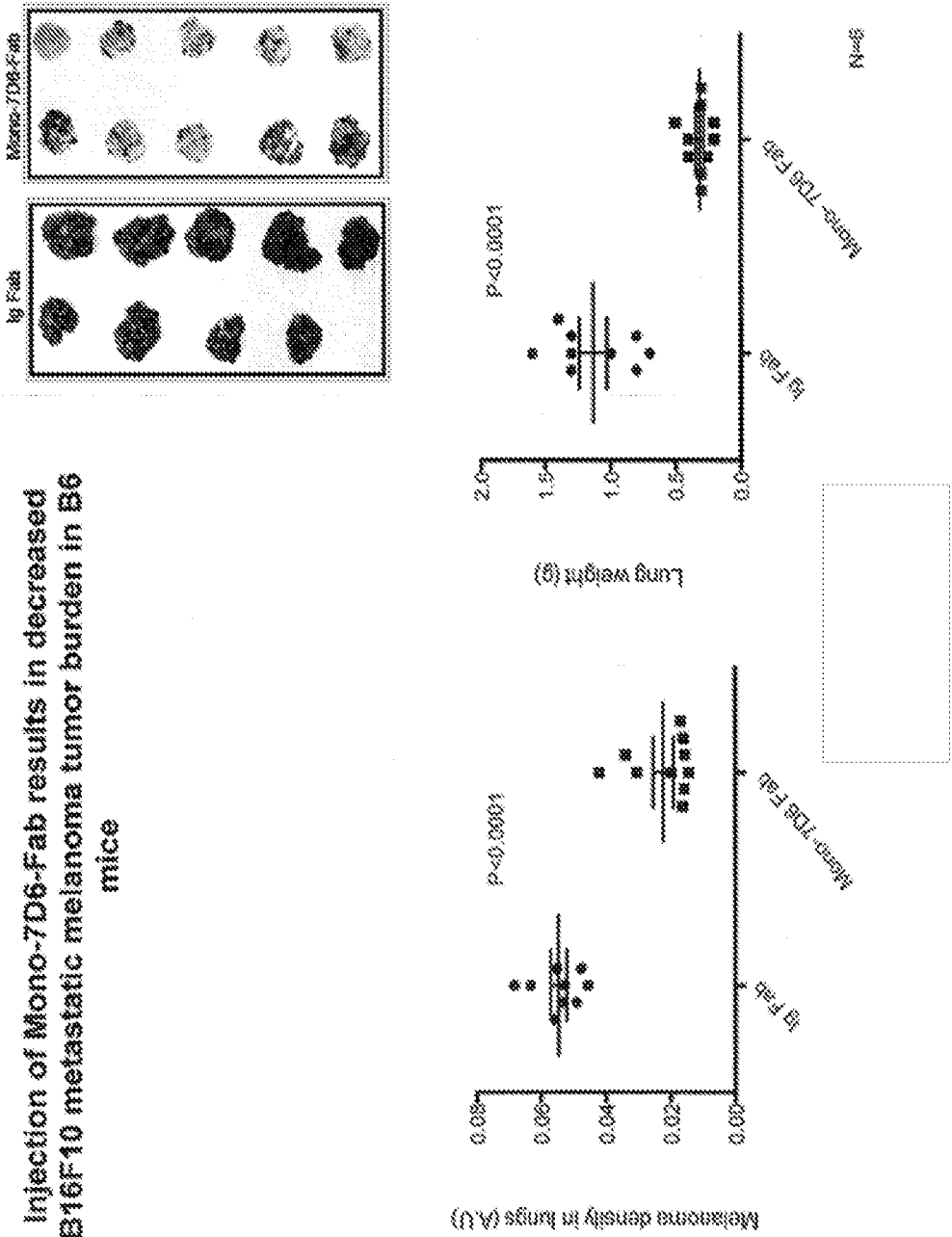


FIG. 22 Mono-7D6-Fab treatment requires T cells

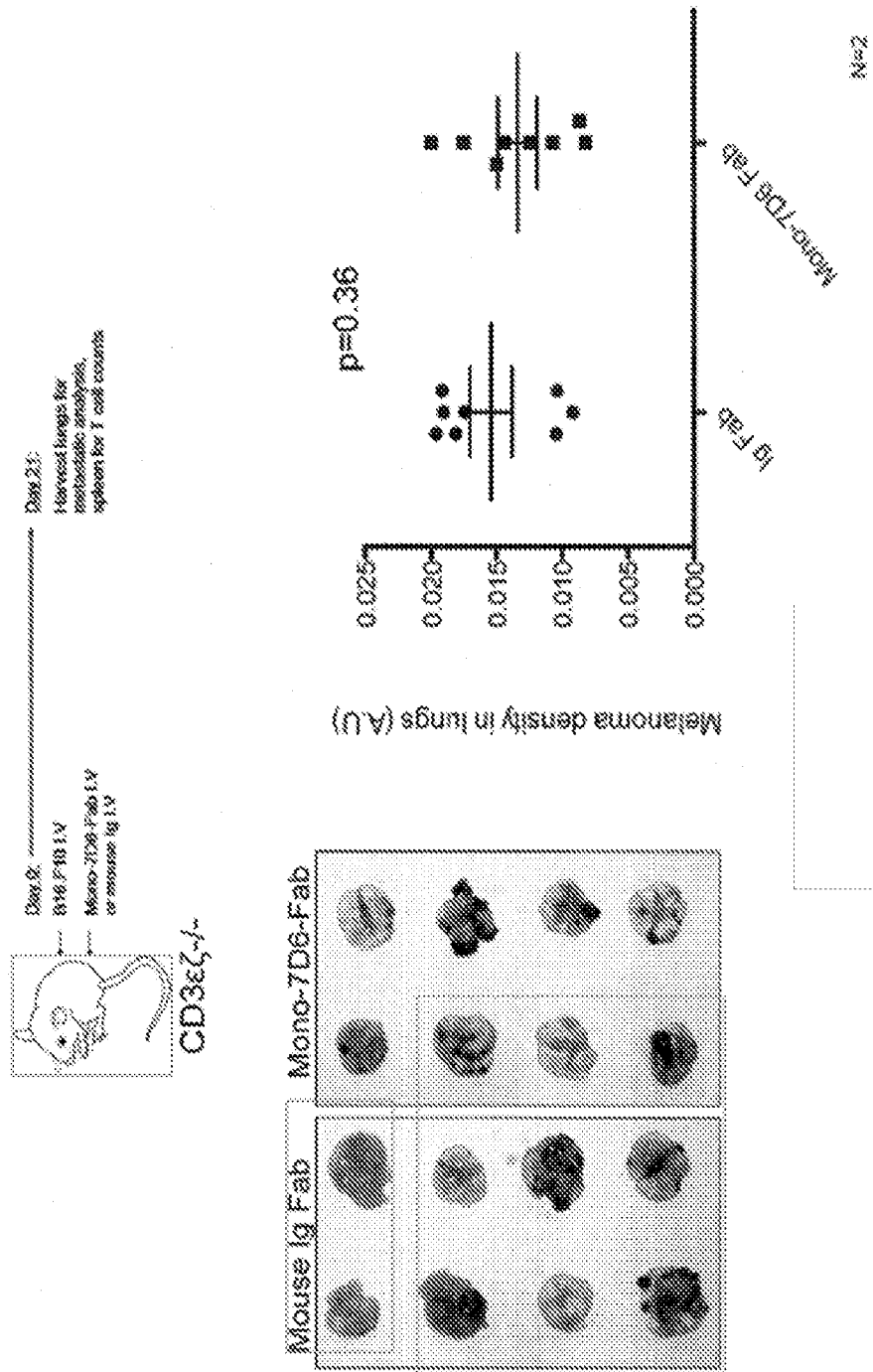


FIG. 23

Both CD4 and CD8 T cells are required for full effect of Mono-7D6-Fab treatment

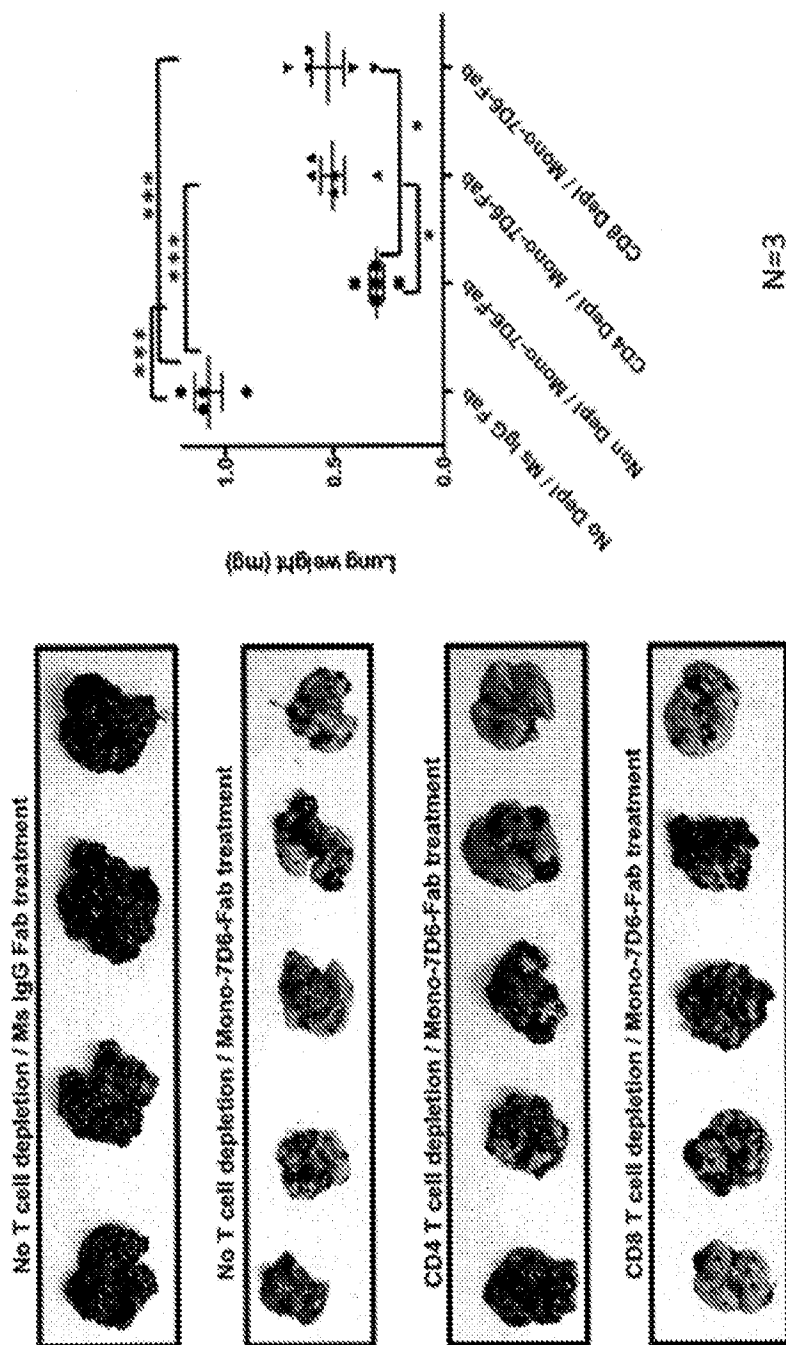


FIG. 24
Mono-7D6-Fab requires tumor specific CD8 T cells

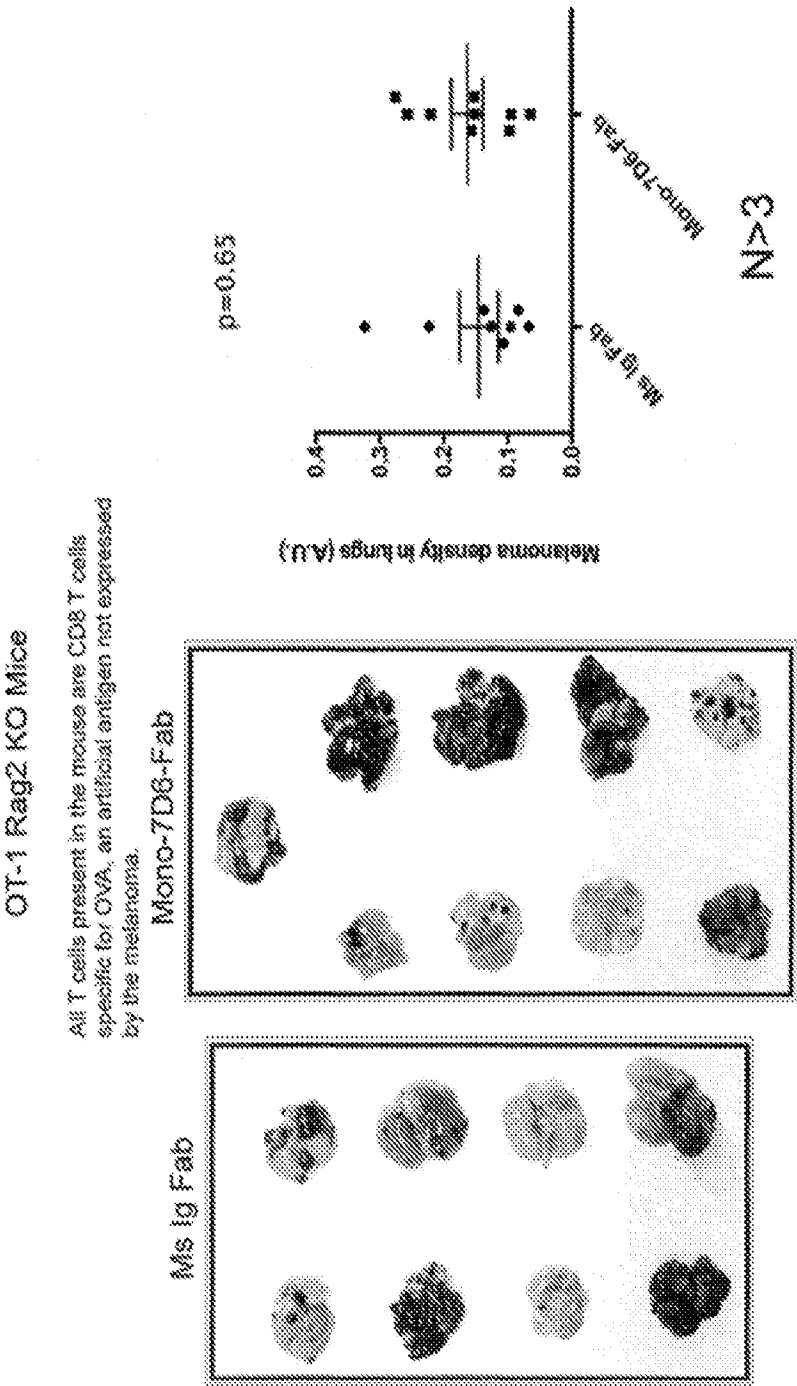


FIG. 25

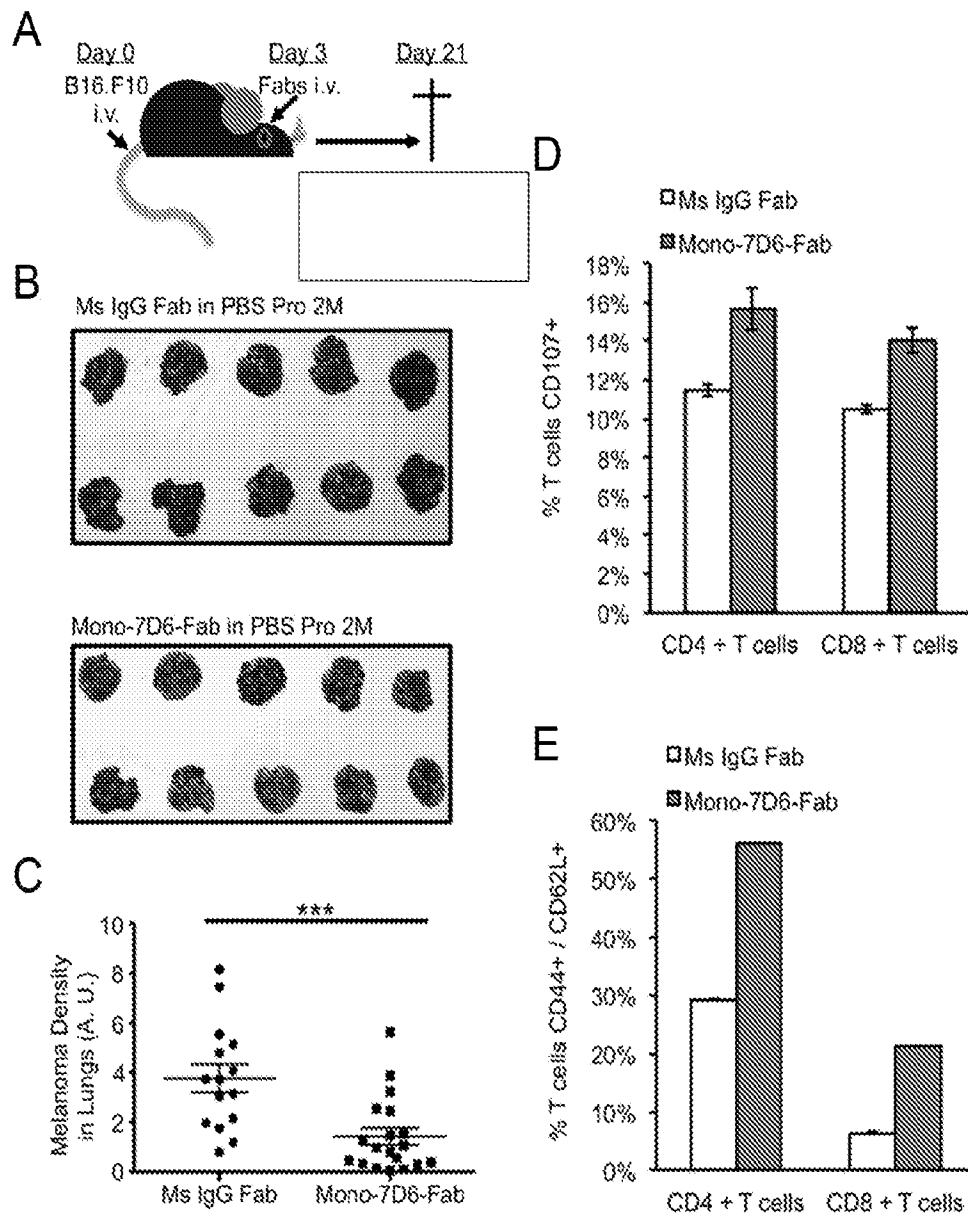
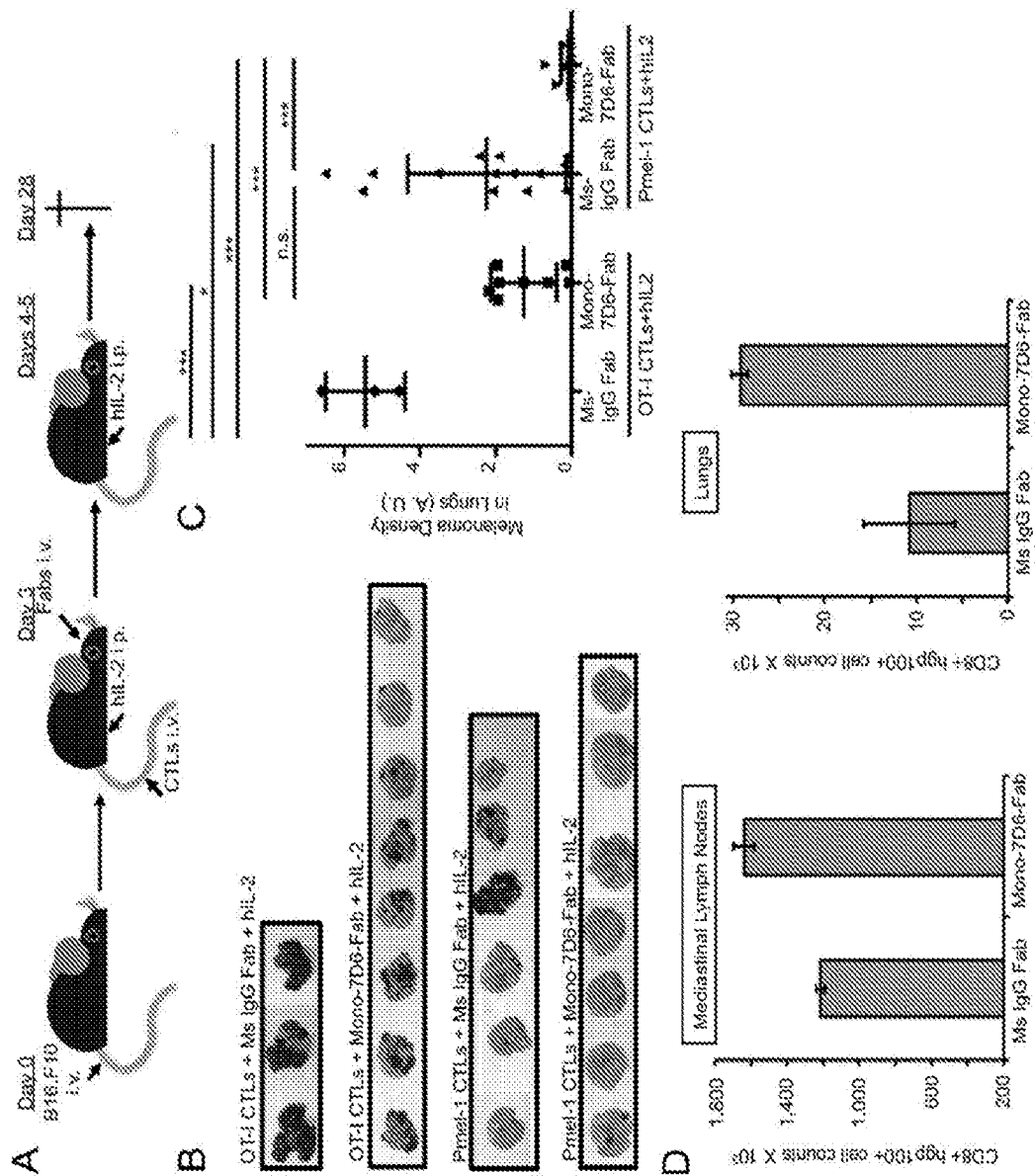


FIG. 26



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FIG. 27

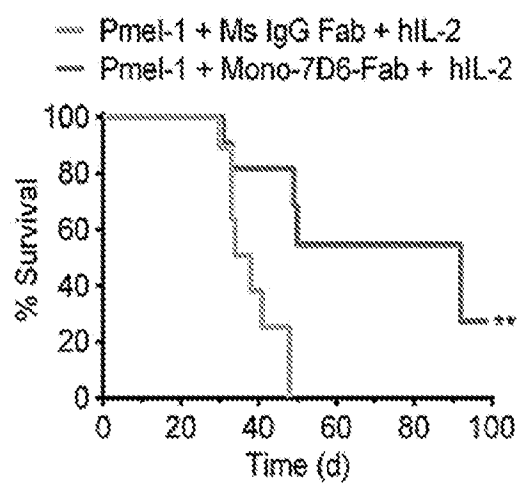
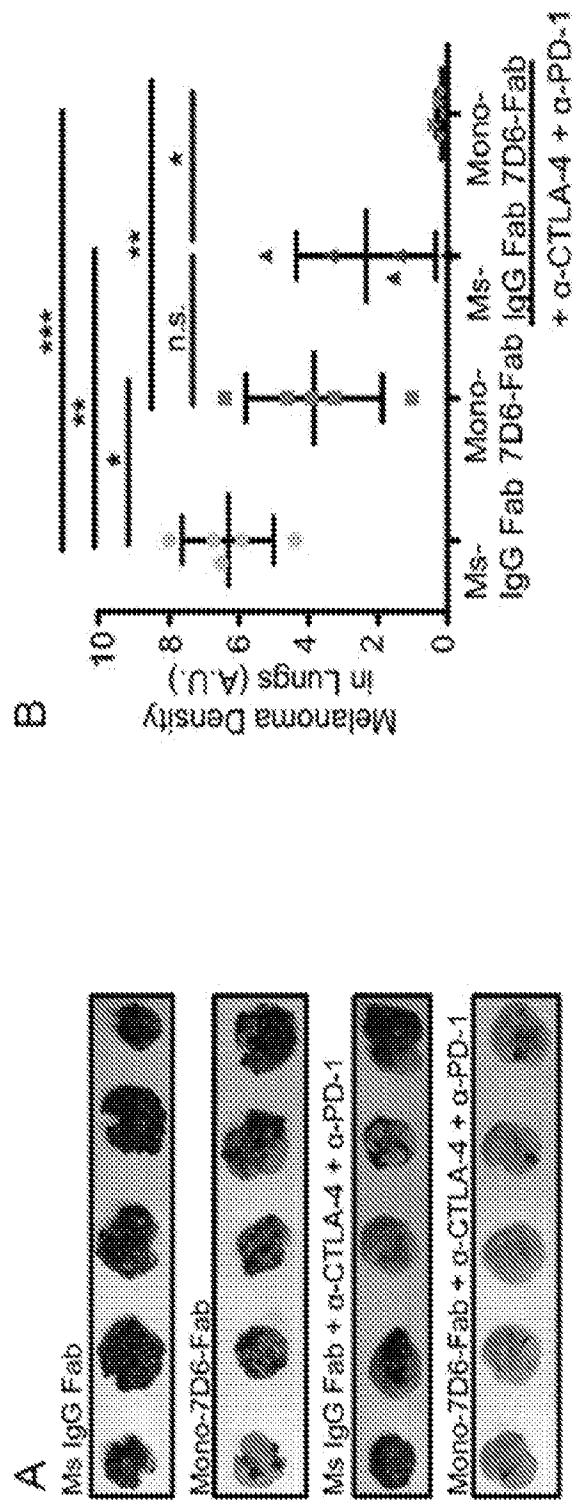


FIG. 28



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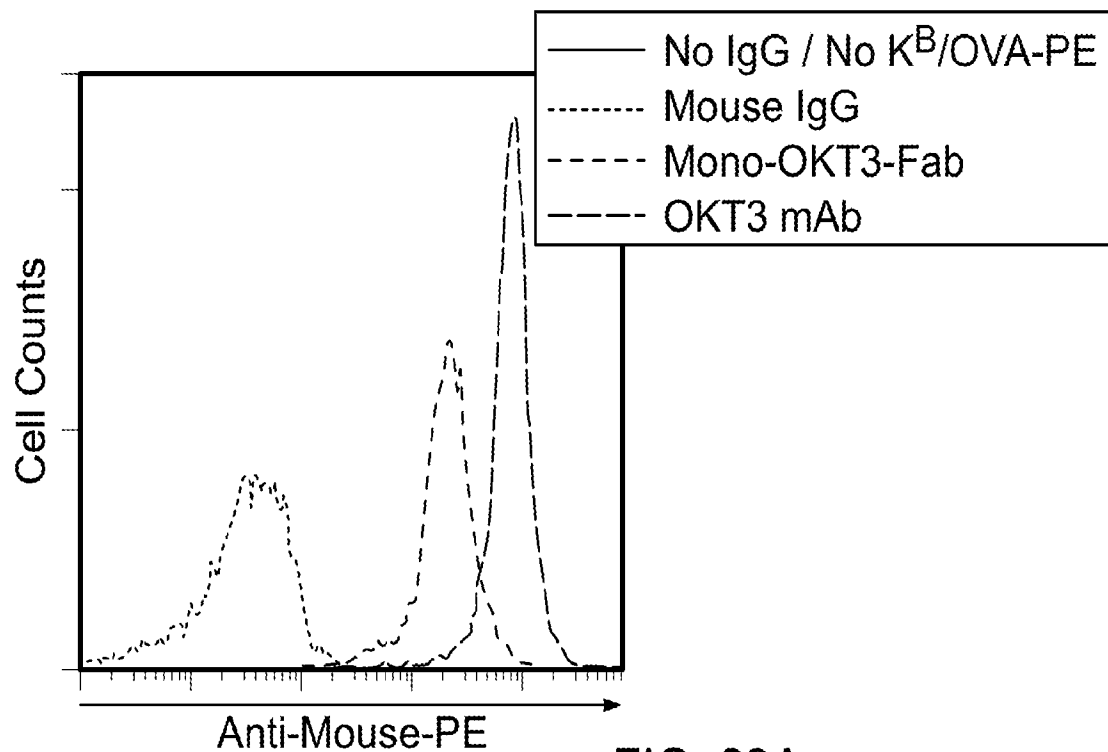


FIG. 29A

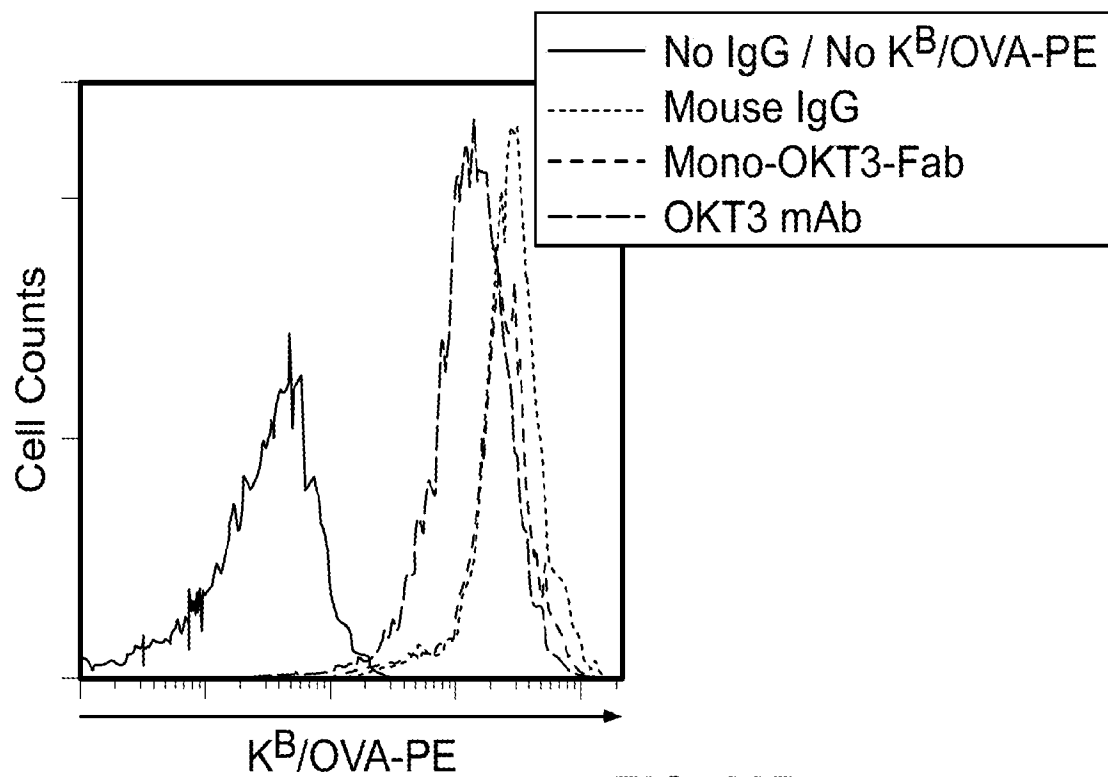


FIG. 29B

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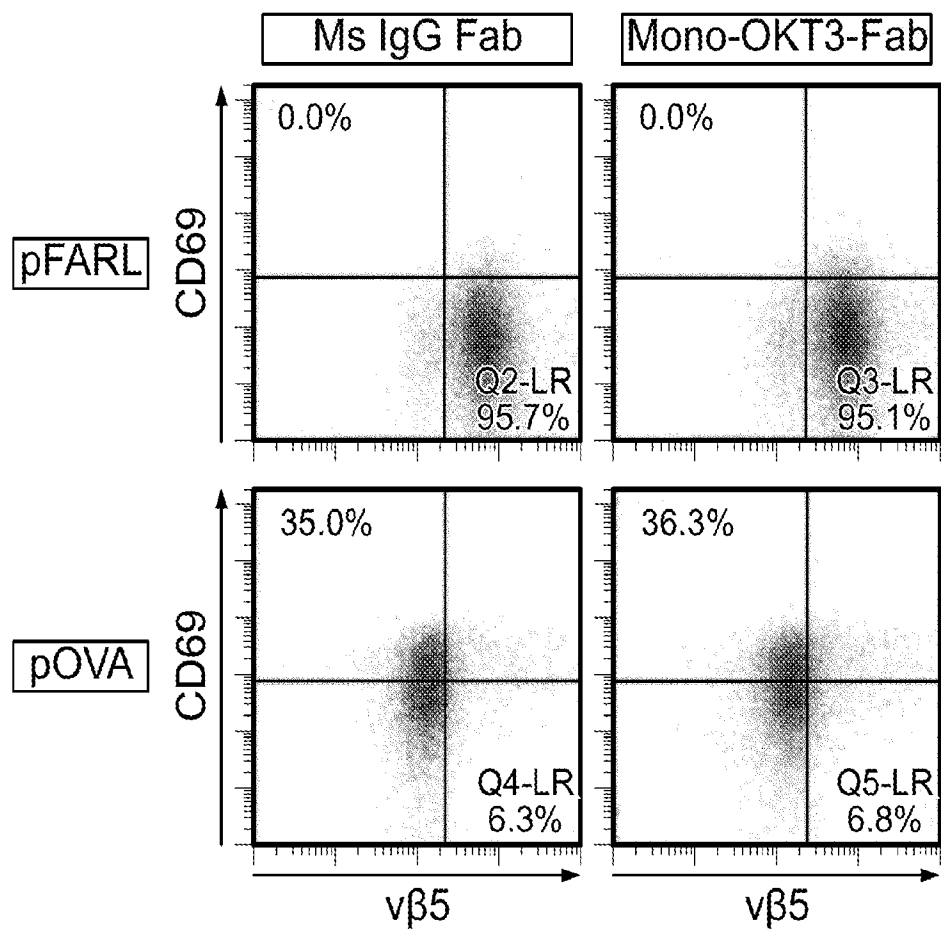


FIG. 29C

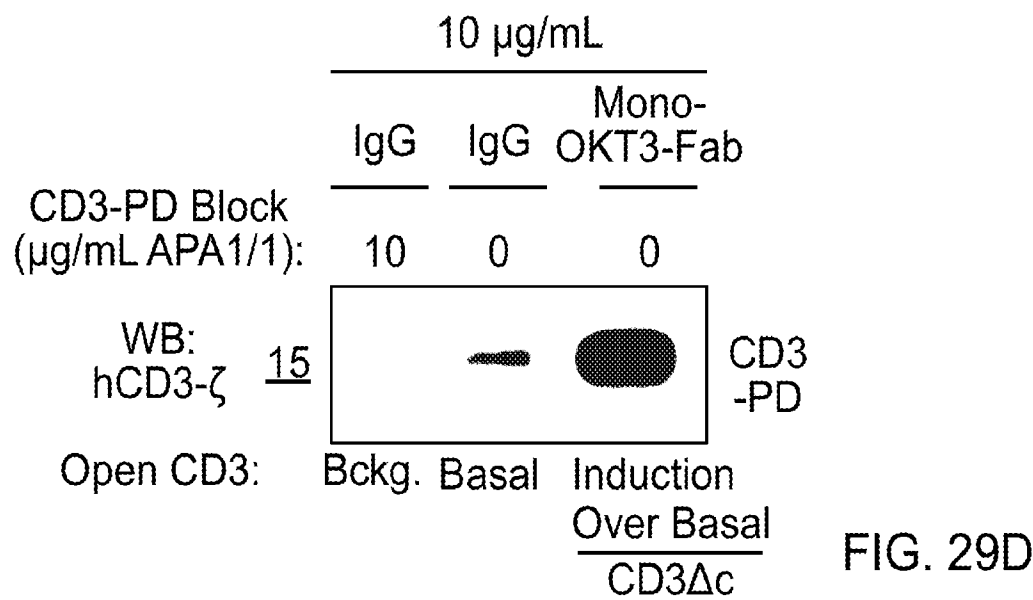
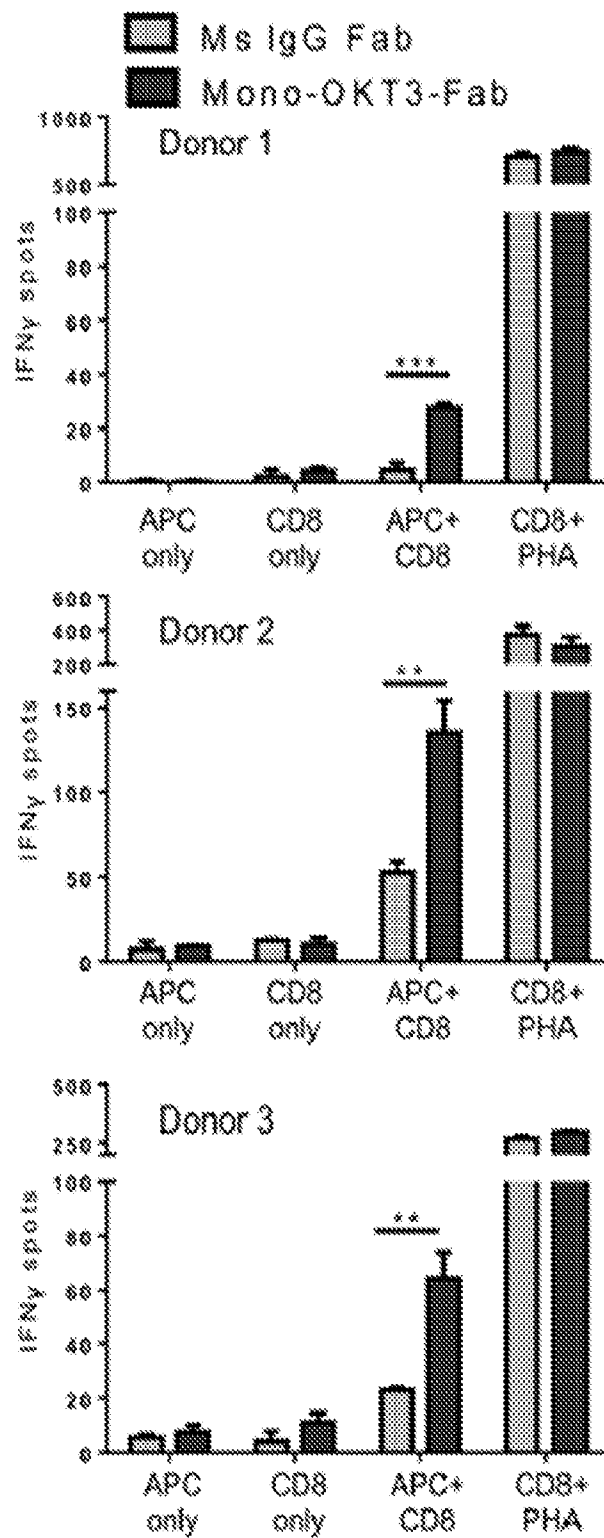


FIG. 29D

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FIG. 30



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FIG. 31

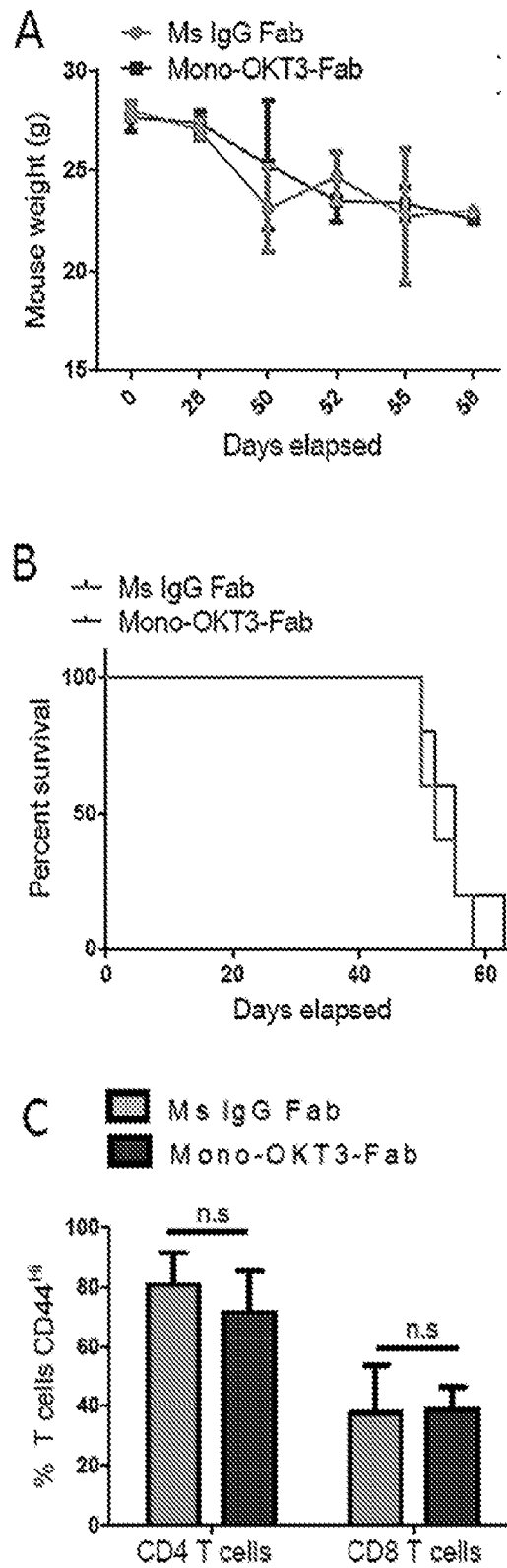


FIG. 32

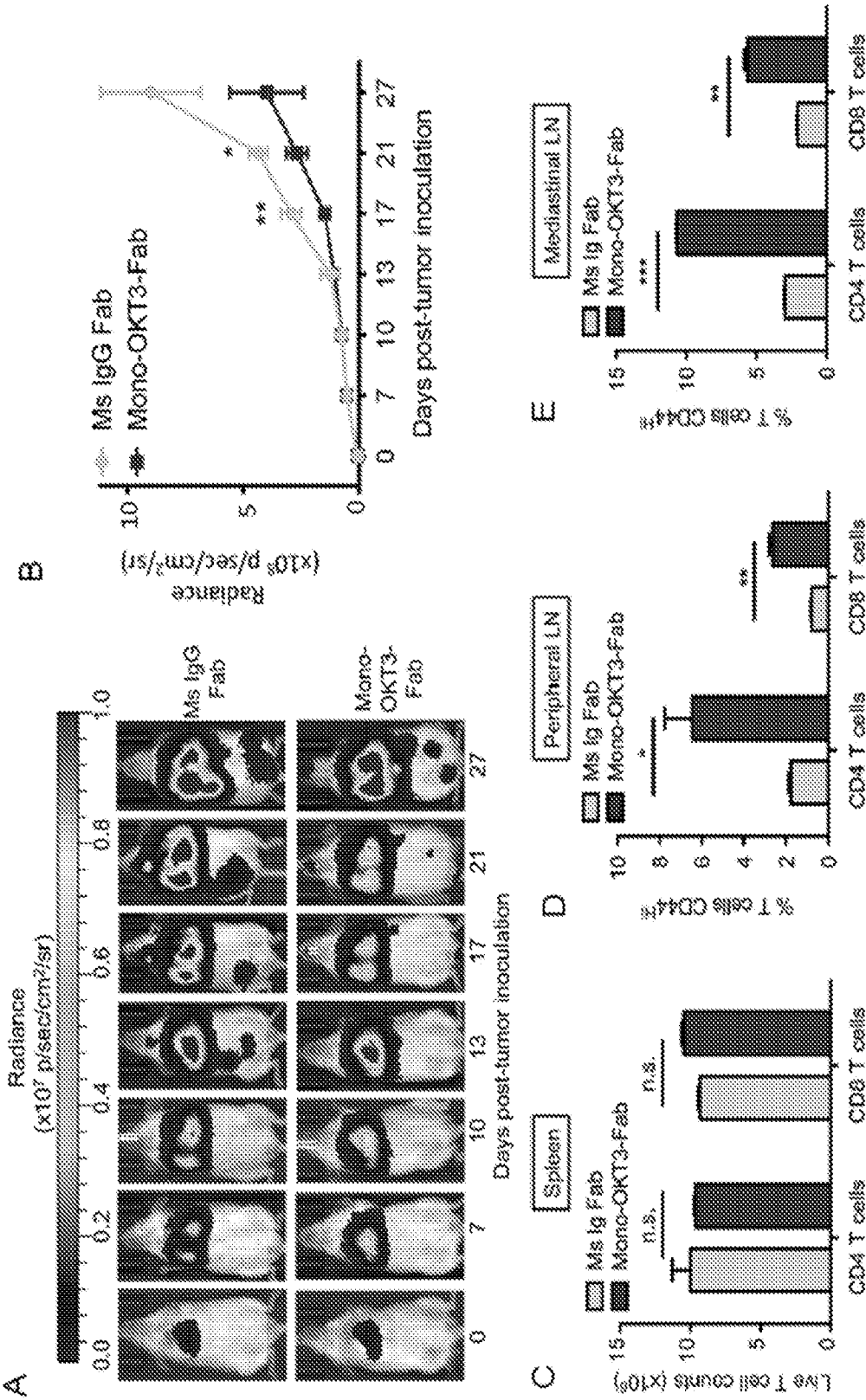
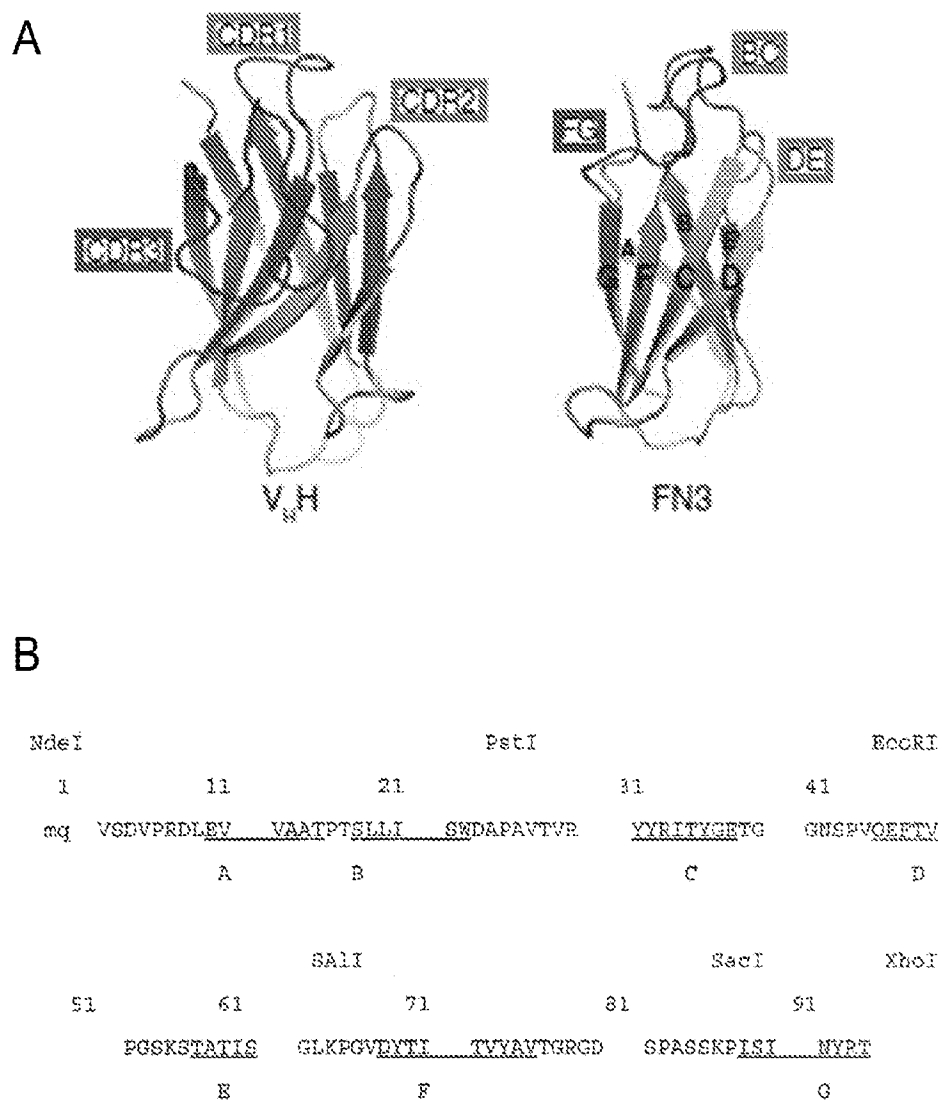


FIG. 33



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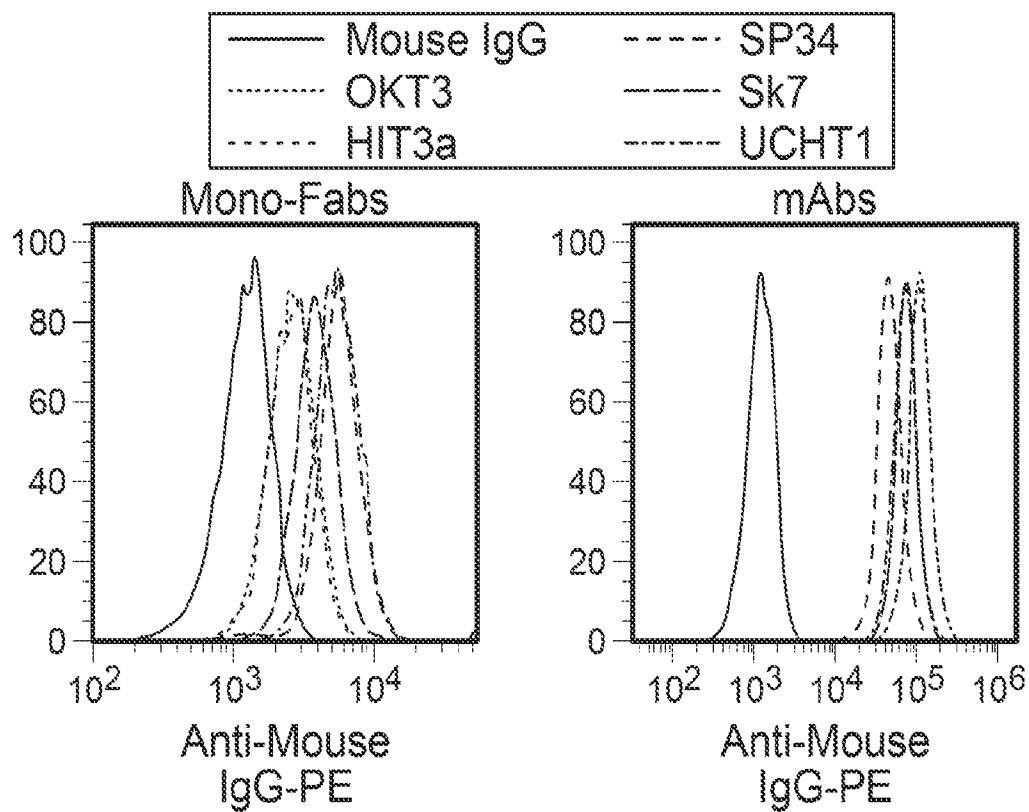


FIG. 34A

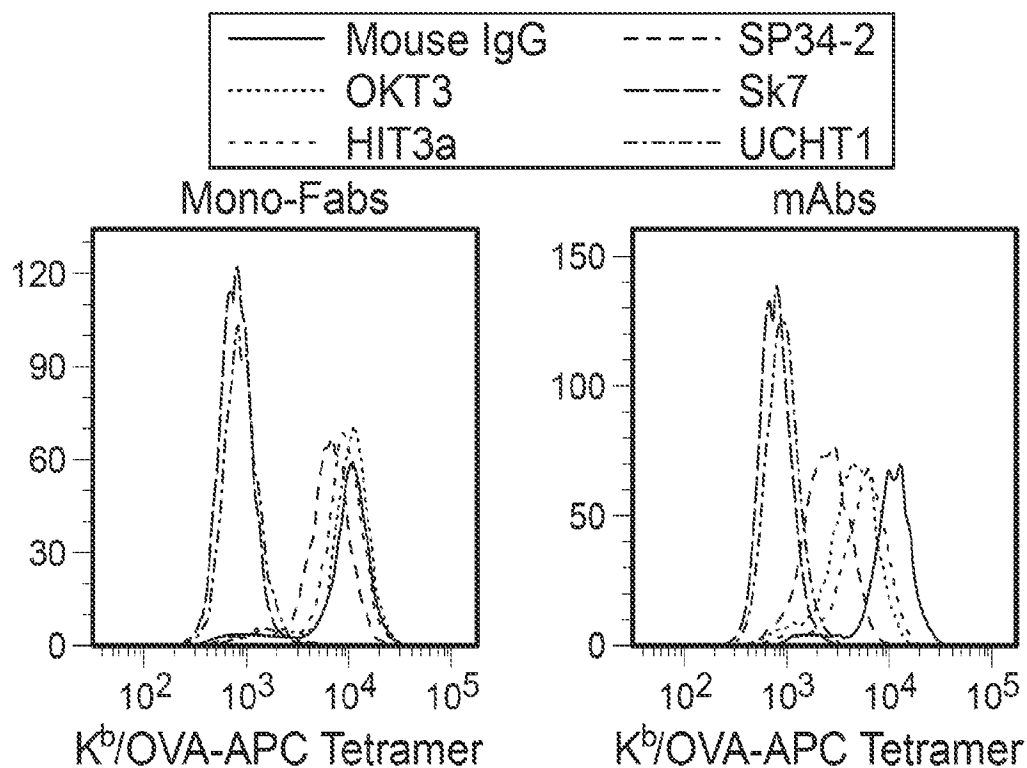


FIG. 34B

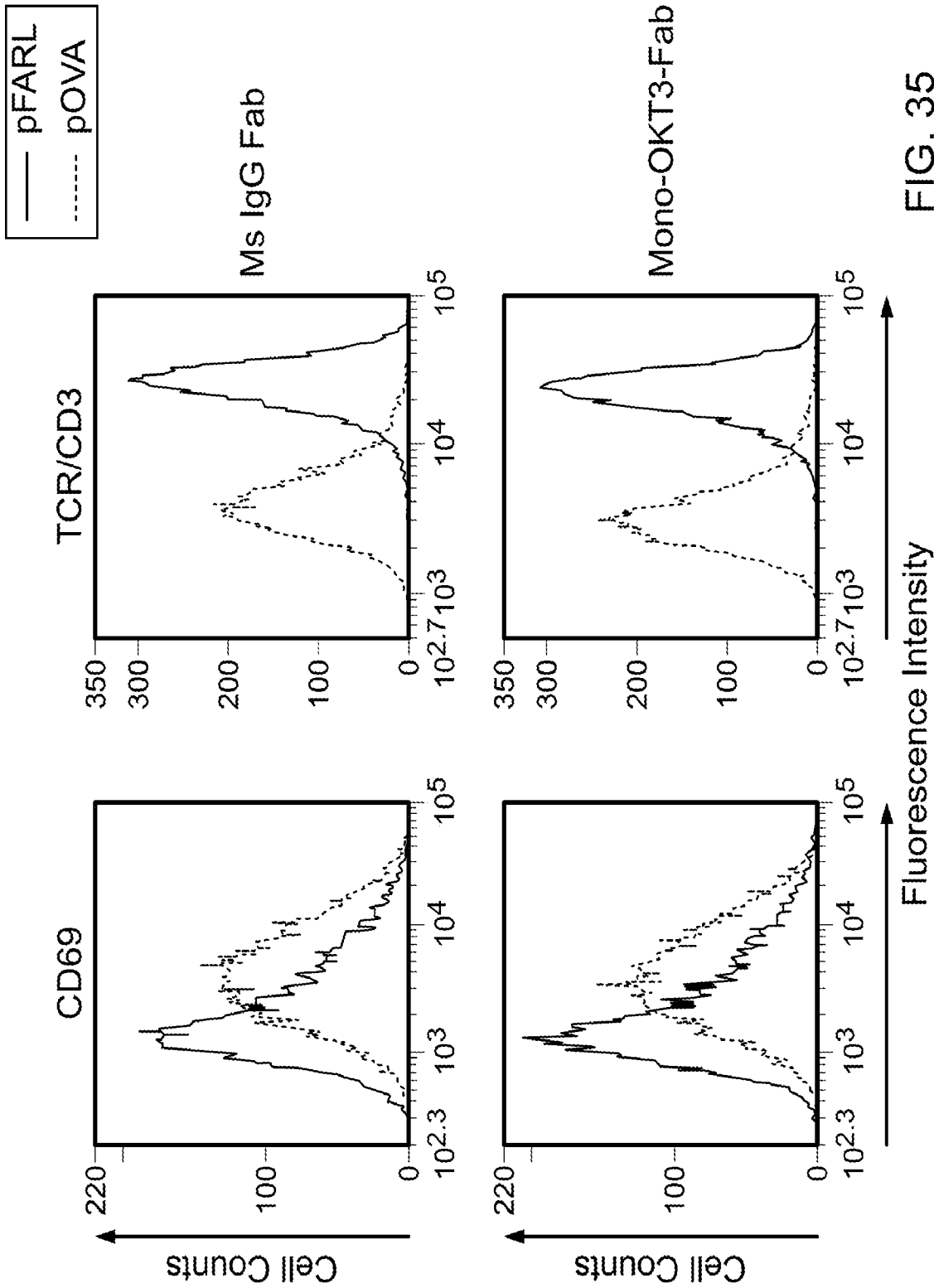


FIG. 35

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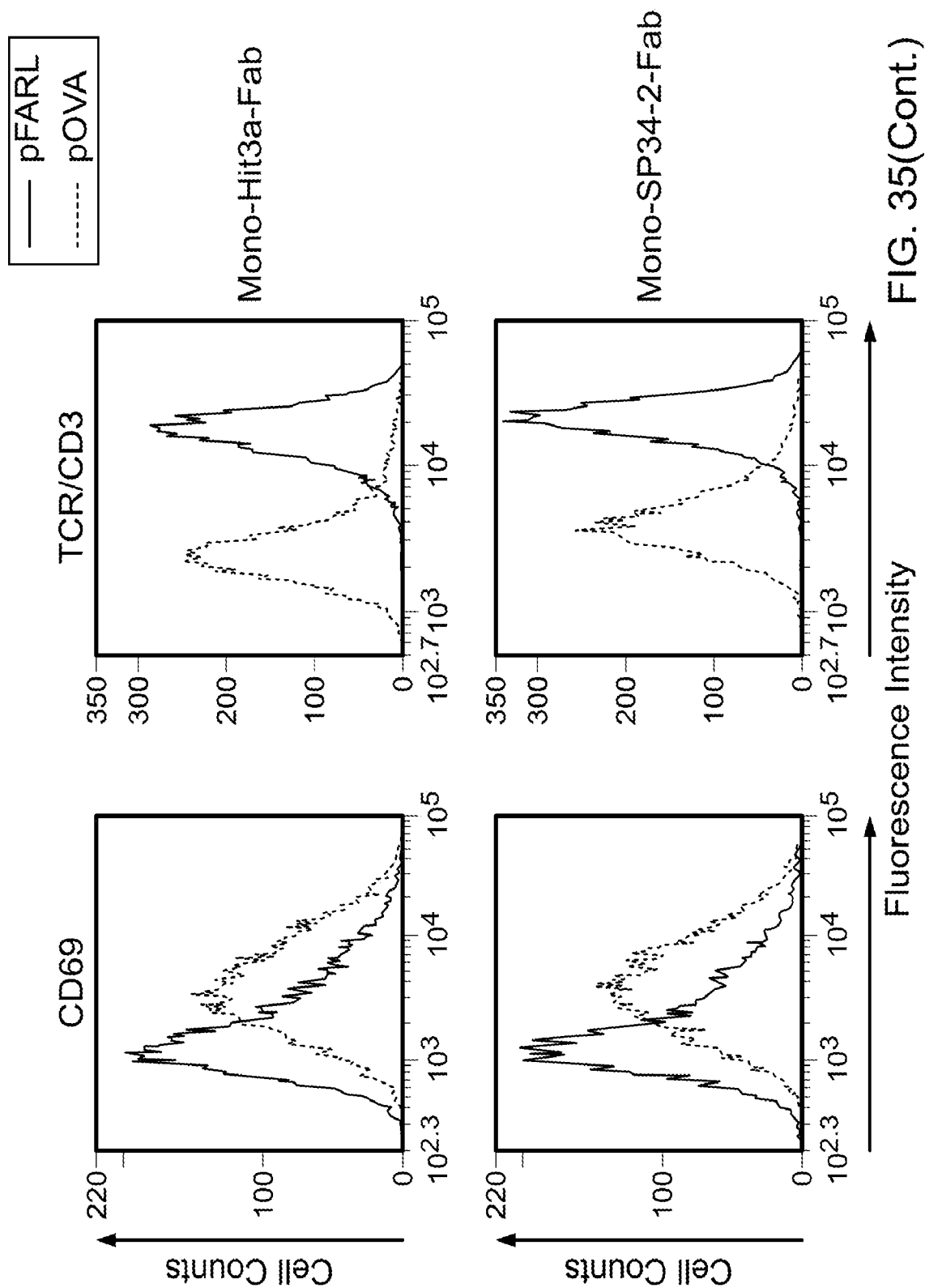


FIG. 35(Cont.)

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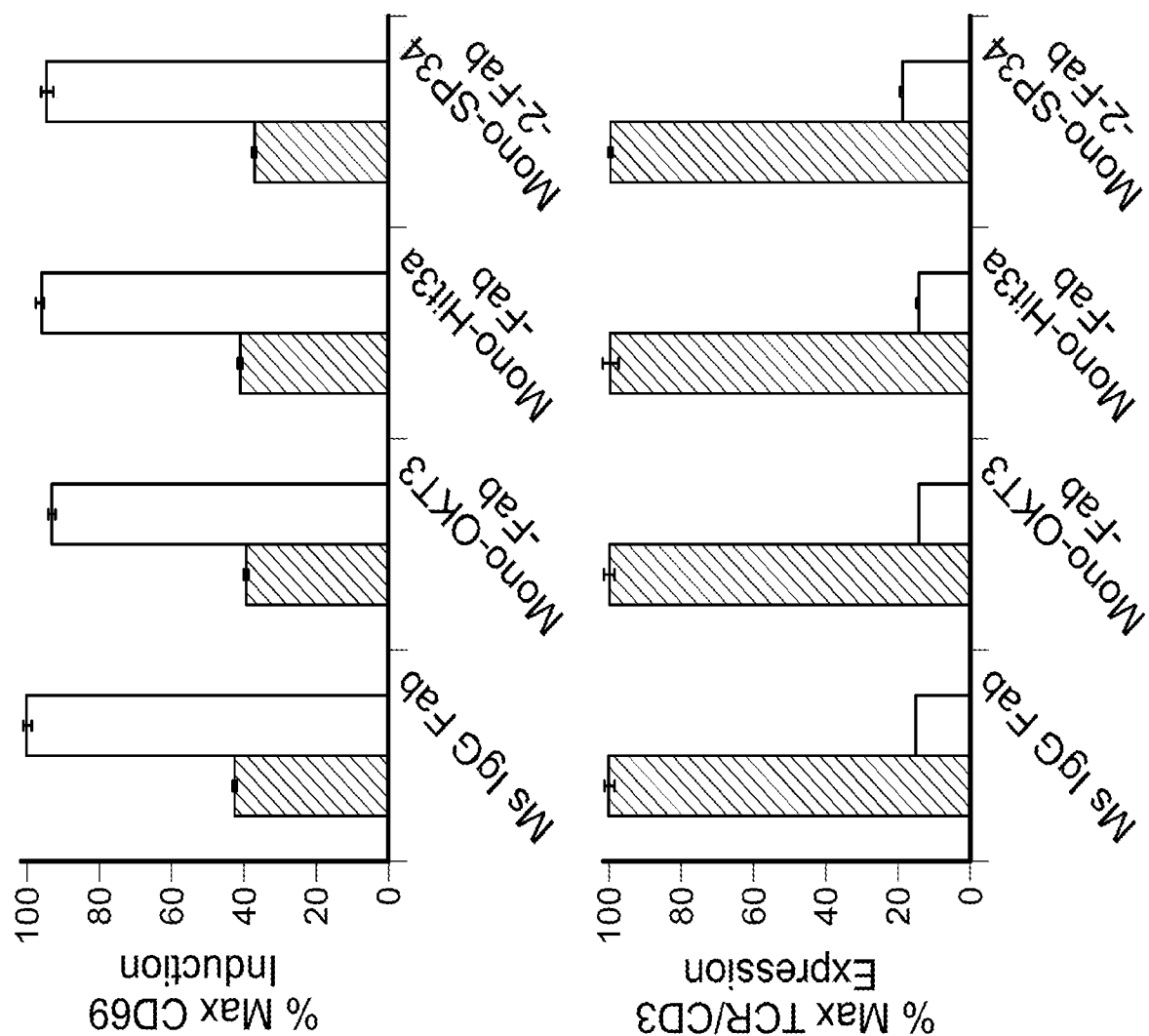


FIG. 35(Cont.)

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FIG. 36

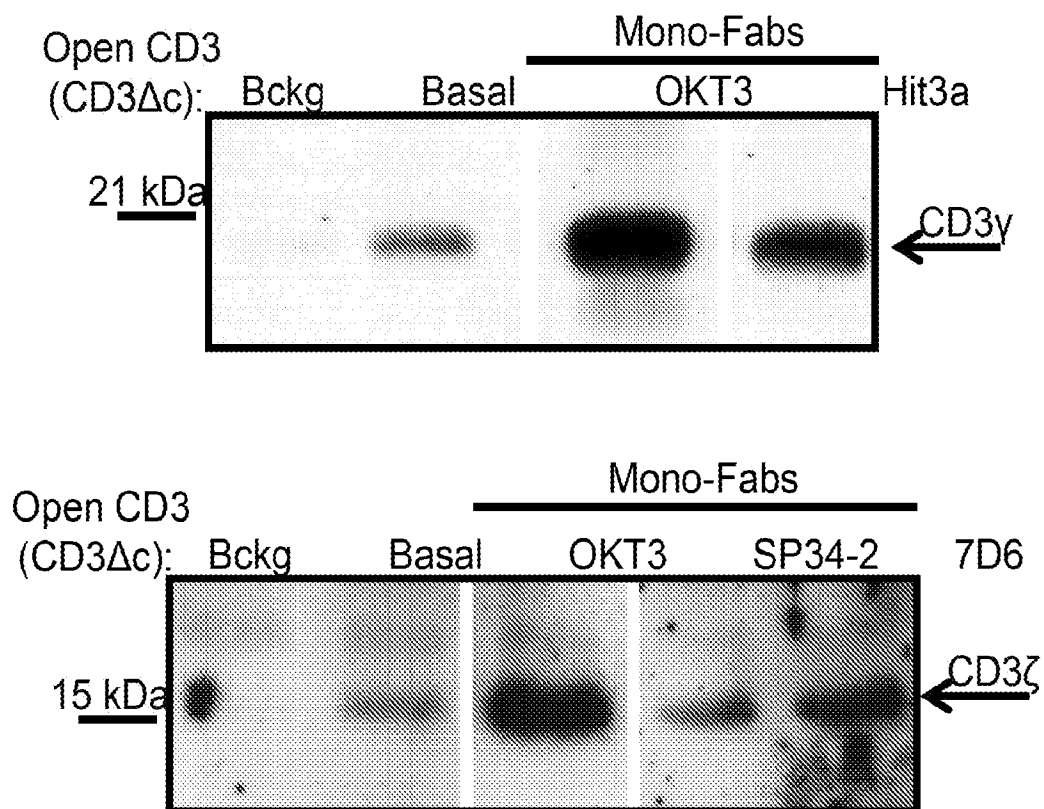
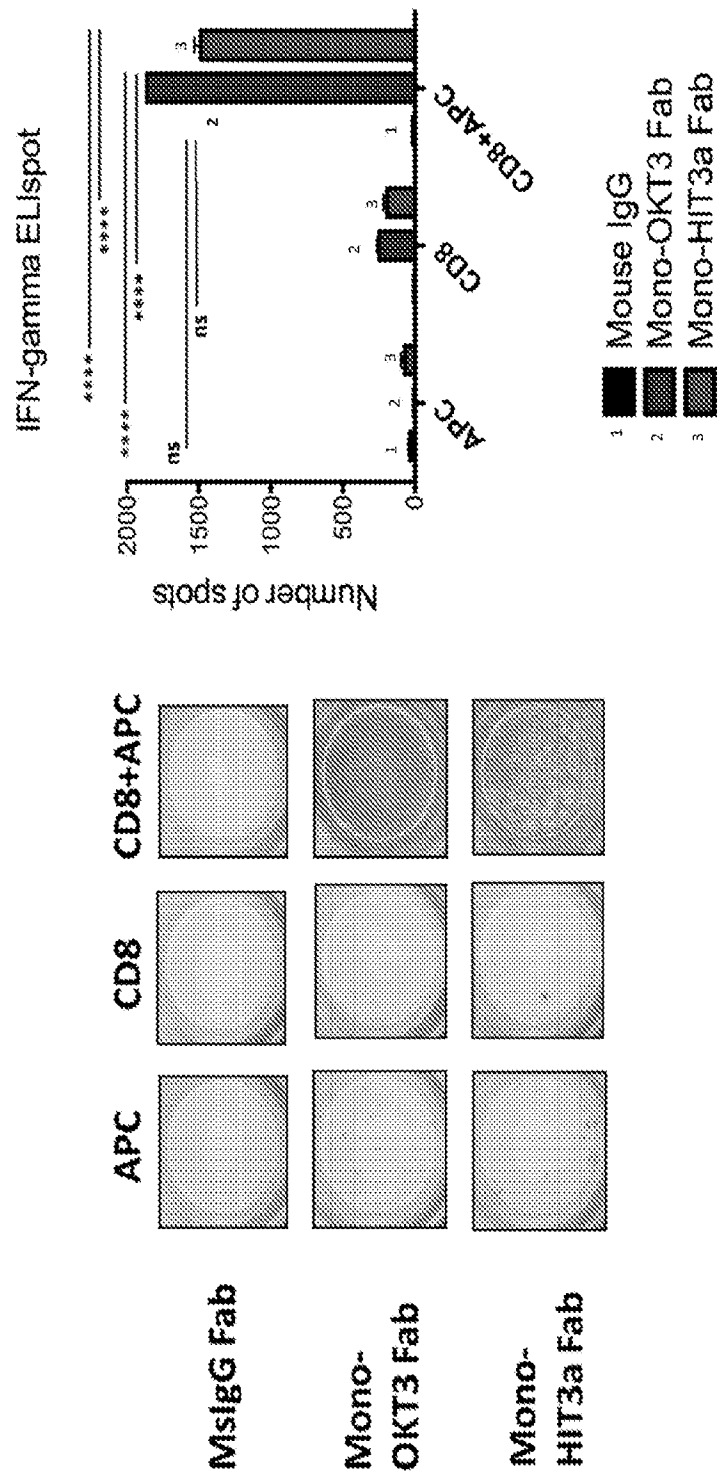


FIG. 37



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/65294

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395, A61K 39/00 (2016.01)

CPC - A61K 39/0011, A61K 39/39

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)

IPC(8) - A61K 39/395, A61K 39/00 (2016.01)

CPC - A61K 39/0011, A61K 39/39

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/133.1, 424/173.1

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

pubWEST; PatBase; Google Scholar

search terms - CD3, gamma, epsilon, fab, OKT3, Hit3a, SP342, SP34-2, monobody, FN3, FN-3, fibronectin 3, fibronectin type-3, monobod*,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2008/0299137 A1 (SVENDSEN et al.) 04 December 2008 (04.12.2008) para [0009]; [0022]; [0036]-[0042]; [0066].	9 ----- 1-8, 10-16
Y	US 2015/0174242 A1 (MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH) 25 June 2015 (25.06.2015) abstract; para [0012]-[0014].	1-8, 10-16

☐

Further documents are listed in the continuation of Box C.

☐

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 January 2017

Date of mailing of the international search report

08 FEB 2017

Name and mailing address of the ISA/US

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