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(54) Title: ANTI-PD-1 ANTIBODIES

(57) **Abrégé/Abstract:**

Antibodies that bind to programmed cell death protein 1 (PD-1), compositions comprising such antibodies, and methods of making and using such antibodies are disclosed.



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(57) Abstract: Antibodies that bind to programmed cell death protein 1 (PD-1), compositions comprising such antibodies, and methods of making and using such antibodies are disclosed.



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ANTI-PD-1 ANTIBODIES

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/095,675, filed on December 22, 2014, U.S. Provisional Application No. 62/220,199, filed on September 17, 2015, U.S. Provisional Application No. 62/251,082, filed on November 4, 2015, and U.S. Provisional Application No. 62/261,118, filed on November 30, 2015. The entire teachings of the above applications are incorporated herein by reference.

INCORPORATION BY REFERENCE OF MATERIAL IN ASCII TEXT FILE

[0002] This application incorporates by reference the Sequence Listing contained in the following ASCII text file being submitted concurrently herewith:

- a) File name: 50911000005SequenceListing.txt; created December 17, 2015, 73 KB in size.

BACKGROUND OF THE INVENTION

[0003] Modulation of the mammalian adaptive immune response (immunomodulation) is a useful therapeutic approach for various diseases and disorders. One way to achieve such immunomodulation is to intervene at one or more immune checkpoints, *e.g.*, the Programmed Death-1 (PD-1) checkpoint. The natural function of immune checkpoints is to suppress the immune response, as necessary, to prevent immune damage to normal tissue. Depending on the disease or disorder, it may be desirable to upregulate or downregulate the immune response. Tumor cells that display non-self-antigens can evade immune attack by secreting cytokines or ligands that activate immune checkpoints. Therefore, in cancer therapy, it is generally desirable to upregulate the immune response against tumor cells. In contrast, in treatment of autoimmune diseases, it is generally desirable to downregulate the immune response in certain tissues.

[0004] “Programmed Death-1” (PD-1) protein (also known as Programmed Cell Death Protein 1 and CD279) is a type I transmembrane receptor that is part of the extended

CD28/CTLA4 family of T cell regulators. Ligands for PD-1 include PD-1 Ligand 1 (PD-L1, also known as B7-H1), and PD-1 Ligand 2 (PD-L2, also known as B7-DC).

[0005] PD-1 is expressed on various cell types, including T cells, B cells, and macrophages. Experimental data implicate the interactions of PD-1 with its ligands in downregulation of central and peripheral immune responses. Proliferation of T cells is inhibited in the presence of PD-L1. Mice with a disrupted PD-1 gene exhibit an autoimmune phenotype. PD-1 deficiency in the C57BL/6 mice results in chronic progressive lupus-like glomerulonephritis and arthritis (Nishimura *et al.*, *J. Exp. Med.* 101(5):891-98, 2000).

[0006] Compounds that modulate PD-1 activity have potential as therapeutic agents for the treatment of various diseases and disorders, including cancer, inflammation, and autoimmune diseases. There is a significant unmet need for immunomodulatory compounds, *e.g.*, antibodies, including PD-1 agonists and PD-1 antagonists.

SUMMARY OF THE INVENTION

[0007] The present invention provides antibodies that bind to PD-1. In some embodiments, the invention provides an isolated antibody that binds to PD-1, comprising a heavy chain variable region (HCVR) selected from the group consisting of SEQ ID NOs: 1-26 and/or a light chain variable region (LCVR) selected from the group consisting of SEQ ID NOs: 27-53. The invention also provides an isolated antibody that binds to PD-1 and competitively inhibits the binding of any of the antibodies disclosed herein to PD-1.

[0008] In some embodiments, the invention also provides an isolated antibody that binds to PD-1, comprising a HCVR selected from the group consisting of SEQ ID NOs: 85-90 and/or a LCVR selected from the group consisting of SEQ ID NOs: 91-96.

[0009] The invention further provides an isolated antibody that binds to PD-1, wherein the antibody binds to a sequence in PD-1 selected from the group consisting of SEQ ID NOs: 54-84.

[0010] The antibodies can be used as therapeutic agents. For use as therapeutic agents, the antibodies disclosed herein can be engineered, *e.g.*, humanized, to reduce or eliminate serum sickness or an undesired immune response when administered to a human patient. Also disclosed are methods of treating diseases and disorders in which the PD-1 signaling pathway plays a significant role ("PD-1-mediated diseases and disorders").

[0011] The present invention includes the surprising discovery that contacting human T cells with an effective amount of an anti-PD-1 antibody that competitively inhibits binding of

PD-L1 or PD-L2 to PD-1 expressed on the surface of T cells, and an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cells increases T cell effector function to a greater extent than an equivalent amount of either anti-PD-1 antibody alone. In some embodiments, the combination yields an additive effect on T cell effector function. In some embodiments, the combination yields a synergistic effect on T cell effector function.

[0012] Accordingly, the present invention provides a method for increasing T cell effector function, comprising contacting a T cell with a combination of: (a) an effective amount of an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell; and (b) an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.

[0013] In some embodiments, the present invention also provides a method for increasing T cell effector function, comprising contacting a T cell with an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.

[0014] Additionally, the present invention provides a method for increasing lymphocyte secretion of a cytokine selected from the group consisting of IL-6, IL-12, IL-18, TNF- α , IL-1 β and GM-CSF in a human patient in need of increased T cell effector function, comprising administering to the patient a therapeutically effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell.

[0015] The present invention provides a method of treating cancer in a mammal, comprising contacting a T cell in a mammal in need thereof with a combination of: (a) an effective amount of an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell; and (b) an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.

[0016] The present invention provides a method of producing anti-PD-1 antibodies comprising a HCVR, a LCVR, or a combination thereof. Accordingly, also provided herein is an isolated nucleic acid comprising a nucleotide sequence encoding the HCVR and/or

LCVR of the present disclosure, as well as a host cell comprising an isolated nucleic acid of the invention.

[0017] The antibodies of the present invention can also be used in diagnostic testing. For example, the invention provides a method of diagnosing a PD-1-mediated disease or disorder, *e.g.*, adaptive immune resistance, in a patient who has cancer.

[0018] These and other aspects and advantages of the invention will become apparent upon consideration of the following figures, detailed description and claims. As used herein, “including” means without limitation, and the examples cited are non-limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The invention can be more completely understood with reference to the following drawings.

[0020] FIG. 1 is an alignment of the sequences of 26 heavy chain variable regions (HCVR) from antibodies that bind to human PD-1. The lower segment of the sequences is a continuation of the upper segment. Framework regions FR1 through FR4 are indicated. Complementarity determining regions CDR1 through CDR3 are also shown.

[0021] FIG. 2 is an alignment of the sequences of 27 light chain variable regions (LCVR) from antibodies that bind to human PD-1. The lower segment of the sequences is a continuation of the upper segment. Framework regions FR1 through FR4 are indicated. Complementarity determining regions CDR1 through CDR3 are also shown.

[0022] FIG. 3 is a bar graph summarizing data on binding of 32 mouse anti-human-PD-1 antibodies to PD-1-HIS. The specific HCVR and LCVR pairings for the 32 antibodies are indicated in the boxed text. Binding was evaluated by ELISA. Data represent average of two experiments.

[0023] FIG. 4 is a bar graph summarizing results from assays to measure the effectiveness of anti-PD-1 antibodies in relieving PD-L1 dependent inhibition of activation of human peripheral blood mononuclear cells (PBMCs). Treatments of cells were carried out in 96-well plates for 3-5 days. All treatments included plate-bound CD3 and soluble CD28 in PBS. Cells were treated additionally with nothing (positive control); with PD-L1 alone; or with PD-L1 plus an anti-PD-L1 antibody (EH12.2H7, pembrolizumab, 246A10 or 244C8). At the end of the treatment period, cells were transferred to a microwell array for IFN γ determination at the level of individual cells, with sample cell populations in the range of approximately 50-100 cells.

[0024] FIG. 5 is a bar graph summarizing the results of differential T cell activation in response to PD-1 blockade. These results indicate that antibodies 388D4, 413E1, 246A10 and 244C8 elicited similar secretion levels or enhanced secretion levels of IFN γ and TNF α as compared to antibody EH12.2H7 or pembrolizumab, under conditions of suboptimal activation (achieved by the treatment with anti-CD3 and anti-CD28), which may mimic activation conditions that occur *in vivo*.

[0025] FIG. 6 is a bar graph summarizing the results of antigen recall assays using cytomegalovirus in human PBMC. These results indicate that anti-PD-1 antibodies 246A10, 244C8, 413D2, 388D4, and 413E1 induce increased levels of IFN γ compared to antibody isotype controls.

[0026] FIGS. 7A and 7B summarize the results of mixed lymphocyte reaction assays using anti-PD-1 antibodies and human PBMCs. Humanized versions of clone 388D4 (“D4-HC3+LC1”; “D4-HC1+LC3”; and “D4-HC3+LC3”) appear to induce cytokine release (FIG. 7A) and CD25 upregulation (FIG. 7B) similar to nivolumab. Humanized versions of clone 244C8 (“C8-HC1+LC1”; “C8-HC1+LC3”; and “C8-HC2+LC1”) appear to induce increased levels of cytokine release (IFN γ) compared with 388D4 or nivolumab (FIG. 7A). T cells incubated with 244C8 also appear to exhibit a higher degree of activation, as inferred from CD25 expression (FIG. 7B).

[0027] FIG. 8 indicates regions within the PD-1 amino acid sequence (SEQ ID NO:97) bound by certain antibodies of the present invention (246A10, 244C8, 388D4, 413D2, and 413E1), as determined by peptide mapping. The PD-1 amino acid sequences corresponding to the sequences of inhibitory peptides are underlined.

[0028] FIGS. 9A-9D show selectivity of anti-PD-1 antibodies for the PD-1 extracellular domain over other immunomodulatory cell surface proteins such as ICOS (inducible T-cell costimulator) (FIG. 9A), CD28 (FIG. 9C), or CTLA4 (FIG. 9D). Binding of anti-PD-1 antibodies to PD-1 is shown in FIG. 9B (EH12.2H7 is an anti-PD-1 antibody commercially available as a laboratory reagent). Anti-PD-1 antibodies 388D4 (100388_D4VH3_100389_D4VK5 in Table 3), 413E1 (100413_E1VH9_100414_E1VK5 in Table 3), 244C8, and 246A10 were tested.

[0029] FIGS. 10A and 10B are amino acid sequence alignments of humanized and mouse antibody sequences. FIG. 10A shows alignments for 100388_D4_VH3 (mouse) with humanized heavy chain variable regions (100388_D4_HC1; 100388_D4_HC2; and

100388_D4_HC3) and 100389_D4_VK5 (mouse) with humanized light chain variable regions (100389_D4_LC1; 100389_D4_LC2; and 100389_D4_LC3). FIG. 10B shows alignments for 100244_C8_VH3 (mouse) with humanized heavy chain variable regions (100244_C8_HC1; 100244_C8_HC2; and 100244_C8_HC3) and 100245_C8_VK5m1 (mouse) with humanized light chain variable regions (100245_C8_LC1; 100245_C8_LC2; and 100245_C8_LC3).

[0030] FIGS. 11A-11D are flow cytometry histogram plots showing that antibody 388D4 blocks binding of soluble PD-L1 to HEK293 cells expressing PD-1, while antibody 244C8 does not. HEK293 cells expressing PD-1 were incubated with 10 µg/ml of an isotype antibody (negative control), commercially available antibody EH12.2H7 (positive control) antibody 388D4, or antibody 244C8. Cells were washed and stained with soluble PD-L1-Ig protein fluorescently labeled with Alexa-488. Cells were washed again, and PD-L1 binding (by displacing previously bound antibody) was assessed by conventional fluorescence activated cell sorting (FACS) analysis. FIG. 11A shows data from FACS analysis of anti-PD-1 antibody EH12.2H7 (positive control); FIG. 11B shows data from FACS analysis of anti-PD-1 antibody mIgG1K (negative control); FIG. 11C shows data from FACS analysis of anti-PD-1 antibody 388D4; FIG. 11D shows data from FACS analysis of anti-PD-1 antibody 244C8.

[0031] FIG. 12 is a histogram showing restoration of T cell function by PD-1 blockade with nivolumab or different humanized forms of antibodies 388D4 and 244C8, *i.e.*, 388D4-2, 388D4-3, 244C8-1, 244C8-2, and 244C8-3. A population of 3×10^5 dissociated and suspended human cells from a non-small cell lung cancer (NSCLC) biopsy, which included 17% lymphocytes (activated as described above) was incubated for 24 hours with anti-PD-1 antibodies at a concentration of 20 µg/mL. IFN γ was measured by ELISA, and the data are expressed in terms of fold-activation relative to treatment with the isotype control antibody. Each of the anti-PD-1 antibodies restored T cell function, increasing IFN γ secretion approximately 7-fold to 7.5 fold, relative to the isotype control.

[0032] FIG. 13 is a histogram summarizing results from an experiment to measure the increase in T cell effector function, as indicated by IFN γ secretion, in response to treatment with antibody 244C8-2 alone versus treatment with 244C8-2 plus 388D4-2, with results normalized relative to the response to treatment with nivolumab. A population of 3×10^5 cells, which included 7.5% lymphocytes sub-optimally activated as described above, was

incubated for 24 hours with anti-PD-1 antibodies at a total antibody concentration of 20 $\mu\text{g/mL}$. As shown in FIG. 13, treatment with 244C8-2 alone increased $\text{IFN}\gamma$ 1.77-fold (± 0.19 sd), while treatment with 244C8-2 in combination with 388D4-2 increased $\text{IFN}\gamma$ secretion 2.11-fold (± 0.21 sd).

[0033] FIG. 14 is a histogram summarizing results from an experiment showing that treatment with the combination of nivolumab and antibody 244C8-2 resulted in greater restoration of T cell effector function than treatment with nivolumab alone, antibody 244C8-2 alone, or antibody 388D4-2 alone. In each treatment, a population of 3×10^5 cells, which included 9% lymphocytes (sub-optimally activated as described above) was incubated for 24 hours with anti-PD-1 antibodies at a total concentration of 20 $\mu\text{g/mL}$. Following PD-1 blockade, cells and supernatants were collected for ELISA measurement of $\text{IFN}\gamma$. Data are expressed in terms of fold-induction of $\text{IFN}\gamma$ secretion, relative to treatment with the isotype control antibody.

[0034] FIGS. 15A-15F are histograms summarizing the results of a mixed lymphocyte reaction (MLR) assay performed on human PBMCs treated with anti-PD-1 antibodies. The MLR assay was performed using commercially available monocyte-derived dendritic cells as stimulator cells and purified CD4^+ T lymphocytes as responder cells from a different healthy blood donor. Supernatants were collected 2.5 days after beginning the assay. Treatment with antibody 244C8-1 (100244_C8_HC1 + 100245_C8_LC1) resulted in increased secretion of cytokines IL-6, IL-12, IL-18, $\text{TNF-}\alpha$, GM-CSF, and IL-1 β , in comparison with antibody 388D4-2 (100388_D4_HC3 + 100389_D4_LC3) or an IgG4 isotype control. (FIG. 15A, IL-6; FIG. 15B, IL-12; FIG. 15C, IL-18; FIG. 15D $\text{TNF-}\alpha$; FIG. 15E, IL-1 β ; FIG. 15F, GM-CSF)

[0035] FIGS. 16A-16F are histograms summarizing the results of an experiment showing alteration of tumor infiltrating lymphocyte (TIL) function by PD-1 blockade with anti-PD-1 antibodies 388D4-2 and 244C8-2. A population of 3×10^5 dissociated and suspended human cells from a non-small cell lung cancer (NSCLC) biopsy, which included 7% stimulated, tumor-infiltrating lymphocytes was incubated for 24 hours with anti-CD3 and anti-CD28 antibodies along with an anti-PD-1 antibody or IgG4 isotype control at a concentration of 10 $\mu\text{g/mL}$. Treatment with antibody 244C8-2 (100244_C8_HC1 + 100245_C8_LC3) resulted in increased secretion of cytokines IL-6, IL-12, IL-18, $\text{TNF-}\alpha$, GM-CSF, and IL-1 β , in comparison with antibody 388D4-2 (100388_D4_HC3 + 100389_D4_LC3) or the IgG4

isotype control. (FIG. 16A, IL-6; FIG. 16B, IL-12; FIG. 16C, IL-18; FIG. 16D TNF- α ; FIG. 16E, IL-1 β ; FIG. 16F, GM-CSF).

[0036] FIGS. 17A-17C show the results from an *in vivo* efficacy experiment involving patient-derived xenograft (PDX) lung tumor growth in humanized mice treated with vehicle control, antibody 388D4-3, antibody 244C8-2, pembrolizumab, or a combination of antibody 244C8-2 and pembrolizumab. Animals received a total of six intra-peritoneal doses of antibody at five-day intervals (Q5D x 6) 5 mg/kg. In the treatment groups that received 388D4-3, 244C8-2 or pembrolizumab, the first dose of the antibody was given as a 10 mg/kg dose, followed by the additional doses at the 5 mg/kg dose. The combination treatment group received a dose of each 5 mg/kg of pembrolizumab and 5 mg/kg of 244C8-2 at each dosing time point. Tumor volumes were measured twice weekly (Day 3, 6, 10, 13, 17, 20, 24 and 28) using a digital caliper to determine length and width of the tumors. All animals were sacrificed at day 28 after dosing initiation. Error bars represent the 95% confidence interval (n=10). All treatment groups showed significant tumor growth inhibition compared to the vehicle control group. As shown in FIG. 17A, no significant difference in tumor growth inhibition was observed among treatment with antibody 388D4-3, antibody 244C8-2, pembrolizumab, or the combination of antibody 244C8-2 with pembrolizumab. FIG. 17B is a boxplot of tumor volumes for each treatment arm at day 28 (end of study) of the experiment described in FIG. 17A. The tumor volume for each treatment group was significantly smaller than that of the vehicle group. Student T-test p values between each treatment group and vehicle group were: 0.00167 (pembrolizumab), 0.00105 (388D4-3), 0.00277 (244C8-2), and 0.00275 (pembrolizumab+244C8), respectively. FIG. 17C is a histogram showing percentage tumor volume of each treatment group relative to vehicle on day 28 of the experiment described in FIGS. 17A and 17B. The calculated percent tumor growth inhibition (%TGI) for each treatment is shown above each bar.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The anti-PD-1 antibodies disclosed herein are based on the antigen binding sites of certain monoclonal antibodies selected on the basis of binding to human Programmed Death-1 (PD-1) protein (UniProt #Q15116). The antibodies contain immunoglobulin variable region CDR sequences that define binding sites for human PD-1.

[0038] By virtue of the PD-1 signal blocking or PD-1 neutralizing activity of certain of these antibodies, they are useful for treating various types of cancer, including inhibiting

tumor growth. In some embodiments (*e.g.*, when used as therapeutic agents), the antibodies can be engineered to minimize or eliminate an immune response when administered to a human patient. Various features and aspects of the invention are discussed in more detail below.

[0039] As used herein, “isolated antibody” means an antibody that is substantially free of its natural environment. For instance, an isolated antibody or nucleic acid is substantially free of cellular material and other proteins from the cell or tissue source from which it is derived.

[0040] As used herein, unless otherwise indicated, “antibody” means an intact antibody or antigen-binding fragment of an antibody, including an intact antibody or antigen-binding fragment that has been modified or engineered, or that is a human antibody. Examples of antibodies that have been modified or engineered are chimeric antibodies, humanized antibodies, multiparatopic antibodies (*e.g.*, biparatopic antibodies), and multispecific antibodies (*e.g.*, bispecific antibodies). Examples of antigen-binding fragments include Fab, Fab', F(ab')₂, Fv, single chain antibodies (*e.g.*, scFv), minibodies and diabodies.

[0041] The antibodies disclosed herein comprise: (a) an immunoglobulin heavy chain variable region comprising the structure CDR_{H1}-CDR_{H2}-CDR_{H3}, and (b) an immunoglobulin light chain variable region comprising the structure CDR_{L1}-CDR_{L2}-CDR_{L3}, wherein the heavy chain variable region and the light chain variable region together define a single binding site for binding human PD-1 protein.

[0042] In some embodiments, the isolated antibody that binds to PD-1 comprises a heavy chain variable region (HCVR) having complementarity determining regions (CDRs) selected from the group consisting of: CDRs 1-3 of SEQ ID NO: 1; CDRs 1-3 of SEQ ID NO: 2; CDRs 1-3 of SEQ ID NO: 3; CDRs 1-3 of SEQ ID NO: 4; CDRs 1-3 of SEQ ID NO: 5; CDRs 1-3 of SEQ ID NO: 6; CDRs 1-3 of SEQ ID NO: 7; CDRs 1-3 of SEQ ID NO: 8; CDRs 1-3 of SEQ ID NO: 9; CDRs 1-3 of SEQ ID NO: 10; CDRs 1-3 of SEQ ID NO: 11; CDRs 1-3 of SEQ ID NO: 12; CDRs 1-3 of SEQ ID NO: 13; CDRs 1-3 of SEQ ID NO: 14; CDRs 1-3 of SEQ ID NO: 15; CDRs 1-3 of SEQ ID NO: 16; CDRs 1-3 of SEQ ID NO: 17; CDRs 1-3 of SEQ ID NO: 18; CDRs 1-3 of SEQ ID NO: 19; CDRs 1-3 of SEQ ID NO: 20; CDRs 1-3 of SEQ ID NO: 21; CDRs 1-3 of SEQ ID NO: 22; CDRs 1-3 of SEQ ID NO: 23; CDRs 1-3 of SEQ ID NO: 24; CDRs 1-3 of SEQ ID NO: 25; and CDRs 1-3 of SEQ ID NO: 26.

[0043] In some embodiments, the isolated antibody that binds to PD-1 comprises a HCVR selected from the group consisting of SEQ ID NOs: 1-26.

[0044] In some embodiments, the isolated antibody that binds to PD-1 comprises a light chain variable region (LCVR) having CDRs selected from the group consisting of: CDRs 1-3 of SEQ ID NO: 27; CDRs 1-3 of SEQ ID NO: 28; CDRs 1-3 of SEQ ID NO: 29; CDRs 1-3 of SEQ ID NO: 30; CDRs 1-3 of SEQ ID NO: 31; CDRs 1-3 of SEQ ID NO: 32; CDRs 1-3 of SEQ ID NO: 33; CDRs 1-3 of SEQ ID NO: 34; CDRs 1-3 of SEQ ID NO: 35; CDRs 1-3 of SEQ ID NO: 36; CDRs 1-3 of SEQ ID NO: 37; CDRs 1-3 of SEQ ID NO: 38; CDRs 1-3 of SEQ ID NO: 39; CDRs 1-3 of SEQ ID NO: 40; CDRs 1-3 of SEQ ID NO: 41; CDRs 1-3 of SEQ ID NO: 42; CDRs 1-3 of SEQ ID NO: 43; CDRs 1-3 of SEQ ID NO: 44; CDRs 1-3 of SEQ ID NO: 45; CDRs 1-3 of SEQ ID NO: 46; CDRs 1-3 of SEQ ID NO: 47; CDRs 1-3 of SEQ ID NO: 48; CDRs 1-3 of SEQ ID NO: 49; CDRs 1-3 of SEQ ID NO: 50; CDRs 1-3 of SEQ ID NO: 51; CDRs 1-3 of SEQ ID NO: 52; and CDRs 1-3 of SEQ ID NO: 53.

[0045] In some embodiments, the isolated antibody that binds to PD-1 comprises a LCVR selected from the group consisting of SEQ ID NOs: 27-53.

[0046] In some embodiments, the isolated antibody that binds to PD-1 comprises a HCVR selected from the group consisting of SEQ ID NOs: 1-26 and a LCVR selected from the group consisting of SEQ ID NOs: 27-53. Examples of pairings of HCVR and LCVR are provided throughout the present disclosure, but additional functional pairings are within the scope of the invention.

[0047] In some embodiments, the antibody comprises a HCVR having the sequence set forth in SEQ ID NO: 4 and a LCVR having the sequence set forth in SEQ ID NO: 28 (designated as 244C7 in Table 3); a HCVR having the sequence set forth in SEQ ID NO: 4 and a LCVR having the sequence set forth in SEQ ID NO: 27 (244C7m1); a HCVR having the sequence set forth in SEQ ID NO: 1 and a LCVR having the sequence set forth in SEQ ID NO: 28 (244C8); a HCVR having the sequence set forth in SEQ ID NO: 1 and a LCVR having the sequence set forth in SEQ ID NO: 27 (244C8m1); a HCVR having the sequence set forth in SEQ ID NO: 3 and a LCVR having the sequence set forth in SEQ ID NO: 31 (246F7); a HCVR having the sequence set forth in SEQ ID NO: 5 and a LCVR having the sequence set forth in SEQ ID NO: 44 (258C1); a HCVR having the sequence set forth in SEQ ID NO: 2 and a LCVR having the sequence set forth in SEQ ID NO: 30 (258F6); a HCVR having the sequence set forth in SEQ ID NO: 2 and a LCVR having the sequence set forth in

SEQ ID NO: 29 (258F6m); a HCVR having the sequence set forth in SEQ ID NO: 6 and a LCVR having the sequence set forth in SEQ ID NO: 34 (392C4); a HCVR having the sequence set forth in SEQ ID NO: 7 and a LCVR having the sequence set forth in SEQ ID NO: 41 (394D5); a HCVR having the sequence set forth in SEQ ID NO: 8 and a LCVR having the sequence set forth in SEQ ID NO: 35 (394G1); a HCVR having the sequence set forth in SEQ ID NO: 12 and a LCVR having the sequence set forth in SEQ ID NO: 39 (388C12A); a HCVR having the sequence set forth in SEQ ID NO: 12 and a LCVR having the sequence set forth in SEQ ID NO: 32 (388C12B); a HCVR having the sequence set forth in SEQ ID NO: 13 and a LCVR having the sequence set forth in SEQ ID NO: 39 (388C16A); a HCVR having the sequence set forth in SEQ ID NO: 13 and a LCVR having the sequence set forth in SEQ ID NO: 32 (388C16B); a HCVR having the sequence set forth in SEQ ID NO: 9 and a LCVR having the sequence set forth in SEQ ID NO: 38 (392C5A); a HCVR having the sequence set forth in SEQ ID NO: 9 and a LCVR having the sequence set forth in SEQ ID NO: 37 (392C5B); a HCVR having the sequence set forth in SEQ ID NO: 17 and a LCVR having the sequence set forth in SEQ ID NO: 40 (392D2); a HCVR having the sequence set forth in SEQ ID NO: 16 and a LCVR having the sequence set forth in SEQ ID NO: 43 (392H4); a HCVR having the sequence set forth in SEQ ID NO: 20 and a LCVR having the sequence set forth in SEQ ID NO: 53 (246A10); a HCVR having the sequence set forth in SEQ ID NO: 18 and a LCVR having the sequence set forth in SEQ ID NO: 47 (388D4); a HCVR having the sequence set forth in SEQ ID NO: 19 and a LCVR having the sequence set forth in SEQ ID NO: 48 (392A6); a HCVR having the sequence set forth in SEQ ID NO: 21 and a LCVR having the sequence set forth in SEQ ID NO: 52 (411C2); a HCVR having the sequence set forth in SEQ ID NO: 22 and a LCVR having the sequence set forth in SEQ ID NO: 51 (413D2); or a HCVR having the sequence set forth in SEQ ID NO: 25 and a LCVR having the sequence set forth in SEQ ID NO: 45 (413E1).

[0048] In some embodiments, the antibody comprises a HCVR having the sequence set forth in SEQ ID NO: 20 and a LCVR having the sequence set forth in SEQ ID NO: 53 (246A10); a HCVR having the sequence set forth in SEQ ID NO: 25 and a LCVR having the sequence set forth in SEQ ID NO: 45 (413E1); a HCVR having the sequence set forth in SEQ ID NO: 22 and a LCVR having the sequence set forth in SEQ ID NO: 51 (413D2); a HCVR having the sequence set forth in SEQ ID NO: 18 and a LCVR having the sequence set forth in SEQ ID NO: 47 (388D4); a HCVR having the sequence set forth in SEQ ID NO: 1 and a

LCVR having the sequence set forth in SEQ ID NO: 28 (244C8); or a HCVR having the sequence set forth in SEQ ID NO: 9 and a LCVR having the sequence set forth in SEQ ID NO: 38 (392C5A).

[0049] In some embodiments, the isolated antibody that binds to PD-1 binds to a sequence in PD-1 selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, and SEQ ID NO: 84.

[0050] As used herein, an “antibody that binds to PD-1, comprising” a HCVR or LCVR, means an antibody comprising the HCVR or LCVR, as opposed to a PD-1 protein comprising the HCVR or LCVR.

[0051] In some embodiments, the antibody binds specifically to PD-1. This means that the antibody binds to PD-1 protein in a sample, with negligible binding to other proteins present in the sample, under a given set of binding reaction conditions.

[0052] Examples of antibody fragments include, a Fab, Fab', F(ab')₂, Fv, scFv, dAb, and a diabody.

[0053] A “Fab fragment” comprises one light chain and the C_H1 and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule.

[0054] An “Fc” region contains two heavy chain fragments comprising the CH2 and CH3 domains of an antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the CH3 domains.

[0055] A “Fab' fragment” contains one light chain and a portion of one heavy chain that contains the VH domain and the CH1 domain and also the region between the CH1 and CH2 domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form a F(ab')₂ molecule.

[0056] A “F(ab')₂ fragment” contains two light chains and two heavy chains containing a portion of the constant region between the C_H1 and C_H2 domains, such that an interchain disulfide bond is formed between the two heavy chains. A F(ab')₂ fragment thus is composed

of two Fab' fragments that are held together by a disulfide bond between the two heavy chains.

[0057] The “Fv region” comprises the variable regions from both the heavy and light chains, but lacks the constant regions.

[0058] A “single-chain Fv antibody” (or “scFv antibody”) refers to antibody fragments comprising the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv, see Pluckthun (1994) *The Pharmacology Of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315. See also, PCT Publication No. WO 88/01649 and U.S. Pat. Nos. 4,946,778 and 5,260,203.

[0059] A “diabody” is a small antibody fragment with two antigen-binding sites. The fragments comprise a heavy chain variable region (VH) connected to a light chain variable region (VL) in the same polypeptide chain (VH-VL or VL-VH). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described in, *e.g.*, patent documents EP 404,097; WO 93/11161; and Holliger *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 6444-6448.

[0060] A “domain antibody fragment” is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. In some instances, two or more VH regions are covalently joined with a peptide linker to create a bivalent domain antibody fragment. The two VH regions of a bivalent domain antibody fragment may target the same or different antigens.

[0061] In some embodiments, the antibody is modified or engineered. Examples of modified or engineered antibodies include chimeric antibodies, multiparatopic antibodies (*e.g.*, biparatopic antibodies), and multispecific antibodies (*e.g.*, bispecific antibodies).

[0062] As used herein, “multiparatopic antibody” means an antibody that comprises at least two single domain antibodies, in which at least one single domain antibody is directed against a first antigenic determinant on an antigen and at least one other single domain antibody is directed against a second antigenic determinant on the same antigen. Thus, for example, a “biparatopic” antibody comprises at least one single domain antibody directed

against a first antigenic determinant on an antigen and at least one further single domain antibody directed against a second antigenic determinant on the same antigen.

[0063] As used herein, “multispecific antibody” means an antibody that comprises at least two single domain antibodies, in which at least one single domain antibody is directed against a first antigen and at least one other single domain antibody is directed against a second antigen (different from the first antigen). Thus, for example, a “bispecific” antibody is one that comprises at least one single domain antibody directed against a first antigen and at least one further single domain antibody directed against a second antigen, *e.g.*, different from the first antigen.

[0064] In some embodiments, the antibodies disclosed herein are monoclonal antibodies, *e.g.*, murine monoclonal antibodies. Methods of producing monoclonal antibodies are known in the art. See, for example, Pluckthun (1994) *The Pharmacology Of Monoclonal Antibodies*, Vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315.

[0065] In some embodiments, antibodies are modified to reduce immunogenicity. When the antibodies are to be administered to a human, the antibodies can be “humanized” to reduce or eliminate antigenicity in humans. Accordingly, in some embodiments, the antibody comprises a humanized or human framework region (FR).

[0066] In some embodiments, the isolated antibody that binds to PD-1 comprises a HCVR selected from the group consisting of SEQ ID NOs: 85-90.

[0067] In some embodiments, the isolated antibody that binds to PD-1 comprises a LCVR selected from the group consisting of SEQ ID NOs: 91-96.

[0068] In certain embodiments, the isolated antibody that binds to PD-1 comprises a HCVR selected from the group consisting of SEQ ID NOs: 85-90 and a LCVR selected from the group consisting of SEQ ID NOs: 91-96. Examples of pairings of HCVRs and LCVRs are provided throughout the present disclosure, but additional functional pairings are within the scope of the invention.

[0069] In some embodiments, the isolated antibody comprises a HCVR having the sequence set forth in SEQ ID NO: 90 and a LCVR having the sequence set forth in SEQ ID NO: 94; a HCVR having the sequence set forth in SEQ ID NO: 88 and a LCVR having the sequence set forth in SEQ ID NO: 96; a HCVR having the sequence set forth in SEQ ID NO: 90 and a LCVR having the sequence set forth in SEQ ID NO: 96; a HCVR having the sequence set forth in SEQ ID NO: 85 and a LCVR having the sequence set forth in SEQ ID

NO: 91; a HCVR having the sequence set forth in SEQ ID NO: 85 and a LCVR having the sequence set forth in SEQ ID NO: 93; or a HCVR having the sequence set forth in SEQ ID NO: 86 and a LCVR having the sequence set forth in SEQ ID NO: 91.

[0070] Methods for reducing or eliminating the antigenicity of antibodies and antibody fragments are known in the art. In one approach, a nucleic acid encoding a PD-1 antibody disclosed herein is modified, for example, by replacing the mouse constant region with human heavy- and light-chain constant regions (*e.g.*, U.S. Pat. No. 4,816,567; Morrison, *et al.*, 1984, *Proc. Natl. Acad. Sci. USA*, 81:6851) to produce what is commonly referred to as a chimeric antibody.

[0071] A humanized antibody generally has one or more amino acid residues from a source that is non-human. The non-human amino acid residues are often referred to as “import” residues, and are typically taken from an “import” variable domain. Humanization can be performed generally following the method of Winter and co-workers (Jones *et al.*, 1986, *Nature* 321:522-525; Riechmann *et al.*, 1988, *Nature*, 332:323-327; Verhoeyen *et al.*, 1988, *Science* 239:1534-1536), by substituting non-human CDRs or CDR sequences for the corresponding sequences of a human antibody. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in non-human, for example, murine antibodies. Preferably, a humanized antibody has the same or substantially the same affinity for the antigen as the non-human, *e.g.*, mouse antibody from which it was derived.

[0072] In an approach known as CDR grafting, the CDRs of the light and heavy chain variable regions are grafted into frameworks from another species. For example, murine CDRs can be grafted into human FRs. In some embodiments, the CDRs of the light and heavy chain variable regions of a PD-1 antibody are grafted into human FRs or consensus human FRs. To create consensus human FRs, FRs from several human heavy chain or light chain amino acid sequences are aligned to identify a consensus amino acid sequence. CDR grafting is described in, *e.g.*, U.S. Pat. No. 7,022,500 (Queen); U.S. Pat. No. 6,982,321 (Winter); U.S. Pat. No. 6,180,370 (Queen); U.S. Pat. No. 6,054,297 (Carter); U.S. Pat. No. 5,693,762 (Queen); U.S. Pat. No. 5,859,205 (Adair); U.S. Pat. No. 5,693,761 (Queen); U.S. Pat. No. 5,565,332 (Hoogenboom); U.S. Pat. No. 5,585,089 (Queen); U.S. Pat. No. 5,530,101 (Queen); Jones *et al.* (1986) *Nature* 321: 522-525; Riechmann *et al.* (1988) *Nature* 332: 323-

327; Verhoeyen *et al.* (1988) *Science* 239: 1534-1536; and Winter (1998) *FEBS Lett* 430: 92-94.

[0073] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is important to reduce antigenicity. According to the so-called “best-fit” method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence that is closest to that of the murine is then accepted as the FR for the humanized antibody (Sims *et al.*, 1987, *J. Immunol.* 151:2296; Chothia *et al.*, 1987, *J. Mol. Biol.* 196:901). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:4285; Presta *et al.*, 1993, *J. Immunol.* 151:2623).

[0074] It is important for humanized antibodies to retain affinity for the antigen and other desirable biological properties. To achieve this result, humanized antibodies can be designed analyzing parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs that illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences are available. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

[0075] Other methods to reduce immunogenicity include “reshaping,” “hyperchimerization,” and “veneering/resurfacing.” See, *e.g.*, Vaswami *et al.*, 1998, *Ann. Allergy & Immunol.* 81:105; Roguska *et al.*, 1996, *Prot. Engineer.* 9:895-904; and U.S. Pat. No. 6,072,035 (Hardman). In the veneering/resurfacing approach, the surface accessible amino acid residues in the murine antibody are replaced by amino acid residues more frequently found at the same positions in a human antibody. This type of antibody resurfacing is described, *e.g.*, in U.S. Pat. No. 5,639,641 (Pedersen).

[0076] Another approach for converting a mouse antibody into a form suitable for medical use in humans is known as ACTIVMAB™ technology (Vaccinex, Inc., Rochester, N.Y.), which involves use of a vaccinia virus-based vector to express antibodies in mammalian cells. High levels of combinatorial diversity of IgG heavy and light chains are said to be produced. *See, e.g.*, U.S. Pat. No. 6,706,477 (Zauderer); U.S. Pat. No. 6,800,442 (Zauderer); and U.S. Pat. No. 6,872,518 (Zauderer).

[0077] Another approach for converting a mouse antibody into a form suitable for use in humans is technology practiced commercially by KaloBios Pharmaceuticals, Inc. (Palo Alto, CA). This technology involves the use of a proprietary human “acceptor” library to produce an “epitope focused” library for antibody selection.

[0078] Another approach for modifying a mouse antibody into a form suitable for medical use in humans is HUMAN ENGINEERING™ technology, which is practiced commercially by XOMA (US) LLC. *See, e.g.*, PCT Publication No. WO 93/11794 and U.S. Pat. No. 5,766,886 (Studnicka); U.S. Pat. No. 5,770,196 (Studnicka); U.S. Pat. No. 5,821,123 (Studnicka); and U.S. Pat. No. 5,869,619 (Studnicka).

[0079] Humanization of antibodies is routine protein engineering. Nearly all murine antibodies can be humanized by CDR grafting, resulting in the retention of antigen binding. *See, e.g.*, Lo, Benny, K. C., editor, in *Antibody Engineering: Methods and Protocols*, Vol. 248, Humana Press, New Jersey, 2004.

[0080] In some embodiments, the antibodies are antagonists. As used herein, “antagonist” in reference to an anti-PD-1 antibody means an antibody that inhibits the PD-1 signaling pathway in a cell (*e.g.*, an immune cell). An antagonist anti-PD-1 antibody might inhibit the PD-1 signaling pathway by blocking the PD-1/PD-L1 or PD-1/PD-L2 interaction, but does not necessarily do so.

[0081] In some embodiments, the antibodies are agonists. As used herein, “agonist” in reference to an anti-PD-1 antibody means an antibody that activates the PD-1 signaling pathway in a cell (*e.g.*, an immune cell). An agonist antibody might influence the PD-1/PD-L1 and/or PD-1/PD-L2 interaction, but does not necessarily do so.

[0082] An antibody that binds to PD-1 and competitively inhibits the binding of an antibody that contains one or more sequences disclosed herein is within the scope of the invention. In certain embodiments, the antibody competitively inhibits the binding of the antibody that comprises a HCVR having the sequence set forth in SEQ ID NO: 20 and a

LCVR having the sequence set forth in SEQ ID NO: 53 (246A10); a HCVR having the sequence set forth in SEQ ID NO: 25 and a LCVR having the sequence set forth in SEQ ID NO: 45 (413E1); a HCVR having the sequence set forth in SEQ ID NO: 22 and a LCVR having the sequence set forth in SEQ ID NO: 51 (413D2); a HCVR having the sequence set forth in SEQ ID NO: 18 and a LCVR having the sequence set forth in SEQ ID NO: 47 (388D4); a HCVR having the sequence set forth in SEQ ID NO: 1 and a LCVR having the sequence set forth in SEQ ID NO: 28 (244C8). In some embodiments, the antibody also binds to a sequence in PD-1 selected from the group consisting of SEQ ID NOs: 54-84.

[0083] Methods for determining whether two or more antibodies compete for binding to the same target are known in the art. For example, a competitive binding, or competition, assay can be used to determine whether one antibody blocks the binding of another antibody to the target. Typically, a competition assay involves the use of purified target antigen (*e.g.*, PD-1) bound to a solid substrate or expressed on cells, an unlabeled test binding molecule (*e.g.*, a test anti-PD-1 antibody), and a labeled reference binding molecule (*e.g.*, an antibody disclosed herein). Competitive inhibition is measured by determining the amount of label bound to the solid substrate or cells in the presence of the test molecule. Usually (but not necessarily) the molecule is present in excess of at least two-fold. A test antibody competes with the reference antibody or ligand (*e.g.*, PD-L1 or PD-L2) for specific binding to the antigen if an excess of one antibody inhibits binding of the other antibody or ligand by at least 50%, as measured in a competition assay.

[0084] In an exemplary competition assay, a reference anti-PD-1 antibody (*e.g.*, an antibody disclosed herein) is biotinylated using commercially available reagents. The biotinylated reference antibody is mixed with serial dilutions of the test antibody or unlabeled reference antibody (self-competition control) resulting in a mixture of various molar ratios of test antibody (or unlabeled reference antibody) to labeled reference antibody. The antibody mixture is added to a PD-1 coated-ELISA plate. The plate is then washed, and horseradish peroxidase (HRP)-streptavidin is added to the plate as the detection reagent. The amount of labeled reference antibody bound to the target antigen is detected following addition of a chromogenic substrate (*e.g.*, TMB (3,3',5,5'-tetramethylbenzidine) or ABTS (2,2''-azino-di-(3-ethylbenzthiazoline-6-sulfonate))), which are known in the art. Optical density readings (OD units) are measured using a spectrophotometer. OD units corresponding to zero percent inhibition are determined from wells without any competing antibody. OD units

corresponding to 100% inhibition, *i.e.*, the assay background, are determined from wells without any labeled reference antibody or test antibody. Percent inhibition of labeled reference antibody to PD-1 by the test antibody (or the unlabeled reference antibody) at each concentration is calculated as follows: $\% \text{ inhibition} = (1 - (\text{OD units} - 100\% \text{ inhibition}) / (0\% \text{ inhibition} - 100\% \text{ inhibition})) * 100$. Persons skilled in the art will appreciate that the competition assay can be performed using various detection systems known in the art.

[0085] Antibodies identified by competition assay (*e.g.*, competing antibodies) include antibodies binding to the same epitope, or similar (*e.g.*, overlapping) epitopes, as the reference antibody. In addition, the competition assay can identify antibodies binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antibody for steric hindrance to occur.

[0086] Two antibodies bind to the same epitope if essentially all amino acid mutations in the antigen that reduce or eliminate binding of one antibody to the antigen reduce or eliminate binding of the other. Two antibodies bind to overlapping epitopes if only a subset of the amino acid mutations that reduce or eliminate binding of one antibody to the antigen reduce or eliminate binding of the other.

[0087] A competition assay may be conducted in both directions to ensure that the presence of the label does not interfere with or otherwise inhibit binding. For example, in the first direction, the reference antibody is labeled and the test antibody is unlabeled, and in the second direction, the test antibody is labeled and the reference antibody is unlabeled.

[0088] In certain embodiments, the present invention provides a method for increasing T cell effector function, comprising contacting a T cell with a combination of: (a) an effective amount of an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell; and (b) an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell. In some embodiments, increasing T cell effector function includes, *e.g.*, increased secretion of effector cytokines, as demonstrated herein.

[0089] In some embodiments, the T cell is contacted with the combination of antibodies *in vivo*. For example, in certain embodiments, the T cell is contacted with the combination in a human patient in need of increased T cell effector function.

[0090] In some embodiments, the present invention also provides a method for increasing T cell effector function, comprising contacting a T cell with an anti-PD-1 antibody that does

not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell. In certain embodiments, the T cell is contacted with an antibody that comprises a heavy chain variable region having complementarity determining regions (CDRs) selected from the group consisting of CDRs 1-3 of SEQ ID NO:85, CDRs 1-3 of SEQ ID NO:86, and CDRs 1-3 of SEQ ID NO:87; and a light chain variable region having CDRs selected from the group consisting of CDRs 1-3 of SEQ ID NO:91, CDRs 1-3 of SEQ ID NO:92, and CDRs 1-3 of SEQ ID NO:93. In some embodiments, the T cell is contacted with an antibody that comprises a heavy chain variable region selected from the group consisting of SEQ ID NOS: 85, 86 and 87 and a light chain variable region selected from the group consisting of SEQ ID NOS: 91, 92 and 93. In certain embodiments, the T cell is contacted with an antibody that is selected from the group consisting of antibody 244C8-1, antibody 244C8-2 and antibody 244C8-3.

[0091] In some embodiments, the present invention provides a method for increasing lymphocyte secretion of a cytokine selected from the group consisting of IL-6, IL-12, IL-18, TNF- α , IL-1 β and GM-CSF in a human patient in need of increased T cell effector function, comprising administering to the patient a therapeutically effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell. In certain embodiments, the patient is administered an antibody that comprises a heavy chain variable region having complementarity determining regions (CDRs) selected from the group consisting of CDRs 1-3 of SEQ ID NO:85, CDRs 1-3 of SEQ ID NO:86, and CDRs 1-3 of SEQ ID NO:87; and a light chain variable region having CDRs selected from the group consisting of CDRs 1-3 of SEQ ID NO:91, CDRs 1-3 of SEQ ID NO:92, and CDRs 1-3 of SEQ ID NO:93. In some embodiments, the patient is administered an antibody that comprises a heavy chain variable region selected from the group consisting of SEQ ID NOS: 85, 86 and 87 and a light chain variable region selected from the group consisting of SEQ ID NOS: 91, 92 and 93. In certain embodiments, the patient is administered an antibody that is selected from the group consisting of antibody 244C8-1, antibody 244C8-2 and antibody 244C8-3.

[0092] In some embodiments, the present invention also provides a method of treating cancer in a mammal, comprising contacting a T cell in a mammal in need thereof with a combination of: (a) an effective amount of an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell; and (b) an

effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.

[0093] The presently disclosed method of treating cancer with a combination of anti-PD-1 antibodies can be used to treat various cancers. In some embodiments, the cancer is selected from the group consisting of: melanoma, renal cancer, prostate cancer, pancreatic adenocarcinoma, breast cancer, colon cancer, lung cancer, esophageal cancer, squamous cell carcinoma of the head and neck, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, and lymphoma.

[0094] In some embodiments, the anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell is selected from the group consisting of: 388D4, nivolumab, pembrolizumab, EH12.2H7 and J105. In some embodiments, the anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell is 388D4.

[0095] In some embodiments, the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell is 244C8. In some embodiments, the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell binds to one or more of the following amino acid sequences: SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 83 and SEQ ID NO: 84. In some embodiments, the anti-PD-1 antibody binds to all of the following amino acid sequences: SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 83 and SEQ ID NO: 84. In some embodiments, the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell binds to a PD-1 epitope bound by 244C8. In some embodiments, the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell competes with 244C8 for binding to PD-1.

[0096] The presently disclosed method of treating cancer with an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of T cells, and an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cells, increases T cell effector function to a greater extent than an equivalent amount of either anti-PD-1 antibody alone. In some embodiments, the combination yields an additive effect on T cell effector

function. In some embodiments, the combination yields a synergistic effect on T cell effector function.

[0097] The present invention provides isolated nucleic acids comprising a nucleotide sequence encoding a HCVR and/or a LCVR disclosed herein, or a fragment thereof. A nucleic acid according to the present invention may comprise DNA or RNA, and may be wholly or partially synthetic. For example, DNA molecules encoding an HCVR and/or LCVR disclosed herein can be chemically synthesized. Synthetic DNA molecules can be ligated to other appropriate nucleotide sequences, including, *e.g.*, constant region coding sequences, and expression control sequences, to produce conventional gene expression constructs encoding the desired antibodies. Production of defined gene constructs is within routine skill in the art. Alternatively, nucleotide sequences can be cloned out of hybridomas, for example, by conventional hybridization techniques or polymerase chain reaction (PCR) techniques, using synthetic nucleic acid probes or primers whose sequences are based on sequence information provided herein, or known sequence information regarding genes encoding the heavy and light chains of murine antibodies in hybridoma cells.

[0098] Techniques and protocols for engineering and production of nucleic acids are known in the art. *See, e.g., Current Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., John Wiley & Sons, 1992.

[0099] A nucleotide sequence encoding an antibody of the invention can be operably linked to a promoter to effect expression of the antibody in a host cell. The sequence may include at its 5' end a leader sequence to facilitate expression in a host cell and/or secretion of the antibody from a host cell. Suitable leader sequences are known in the art and can be selected by the skilled person, taking account of the host cell.

[00100] In some embodiments, the nucleic acid is incorporated into a vector. Suitable vectors containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate, can be obtained commercially or constructed by persons of skill in the art. For further details *see, e.g., Molecular Cloning: a Laboratory Manual*, 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press. Examples of vectors include plasmids, phages, phagemids, and cosmids, as well as transcription and expression cassettes.

[00101] Nucleic acids encoding a HCVR and/or a LCVR disclosed herein can be incorporated (ligated) into expression vectors, which can be introduced into host cells through conventional transfection or transformation techniques. Accordingly, a host cell can be transformed with an expression vector comprising a nucleotide sequence encoding a HCVR and/or a LCVR, or a fragment thereof. Examples of host cells include *E. coli* cells, Chinese hamster ovary (CHO) cells, human embryonic kidney 293 (HEK 293) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), and human hepatocellular carcinoma cells (*e.g.*, Hep G2).

[00102] Methods of producing an HCVR and/or LCVR, or a fragment thereof, disclosed herein are within the scope of the invention. In some embodiments, the method comprises: (a) growing a host cell containing an expression vector encoding the HCVR and/or LCVR under conditions so that the host cell expresses the antibody comprising the HCVR and/or LCVR, or a fragment thereof; and (b) isolating the antibody comprising the HCVR and/or LCVR, or a fragment thereof.

[00103] Suitable conditions for antibody expression and isolation or purification depend on the expression system employed. For example, if a gene is to be expressed in *E. coli*, it is first cloned into an expression vector by positioning the engineered gene downstream from a suitable bacterial promoter, *e.g.*, Trp or Tac, and a prokaryotic signal sequence. The expressed secreted protein accumulates in refractile or inclusion bodies, and can be harvested after disruption of the cells by French press or sonication. The refractile bodies then are solubilized, and the proteins refolded and cleaved by methods known in the art.

[00104] If the engineered gene is to be expressed in eukaryotic host cells, *e.g.*, CHO (Chinese hamster ovary) cells, it is first inserted into an expression vector containing a suitable eukaryotic promoter, a secretion signal, a poly A sequence, and a stop codon. Optionally, the vector or gene construct contains enhancers and introns. This expression vector optionally contains sequences encoding all or part of a constant region, enabling an entire, or a part of, a heavy or light chain to be expressed. The gene construct can be introduced into eukaryotic host cells using conventional techniques. The host cells express VL or VH fragments, VL-VH heterodimers, VH-VL or VL-VH single chain polypeptides, complete heavy or light immunoglobulin chains, or portions thereof, each of which may be attached to a moiety having another function (*e.g.*, cytotoxicity).

[00105] In some embodiments, a host cell is transfected with a single vector expressing a polypeptide expressing an entire, or part of, a heavy chain (*e.g.*, a heavy chain variable region) or a light chain (*e.g.*, a light chain variable region). In some embodiments, a host cell is transfected with a single vector encoding (a) a polypeptide comprising a heavy chain variable region and a polypeptide comprising a light chain variable region, or (b) an entire immunoglobulin heavy chain and an entire immunoglobulin light chain. In some embodiments, a host cell is co-transfected with more than one expression vector (*e.g.*, one expression vector expressing a polypeptide comprising an entire, or part of, a heavy chain or heavy chain variable region, and another expression vector expressing a polypeptide comprising an entire, or part of, a light chain or light chain variable region).

[00106] A polypeptide comprising an immunoglobulin heavy chain variable region or light chain variable region can be produced, for example, by growing (culturing) a host cell transfected with an expression vector encoding such a variable region, under conditions that permit expression of the polypeptide. Following expression, the polypeptide can be harvested and purified or isolated using techniques known in the art, *e.g.*, affinity tags such as Protein A, Protein G, glutathione-S-transferase (GST), or histidine tags.

[00107] The antibodies of the present invention can be produced by growing (culturing) a host cell transfected with, for example: (a) an expression vector that encodes a complete or partial immunoglobulin heavy chain, and a separate expression vector that encodes a complete or partial immunoglobulin light chain; or (b) a single expression vector that encodes both chains (*e.g.*, complete or partial heavy and light chains), under conditions that permit expression of both chains. The intact antibody (or antigen-binding fragment) can be harvested and purified or isolated using techniques known in the art, *e.g.*, Protein A, Protein G, affinity tags such as glutathione-S-transferase (GST) or histidine tags. It is within ordinary skill in the art to express the heavy chain and the light chain from a single expression vector or from two separate expression vectors.

[00108] In some embodiments, anti-PD-1 antibodies are linked to a different functional molecule or moiety, *e.g.*, a peptide, protein, toxin, radioisotope, or cytostatic agent, for various purposes such as *in vivo* diagnostic imaging or a diagnostic assay. The antibodies can be linked by chemical cross-linking or by recombinant methods. The antibodies also can be linked to any of various nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689;

4,301,144; 4,670,417; 4,791,192; or 4,179,337. The antibodies can be chemically modified by covalent conjugation to a polymer, for example, to increase their circulating half-life. Examples of polymers and methods to attach them are described in U.S. Pat. Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546.

Pharmaceutical Formulations

[00109] In some embodiments, the antibodies are formulated into pharmaceutical compositions suitable for administration to a mammal, e.g., a human patient. The compositions typically comprise one or more antibodies of the present invention and a pharmaceutically acceptable excipient. The term “pharmaceutically acceptable excipient” includes suitable solvents, dispersion media, coatings, antibacterial agents and antifungal agents, isotonic agents, and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is known in the art. The compositions also can contain other active compounds providing supplemental, additional, or enhanced therapeutic functions. The pharmaceutical compositions also can be included in a container, pack, or dispenser together with instructions for administration.

[00110] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Methods to accomplish the administration are known in the art. The administration may be, for example, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, intradermal, topical, inhalation, transmucosal, rectal or transdermal.

[00111] Solutions or suspensions used for intradermal or subcutaneous application typically include one or more of the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol, or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, as necessary. Such preparations may be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00112] Pharmaceutical compositions suitable for injection include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Sterilization can be accomplished, for example, by filtration through sterile filtration membranes. For intravenous administration, suitable carriers include, for example, physiological saline, bacteriostatic water, Cremophor EL (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). Preferably, the pharmaceutical composition is stable under the conditions of manufacture and storage and is preserved against contamination by microorganisms such as bacteria and fungi. Avoidance of microorganisms can be achieved by inclusion of antibacterial and/or antifungal agents. Examples include: parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol such as glycerol, propylene glycol, liquid polyethylene glycol, and the like, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. Prolonged absorption of the injectable compositions can be achieved by including in the composition an agent that delays absorption, *e.g.*, aluminum monostearate or gelatin.

[00113] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For oral administration, the antibodies can be combined with excipients and used in the form of tablets, troches, or capsules.

[00114] For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated can be used in the formulation. Such penetrants are known in the art, and include, for example, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration may be accomplished, for example, through the use of lozenges, nasal sprays, inhalers, or suppositories. For example, in case of antibodies that comprise the Fc portion, compositions may be capable of transmission across mucous membranes in intestine, mouth, or lungs (*e.g.*, via the FcRn receptor-mediated pathway as described in U.S. Pat. No. 6,030,613). For transdermal administration, the active compounds may be formulated into ointments, salves, gels, or creams as generally known in the art. For administration by

inhalation, the antibodies may be delivered in the form of an aerosol spray from pressured container or dispenser, which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

[00115] In some embodiments, the presently disclosed antibodies are formulated with carriers that protect the antibody against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used. Exemplary polymers include ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. Liposomal suspensions containing the presently disclosed antibodies can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known in the art. *See, e.g.*, U.S. Pat. No. 4,522,811.

[00116] In some embodiments, pharmaceutical compositions contain, in addition to an antibody of the invention, a cytotoxic agent, cytostatic agent, anti-angiogenic agent, a tumor targeted agent, an immune stimulating agent or immune modulating agent, or an antibody conjugated to a cytotoxic, cytostatic, or otherwise toxic agent. The pharmaceutical composition optionally can be employed with other therapeutic modalities such as surgery, chemotherapy, and radiation.

[00117] Toxicity and therapeutic efficacy of the composition of the invention can be determined by conventional pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compositions that exhibit large therapeutic indices are preferred.

[00118] A therapeutically effective dose of a therapeutic antibody can be estimated initially, *e.g.*, from cell culture assays. Examples of suitable bioassays include DNA replication assays, cytokine release assays, transcription-based assays, PD-1/PD-L1 binding assays, creatine kinase assays, assays based on the differentiation of pre-adipocytes, assays based on glucose uptake in adipocytes, immunological assays other assays as, for example, described in the Examples. The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (*i.e.*,

the concentration of the antibody that achieves a half-maximal inhibition of symptoms). Circulating levels in plasma may be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage lies preferably within a range of circulating concentrations with little or no toxicity. The dosage may vary depending upon the dosage form employed and the route of administration.

[00119] Generally, a therapeutically effective amount of an antibody or a composition described herein is in the range of 0.1 mg/kg to 100 mg/kg, preferably 0.1 mg/kg to 50 mg/kg. The amount administered will depend on variables such as the type and extent of disease or indication to be treated, the overall health of the patient, the *in vivo* potency of the antibody, the pharmaceutical formulation, the serum half-life of the antibody, and the route of administration.

[00120] Administration frequency can vary, depending on factors such as route of administration, dosage amount, serum half-life of the antibody or fusion protein, and the disease being treated.

Therapeutic Uses

[00121] The invention provides methods of treating PD-1-mediated diseases or disorders in a mammal, *e.g.*, a human patient, comprising administering an effective amount of an antibody of the present invention to a mammal in need thereof. In some embodiments, the method is a method of treating cancer. In some embodiments, the method is a method of treating inflammation. In some embodiments, the method is a method of treating an autoimmune disease, *e.g.*, Crohn's disease.

[00122] As used herein, "treat", "treating" or "treatment" means inhibiting or relieving a disease or disorder. For example, treatment can include a postponement of development of the symptoms associated with a disease or disorder, and/or a reduction in the severity of such symptoms that will, or are expected, to develop with said disease. The terms include ameliorating existing symptoms, preventing additional symptoms, and ameliorating or preventing the underlying causes of such symptoms. Thus, the terms denote that a beneficial result is being conferred on at least some of the mammals, *e.g.*, human patients, being treated. Many medical treatments are effective for some, but not all, patients that undergo the treatment.

[00123] As used herein, the term “effective amount” means an amount of an anti-PD-1 antibody, that when administered alone or in combination with an additional therapeutic agent to a cell, tissue, or subject, is effective to achieve the desired therapeutic or prophylactic effect under the conditions of administration. For example, an effective amount is one that would be sufficient to enhance or diminish the immune response to bring about effectiveness of a therapy. The effectiveness of a therapy (*e.g.*, activation of a suppressed or deficient immune response, increased cytolytic activity of T cells, increased T cell effector function, alteration of PD-1 activity associated with the negative regulation of T-cell mediated immune response, or reduction in tumor growth) can be determined by suitable methods known in the art.

[00124] When used to treat cancer, antibodies of the invention can be used alone or in combination with another therapeutic agent. Examples of other therapeutic agents include other checkpoint inhibitors, immunogenic agents, attenuated cancerous cells, tumor antigens (*e.g.*, recombinant proteins, peptides, and carbohydrate molecules), antigen presenting cells such as dendritic cells pulsed with tumor-derived antigen or nucleic acids, immune stimulating cytokines (*e.g.*, IL-2, IFN α 2, GM-CSF), and cells transfected with a gene encoding an immune stimulating cytokine (*e.g.*, GM-CSF); chemotherapy, radiotherapy, and surgery.

[00125] In some embodiments, an antibody of the invention is administered to a cancer patient in combination with another checkpoint inhibitor. The other checkpoint inhibitor can be targeted against PD-1 or against a different checkpoint molecule, *e.g.*, TIM3, CEACAM1, TIGIT, LAG3 or VISTA. The other checkpoint inhibitor can be a small molecule or a monoclonal antibody. When the other checkpoint inhibitor is a second PD-1 inhibitor, preferably, the mechanism of action of the second PD-1 inhibitor differs from the mechanism of action of the first PD-1 inhibitor. For example, the two PD-1 inhibitors can be two anti-PD-1 monoclonal antibodies that bind to different epitopes on the PD-1 molecule.

[00126] When used to treat cancer, antibodies of the invention can be used alone or in combination with other checkpoint inhibitors, anti-neoplastic agents or immunogenic agents. Examples include attenuated cancerous cells, tumor antigens (including, *e.g.*, recombinant proteins, peptides, and carbohydrate molecules), antigen presenting cells such as dendritic cells pulsed with tumor-derived antigen or nucleic acids, immune stimulating cytokines (*e.g.*, IL-2, IFN α 2, GM-CSF), and cells transfected with genes encoding immune stimulating

cytokines (*e.g.*, GM-CSF; cancer treatments such as chemotherapy, radiotherapy, and surgery).

[00127] In treating certain diseases or disorders, it is desirable to diminish or suppress a patient's immune response, at least in certain tissues of the body. Such diseases and disorders include allergies and various autoimmune diseases. Examples of autoimmune diseases include rheumatoid arthritis, type I diabetes mellitus, multiple sclerosis, inflammatory bowel disease, Crohn's disease, and systemic lupus erythematosus, Hashimoto's thyroiditis, ankylosing spondylitis, and graft-versus-host disease (GVHD). It is also desirable to suppress a patient's immune response to avoid transplant rejection following tissue, skin or organ transplant.

[00128] In some embodiments, anti-PD-1 antibodies of the invention are administered with one or more additional therapeutic agents, *e.g.*, a cytotoxic agent, a radiotoxic agent, or an immunosuppressive agent. The antibody can be linked to the agent (as an immunocomplex) or administered separately. In some embodiments, the additional therapeutic agent is an immunomodulatory agent or an anti-cancer agent (*e.g.*, a chemotherapeutic agent). In separate administration, the antibody can be administered before, after or concurrently with the agent or can be co-administered with other known therapies. Combination therapies are known in the art. See, *e.g.*, Hardman, *et al.* (eds.) (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 10th ed., McGraw-Hill, New York, N.Y.; Poole and Peterson (eds.) (2001) *Pharmacotherapeutics for Advanced Practice: A Practical Approach*, Lippincott, Williams & Wilkins, Phila., PA; Chabner and Longo (eds.) (2001) *Cancer Chemotherapy and Biotherapy*, Lippincott, Williams & Wilkins, Philadelphia, PA.

[00129] In some embodiments, an antibody disclosed herein is used as a targeting agent for delivery of a payload, *e.g.*, a toxin, to a cell expressing PD-1. The method includes administering an anti-PD-1 antibody conjugated to a payload moiety. Suitable conjugation methods are known in the art.

Non-therapeutic Uses

[00130] In some embodiments, antibodies of the invention are used for non-therapeutic purposes, such as diagnostic tests and assays. For example, the invention provides a method of diagnosing a PD-1-mediated adaptive immune resistance in a patient who has cancer. The

method comprises contacting a tumor microenvironment in the patient with an antibody disclosed herein that has been labeled with a detectable moiety; and detecting expression of PD-1 on immune cells, *e.g.*, CD8⁺ T cells; B cells; and macrophages, within the tumor microenvironment.

[00131] Adaptive immune resistance includes suppression of a host immune response as a result of activation of a PD-1 signaling pathway in immune cells of the host. For example, cancer tissue suppresses a host immune response by upregulation of PD-L1 and its binding to PD-1 on immune cells on T cells (such as CD8⁺ T cells); B cells; and macrophages.

[00132] A diagnostic method utilizing an antibody of the invention to detect PD-1 expression also can comprise an agent for detecting expression of PD-L1 on immune cells within the tumor microenvironment. Such a diagnostic method can be performed *in vivo*, or on a biopsy sample from a patient, wherein the tumor microenvironment is present in a tumor biopsy.

[00133] Modifications of antibodies for diagnostic purposes are well known in the art. For example, antibodies may be modified with a ligand group such as biotin, or a detectable marker group such as a fluorescent group, a radioisotope, or an enzyme. Antibodies of the invention can be labeled using conventional techniques. Suitable detectable labels include: fluorophores, chromophores, radioactive atoms, electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes typically are detected by their reaction products. For example, horseradish peroxidase can be detected through conversion of tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. For detection, suitable binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art.

[00134] Antibodies of the invention also can be used to detect the presence of PD-1 in biological samples. The amount of PD-1 detected can be correlated with the expression level of PD-1, which, in turn, is correlated with the activation status of immune cells, *e.g.*, activated T cells, B cells, and monocytes, in the subject.

[00135] Detection methods that employ antibodies are known in the art, and include ELISA, radioimmunoassay, immunoblot, Western blot, immunofluorescence, and immunoprecipitation techniques. Antibodies of the invention can be provided in a diagnostic

kit that incorporates one or more of these techniques to detect PD-1. Such a kit may contain other components, packaging, instructions, or material to aid in the detection of PD-1 protein.

EXAMPLES

[00136] The following Examples are merely illustrative, and are not intended to limit the scope or content of the invention in any way.

[00137] Example 1. Identification of PD-1 Antibodies

[00138] A. Immunization of Mice with PD-1

[00139] Balb/C, C57BL/6 or NZW/B female mice aged 4-8 weeks were immunized in a standard prime/boost regimen employing standard adjuvant mixtures. A soluble extracellular domain of human PD-1 (AA1-167) expressed with a C-terminal polyhistidine sequence (SinoBiological #10377-H08H) was used for immunizations. Cohorts of mice were primed with 50 µg of recombinant PD-1 and (1) complete Freund's adjuvant (Sigma-Aldrich #263810) or (2) alhydrogel (Invivogen). Two to three weeks later, animals in each group were boosted with 50 µg of soluble PD-1 with (1) incomplete Freund's adjuvant (Sigma-Aldrich #263910) or (2) alhydrogel. Serum titres were collected after each antigen boost and assayed by ELISA for reactivity and antibody isotype class switching. The same protein used for immunizations was immobilized onto 96 well assay plates (Nunc MAXISORP) at a concentration of 1 µg/mL. Serial dilutions of the sera from immunized animals were then tested for binding to PD-1.

[00140] B. Screening of Antibodies for Binding to Human PD-1

[00141] Cells, fresh or thawed from cryopreserved samples, from bone marrow, lymph nodes or the spleen were carried in standard tissue culture medium (LifeTech RPMI with 10% low IgG). Cells were interrogated for antigen-specific B cells, unstimulated or stimulated, using LPS (Invivogen) at a concentration of 20 ng/mL. Cells, unstimulated or stimulated, were then loaded at a stochastic cellular density to favor single cell per well loading onto microwell arrays (MWAs) as described in U.S. Patent Nos. 7,776,553 and 8,772,049.

[00142] A functionalized capture surface, coated with two mixtures of polyclonal anti-mouse IgG antibodies (Jackson ImmunoResearch #715-005-150, #115-005-146), was then used to hermetically seal the ordered microwell array. After two hours, the capture surface(s) was removed from the microdevices and processed as described previously (Ogunnyi *et al.*

Nature Protocols, 2009). The microarray capture surface, representing a mirror image of the cells in the microwell array, contained the secreted output of the B cells. The antibodies secreted by the B cells in nanowells and captured were then assayed for reactivity against human PD-1, or an unrelated antigen, and also assayed for IgG versus IgM reactivity (JacksonImmunoResearch #115-005-044, #115-005-164).

[00143] After the protein microarrays were scanned, putative antibody clones with the desired specificity and antibody isotype class were bioinformatically identified by standard data quality metrics. According to methods previously described (Ogunnyi *et al.*, *Vaccine* 2014), microarray images were analyzed using Molecular Devices GenePix software. Microarray features were analyzed for false positivity, co-variance, and signal-to-noise ratios. Features with the correct attributes, *e.g.*, specific for PD-1 and IgG, were then nominated for cellular retrieval. This automated pick-list was then generated from 4 to 12 microdevices, and cells that had secreted antibodies with desired characteristics were isolated from the microdevices and placed into standard SBS microtitre assay plates for further processing.

[00144] C. Isolation of Antibodies that Bind PD-1

[00145] Single antibody-producing cells identified from screening were used for single cell molecular biology in order to isolate the genetic sequences encoding antibody heavy and light chains (Tiller *et al.*, *J. Immunol. Methods* 350:183-93 (2009)). The genes encoding the specific antibodies that recognize human PD-1 were retrieved using single cell RT-PCR. Retrieved cells were placed into reverse transcription buffer, and the mRNA from each individual cell was reverse transcribed (LifeTech SuperScript III) into cDNA. After generating these amplicons by standard nested polymerase chain reaction(s) (PCR), these amplicons were subjected to direct sequencing. After analysis by Phred software using a Phred 0.05 cut-off value, sequences were sub-cloned into PCR 2.1 (LifeTech) or other standard vector backbones for further propagation. Phred is software that reads DNA sequencing trace files, calls bases, and assigns a quality value to each called base. See, *e.g.*, Ewing and Green, *Genome Research* 8:186-94 (1998).

[00146] These DNA sequences were then bioinformatically filtered for sequence quality and organized into a sequence database for cladistics analysis (distance or parsimony), in order to identify how many unique antibody clades were isolated that recognize PD-1. These analyses identified approximately 20 unique clades (groups) of sequences that recognize human PD-1.

[00147] D. Antibody Construction

[00148] Complete antibody variable regions comprising a pair of heavy and light chain variable regions (Tables 1 and 2) were reformatted into plasmids with the proper elements for transient ectopic expression in mammalian cell lines, *e.g.*, HEK293 or CHO, using standard molecular biology techniques. For example, the variable heavy (VH) and variable light (VL) cDNA sequences were sub-cloned into the vector backbone pFUSE-CHlg-mG1 (InVivoGen), which contains an IL2 signal sequence, as well as an in-frame murine Fc-domain. A sequence verified consensus sequence for each antibody VH and VL gene was engineered, using PCR primers, with restriction sites. The expression vector of choice and the PCR amplicons were then digested with restriction enzymes and then ligated together for transformation of *E. Coli*. Resulting expression clones were sequence verified.

[00149] The sequences of the individual heavy chain and light chain variable regions are shown in FIG. 1 (HCVR) and FIG. 2 (LCVR). The complementarity determining regions (CDRs) and framework regions (FRs) are indicated. Tables 1 and 2 list each HCVR or LCVR by clone name and corresponding sequence identifier. The corresponding sequences are shown in FIGS. 1 and 2.

[00150] Table 1. Heavy Chain Variable Region Sequence Designations and Identifiers

Heavy Chain Variable Region	SEQ ID NO:
100244_C8VH3	1
100258_F6VH4	2
100246_F7VH3	3
100244_C7VH10	4
100258_C1VH4	5
100392_C4VH5	6
100394_D5VH9	7
100394_G1VH1	8
100392_C5VH6	9
100392_H3VH6	10
100394_F1VH8	11
100388_C1VH2	12
100388_C1VH6	13

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Heavy Chain Variable Region	SEQ ID NO:
100388_G1VH5	14
100392_B4VH10	15
100392_H4VH9	16
100392_D2VH7	17
100388_D4VH3	18
100392_A6VH8	19
100246_A10VH1	20
100411_C2VH3	21
100413_D2VH9	22
100388_H4VH1	23
100392_C3VH7	24
100413_E1VH9	25
100413_H1VH3	26

[00151] Table 2. Light Chain Variable Region Sequence Designations and Identifiers

Light Chain Variable Region	SEQ ID NO:
100245_C8VK5ml	27
100245_C8VK5	28
100259_F6VK1ml	29
100259_F6VK1	30
100247_F7VK2	31
100389_C1VK2	32
100395_F1VK4	33
100393_C4VK8	34
100395_G1VK4	35
100389_G1VK1	36
100393_C5VK10	37
100393_C5VK7	38
100389_C1VK1	39
100393_D2VK6	40

Light Chain Variable Region	SEQ ID NO:
100395_D5VK7	41
100393_H3VK2	42
100393_B4VK10/H4VK6	43
100259_C1VK1	44
100414_E1VK5	45
100414_H1VK6	46
100389_D4VK5	47
100393_A6VK5	48
100389_H4VK4	49
100393_C3VK10	50
100414_D2VK6	51
100412_C2VK7	52
100247_A10VK3	53

[00152] Example 2. Antibody Characterization

[00153] A. Hit Confirmation and Specificity

[00154] Supernatants from transiently transfected mammalian cell lines were used to test for immunoglobulin (Ig) expression, antigen specificity, and antigen affinity. These assays were ELISA-based, using reagents that recognize IgG, as well as the soluble ECD of PD-1 as an Fc-fusion protein (SinoBiological “CD279-Fc”). While the screening immunogen was the soluble form of PD-1, the form used for binding confirmation was a fusion protein comprising the ECD of human PD-1 with a human Fc domain. Other immune checkpoint proteins in the same biochemical configurations were used as specificity controls, *e.g.*, CD28, GITR. Proteins were immobilized in wells of a 96 well assay plate (Nunc MAXISORB) and supernatants from transfected HEK293 cells were used to assess binding of reformatted anti-PD-1 antibodies, produced as described above. These experiments enabled determination of binding specificity and affinity.

[00155] FIG. 3 shows an example of an ELISA-based binding assay. Various VH and VK were paired to yield 32 clones (clone pairings shown in box of FIG. 3), which were expressed in HEK293 cells and assayed for binding to PD-1-HIS(tag). The results represent an average of two experiments.

[00156] B. Cellular Binding

[00157] Recombinant antibodies from cultured supernatants were also used in cell-based binding studies. HEK293 cells were transfected (Qiagen, SuperFect) with expression plasmids encoding the Ig heavy and light chains of anti-PD-1 antibodies. After 3-5 days, recombinant antibodies in the supernatants of transfected cells were harvested. Stable HEK293 cells expressing human PD-1 or primary cells were used for the cell-based binding confirmation studies. Antibodies from cultured supernatants were assessed for binding to cell surface PD-1 using fluorescently labeled anti-mouse IgG (polyclonal) antibody (Jackson ImmunoResearch).

[00158] Fluorescence microscopy studies revealed that a number of anti-PD-1 antibodies obtained from transfection supernatants bound to PD-1 expressed on the surface of HEK293 cells. Binding of four mouse anti-PD-1 antibodies including, 100244_C7VH10_100245_C8VK5m1 (comprising the HCVR corresponding to SEQ ID NO: 4 and LCVR corresponding to SEQ ID NO: 27), were detected with anti-mouse κ -PE (secondary antibody). Similarly, in a separate study, binding of five mouse anti-PD-1 antibodies including, 100392_C5VH6_100393_C5VK7, comprising the HCVR corresponding to SEQ ID NO: 9 and LCVR corresponding to SEQ ID NO: 38, were detected with anti-mouse IgG1-AF488 (secondary antibody). In both studies, commercially available mouse anti-PD-1 antibody and isotype control mouse IgG1 anti-PD-1 were used as controls. Table 3 summarizes results of tests of binding of various anti-PD-1 antibodies to PD-1 expressed on the surface of HEK293 cells. Strong binding is denoted as “+++”, medium binding is denoted as “++”, and weak binding is denoted as “+”.

[00159] Table 3. Summary of PD-1 mAb binding to cell-surface expressed PD-1

Mouse anti-human PD-1 Abs	Binding to Human PD-1 Expressed on HEK293 Surface	mAb Name
100244_C7VH10_100245_C8VK5	++	244C7
100244_C7VH10_100245_C8VK5m1	++	244C7m1
100244_C8VH3_100245_C8VK5	++	244C8
100244_C8VH3_100245_C8VK5m1	++	244C8m1
100246_F7VH3_100247_F7VK2	++	246F7
100258_C1VH4_100259_C1VK1	+	258C1
100258_F6VH4_100259_F6VK1	++	258F6
100258_F6VH4_100259_F6VK1m1	++	258F6m
100392_C4VH5_100393_C4VK8	+++	392C4
100394_D5VH9_100395_D5VK7	++	394D5

Mouse anti-human PD-1 Abs	Binding to Human PD-1 Expressed on HEK293 Surface	mAb Name
100394 G1VH1 100395 G1VK4	++	394G1
100388 C1VH2 100389 C1VK1	++	388C12A
100388 C1VH2 100389 C1VK2	++	388C12B
100388 C1VH6 100389 C1VK1	++	388C16A
100388 C1VH6 100389 C1VK2	++	388C16B
100388 G1VH5 100389 G1VK1	-	388G1
100392 B4VH10 100393 B4VK10	-	392B4
100392 C5VH6 100393 C5VK7	++	392C5A
100392 C5VH6 100393 C5VK10	++	392C5B
100392 D2VH7 100393 D2VK6	++	392D2
100392 H3VH6 100393 H3VK2	-	392H3
100392 H4VH9 100393 H4VK6	+	392H4
100394 F1VH8 100395 F1VK4	-	394F1
100246 A10VH1 100247 A10VK3	+++	246A10
100388 D4VH3 100389 D4VK5	+++	388D4
100388 H4VH1 100389 H4VK4	-	388H4
100392 A6VH8 100393 A6VK5	++	392A6
100392 C3VH7 100393 C3VK10	-	392C3
100411 C2VH3 100412 C2VK7	++	411C2
100413 D2VH9 100414 D2VK6	++	413D2
100413 E1VH9 100414 E1VK5	++	413E1
100413 H1VH3 100414 H1VK6	-	413H1
mouse anti-PD-1 cl. EH12.2H7 (1µg/mL)	++	n/a
isotype control mouse IgG1(1µg/mL)	-	n/a

[00160] C. Cell-based Activity

[00161] Antibodies that recognize PD-1 with high affinity were selected for use in cell-based assays to test for agonist and antagonist activity. Using standard *ex vivo* activation conditions with primary human cells, selected anti-PD-1 antibodies were used for cell-based assays in microwell array devices, using conditions that have been previously characterized (see, *e.g.*, Varadarajan *et al.*, 2012, *Proc. Nat'l Acad. Sci.* 109:3885-3890) to assess effects of these antibodies on effector cell function.

[00162] Peripheral blood mononuclear cells (PBMCs) obtained from de-identified donors via a commercial source were used in these studies. PBMCs were placed into wells of a 96-well assay plate previously coated with plate bound anti-CD3 (OKT3) at a concentration of 1 µg/mL. In addition to TCR-mediated stimulation, cells were treated with PBS (control), soluble recombinant human PD-L1 (shPD-L1) (SinoBiological) at 20 µg/mL, or shPD-L1 with anti-PD-1 antibody (10 µg/mL). Cellular proliferation was assayed by ELISA for IL-2

(R&D Systems, #D2050) in the supernatant of cell cultures, or by direct measurement of secreted IFN γ at the single cell level (Varadarajan, *supra*) using microwell array devices.

[00163] FIG. 4 summarizes results from assays to measure the effectiveness of anti-PD-1 antibodies in relieving PD-L1 dependent inhibition of activation of human peripheral blood mononuclear cells (PBMCs). Treatments of cells were carried out in 96-well plates for 3-5 days. All treatments included plate-bound CD3 and soluble CD28 in conventional media. Cells were treated additionally with nothing (positive control); with PD-L1 alone; or with PD-L1 plus an anti-PD-1 antibody (EH12.2H7 (BioLegend Products, San Diego, CA), j105 (eBioscience, San Diego, CA), pembrolizumab, 246A10 or 244C8). At the end of the treatment period, cells were transferred to a microwell array device for IFN γ determination at the level of individual cells, with sample cell populations in the range of approximately 50-100 cells. The results shown in FIG.4 illustrate antagonism of PD-1 activity by anti-PD-1 antibodies. These anti-PD-1 antibodies blocked the inhibitory effect of PD-L1, thus decreasing PD-1/PD-L1 (or PD-1/PD-L2) mediated inhibition of cellular immune response.

[00164] D. Differential T Cell Activation

[00165] In a first type of cell-based activation assay, commercially-sourced human PBMCs (peripheral blood mononuclear cells) (Research Blood Components, Allston, MA) were analyzed by flow cytometry to test for differential T cell activation in response to PD-1 blockade by different anti-PD-1 antibodies *in vitro*. CD4 and CD8 were used as T cell markers. The relative extent of T cell activation was inferred from measuring production of the effector cytokines, interferon gamma (IFN γ) and tumor necrosis factor-alpha (TNF α). The experiments were conducted essentially as follows. Approximately 500,000 to 750,000 PBMCs were incubated for three days in the presence of 1 μ g/mL anti-CD3 (clone HIT3a), 50 ng/mL anti-CD28 (clone CD28.2) and 20 μ g/mL anti-PD-1 antibody or isotype control. At the end of the 3-day incubation period, cells were treated with Brefeldin A for 6 hours and then subjected to extracellular staining for CD4, CD8, CD69, CD25, PD-L1, or other extracellular markers conjugated to fluorophores. Cells were then fixed, permeabilized, and stained for intracellular markers including IFN γ (antibody clone 4S.B3). Data were collected by flow cytometry using a FACSCALIBUR™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and analyzed using FLOWJO™ software (FlowJo, LLC, Ashland, OR). The antibodies tested were pembrolizumab, clone EH12.2H7 (BioLegend), and anti-PD-1 antibodies 388D4, 413E1, 246A10 and 244C8 of the present invention. Under conditions of

suboptimal activation (achieved by the treatment with anti-CD3 and anti-CD28), which may mimic activation conditions that occur *in vivo*, antibodies 388D4, 413E1, 246A10 and 244C8 in these tests elicited similar secretion levels, or enhanced secretion levels of IFN γ and TNF α , as compared to EH12.2H7 or pembrolizumab (Table 4). The data in Table 4 are compared graphically in FIG. 5.

[00166] Table 4. Summary of differential T cell activation in response to PD-1 blockade by different anti-PD-1 antibodies

	CD8 IFN γ % positive	CD4 IFN γ % positive	CD8 TNF α % positive
Negative control	4.6	1.64	3.73
CD3/CD28	18.2	6.31	6.19
pembrolizumab	28.4	14.1	13
388D4	31.2	21.2	8.56
413E1	27	20.2	11.1
246A10	26.2	20.3	14.9
244C8	19.9	9.64	14.5
EH12.2H7	19.5	12.8	14.1
Positive control	73.7	61.5	37.3

[00167] In a second set of experiments, human PBMCs were tested for reactivity in antigen recall assays using CMV (cytomegalovirus) (IMMUNOSPOT®, Shaker Heights, OH). PBMCs were purified from whole blood of healthy donors (Research Blood Components, Allston, MA). The PBMCs were incubated with ready-to-use peptide antigen solutions (Astarte Biologics, Bothell, WA) without or with anti-PD-1 antibodies. Two commercial stage anti-PD-1 antibodies, pembrolizumab and nivolumab, were used as benchmarks for this experiment. Compared to antibody isotype control(s), anti-PD-1 antibodies induce increased levels of IFN γ , a key effector cytokine in T cell antigen recall biology (FIG. 6).

[00168] In a third set of experiments, anti-PD-1 antibodies were used with PBMCs in MLR (mixed lymphocyte reaction) assays. In these assays, secretion of IL-2 or IFN γ was the

experimental cytokine readout (FIG. 7A). Activation markers such as CD25 were also evaluated (FIG. 7B). Results are summarized in FIGS. 7A and 7B. In this assay, clone 388D4 appears to induce cytokine release and CD25 upregulation similar to nivolumab. In multiple assays, clone 244C8 appears to induce increased levels of cytokine release (IFN γ) compared with 388D4 or nivolumab. T cells incubated with 244C8 also appear to exhibit a higher degree of activation as inferred from CD25 expression (FIG. 7B).

[00169] These data indicate that some of the anti-PD-1 antibodies of the present invention, e.g., 388D4, induce increased cytokine release in a manner similar to pembrolizumab and nivolumab, while other antibodies, e.g., 244C8, elicit physiological responses that are measurably different from the responses elicited by pembrolizumab and nivolumab.

[00170] E. Peptide-based epitope mapping

[00171] Synthetic overlapping peptides based on the human PD-1 sequence (15-mers) (Sigma-Aldrich PEPscreen, Saint Louis, MO) were used in epitope mapping experiments. The peptides used are listed in Table 5 below.

[00172] Table 5. Human PD-1 peptide sequences used for epitope mapping

Peptide Name	N-term mod	Sequence	C-term Mod	SEQ ID NO
PD101	[H]	MQIPQAPWPVWVAVL	[OH]	54
PD102	[H]	APWPVWVAVLQLGWR	[OH]	55
PD103	[H]	VWAVLQLGWPRGWFL	[OH]	56
PD104	[H]	QLGWPRGWFLDSPDR	[OH]	57
PD105	[H]	PGWFLDSPDRPWNPP	[OH]	58
PD106	[H]	DSPDRPWNPPTFSPA	[OH]	59
PD107	[H]	PWNPPTFSPALLVVT	[OH]	60
PD108	[H]	TFSPALLVVTEGDNA	[OH]	61
PD109	[H]	LLVVTEGDNATFTCS	[OH]	62
PD110	[H]	EGDNATFTCSFSNTS	[OH]	63
PD111	[H]	TFTCSFSNTSESVL	[OH]	64
PD112	[H]	FSNTSESVLWYRM	[OH]	65
PD113	[H]	ESVWLNWYRMSPSNQ	[OH]	66
PD114	[H]	NWYRMSPSNQTDKLA	[OH]	67
PD115	[H]	SPSNQTDKLAAFPED	[OH]	68
PD116	[H]	TDKLAAFPEDRSQPG	[OH]	69
PD117	[H]	AFPEDRSQPGQDCRF	[OH]	70
PD118	[H]	RSQPGQDCRFRTQL	[OH]	71
PD119	[H]	QDCRFRTQLPNGRD	[OH]	72
PD120	[H]	RVTQLPNGRDFHMSV	[OH]	73
PD121	[H]	PNGRDFHMSVVRARR	[OH]	74
PD122	[H]	FHMSVVRARRNDSGT	[OH]	75

Peptide Name	N-term mod	Sequence	C-term Mod	SEQ ID NO
PD123	[H]	VRARRNDSGTYLCGA	[OH]	76
PD124	[H]	NDSGTYLCGAISLAP	[OH]	77
PD125	[H]	YLCGAISLAPKAQIK	[OH]	78
PD126	[H]	ISLAPKAQIKESLRA	[OH]	79
PD127	[H]	KAQIKESLRAELRVT	[OH]	80
PD128	[H]	ESLRAELRVTERRAE	[OH]	81
PD129	[H]	ELRVTERRAEVPTAH	[OH]	82
PD130	[H]	ERRAEVPTAHPSPSP	[OH]	83
PD131	[H]	VPTAHPSPSPRPAGQF	[OH]	84

[00173] Each peptide was incubated with each antibody for one hour, to allow peptide-antibody complex formation. Then each of these antibody-peptide mixtures was used in a conventional ELISA, in which human PD-1 was immobilized on 96-well plates. ELISA plates were coated with 100 ng/well of PD-1-His Tag (Sino Biological, North Wales, PA; #10377-H08H-50) in carbonate buffer, pH 9.6. After washing, plates were blocked with 4% milk PBS, 0.05% Tween-PBS (blocking buffer). After blocking and washing of the plates, the peptide-antibody mixtures were incubated with the immobilized human PD-1. After washing, the plates were developed by incubation for 1 hour with goat HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA; #115-035-071) and addition of 100 μ l of TMB solution (ThermoScientific, Waltham, MA; #PI-34022). Optical densities were measured at the appropriate wavelength, using an ELISA microplate reader. This enabled quantitative assessment of which peptides complexed with the antibody and then inhibited antibody binding to human PD-1. FIG. 8 summarizes the binding results of five antibody clones (246A10, 244C8, 388D4, 413D2, and 413E1).

[00174] F. Biophysical characterization of anti-PD-1 antibodies

[00175] Biophysical characteristics of certain anti-PD-1 antibodies were analyzed by biolayer interferometry (BLI), using the ForteBio Octet Red system (Pall Corporation, Menlo Park, CA). The antibodies were immobilized on BLI biosensors and then incubated with the soluble extracellular domain of human PD-1. Using standard biophysics methods, apparent on-rates and off-rates were then inferred for PD-1 with anti-PD-1 antibodies. These values were used to generate apparent affinity values (K_D values), which are listed in Table 6.

[00176] Table 6.

IgG	K_D (nM)	k_{on} (1/Ms)	k_{dis} (1/s)	R_{max}	Full X²	Full R²
246A10	76.4	1.61 x 10 ⁵	1.23 x 10 ⁻²	0.10	0.01	0.99
244C8	15.1	2.13 x 10 ⁵	3.22 x 10 ⁻³	0.12	0.01	1.00
413D2	8.20	2.75 x 10 ⁵	2.26 x 10 ⁻³	0.15	0.02	0.99
393C5	2.44	4.75 x 10 ⁵	1.16 x 10 ⁻³	0.15	0.01	1.00
388D4	2.69	3.71 x 10 ⁵	9.99 x 10 ⁻⁴	0.16	0.01	1.00
413E1	58.9	5.07 x 10 ⁵	2.99 x 10 ⁻²	0.12	0.01	0.99

[00177] G. Selectivity of anti-PD-1 antibodies

[00178] To assess selectivity of the anti-PD-1 antibodies, ELISA was used to generate dose response curves for binding of the anti-PD-1 antibodies to several immunomodulatory cell surface proteins. Recombinant soluble extracellular domains (ECDs) of ICOS (inducible T-cell costimulator), PD-1, CD28 or CTLA4 (R&D Systems, Minneapolis, MN) were coated onto ELISA assay plates. Binding of each anti-PD-1 antibody and each control antibody to each target protein was then assessed over a range of antibody concentrations (FIGS. 9A-9D).

[00179] These experiments demonstrated that anti-PD-1 antibodies 388D4, 413E1, 244C8 and 246A10 bind to the PD-1 ECD with high specificity, showing no binding to three structurally related Ig-superfamily protein members

[00180] **EXAMPLE 3. Humanization of Anti-PD-1 Antibodies**

[00181] Humanization of selected anti-PD-1 antibodies was performed in order to reduce the apparent immunogenicity of the mouse-based antibodies. Using antibody engineering information well known in the art, and conventional bioinformatics tools, amino acid sequences of certain murine anti-PD-1 antibodies of the invention were analyzed and compared against known human antibody sequences. Based on these analyses and comparisons, certain human sequences were chosen for conventional murine CDR grafting, and inclusion of suitable back mutations. In tests for binding to human PD-1, these humanized antibodies were evaluated with respect to criteria such as affinity, avidity, binding kinetics, and biochemical behavior such as aggregation as well as expression levels. The HCVR and LCVR amino acid sequences of certain humanized antibodies displaying desirable characteristics (*e.g.*, binding to PD-1) are shown in Tables 7 and 8, respectively. FIGS. 10A and 10B show amino acid sequence alignments of the humanized heavy or light chain variable region sequences with the indicated murine heavy (100388_D4_VH3 or

100244_C8_VH3) or light chain (100389_D4_VK5 or 100245_C8_Vk5m1) variable region sequences.

[00182] Table 7. Humanized heavy chain variable region sequences

HCVR designation	Amino Acid Sequence	SEQ ID NO.
100244_C8_HC1	QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYWMHWVRQAPGQGLEWMGMIDPSNSETS LNQKFQGRVTMTVDKSTNTVYMELSSLRSE DTAVYYCARSRGNYAYEMDYWGQGTLVTV SS	85
100244_C8_HC2	EVQLVQSGAEVKKPGASVKVSCKASGYTFTS YWMHWVRQAPGQGLEWMGMIDPSNSETSL NQKFQGRVTNLVDKSTNTAYMELSSLRSEDT AVYYCARSRGNYAYEMDYWGQGTLVTVSS	86
100244_C8_HC3	EVQLVQSGTEVTKPGASVKVSCKASGYTFTS YWMHWVRQAPGQGLEWLGMIDPSNSETTL NQKFQGRVTMTVDKSTNTVYMELTSLRSED TAVYYCARSRGNYAYEMDYWGQGTLVTVS S	87
100388_D4_HC1	EVQLVQSGAEVKKPGASVKVSCKASGYTFT DYEMHWVRQAPGQGLEWMGIIDPGTGGTA YNQKFQGRVTMTADKSTSTVYMELSSLRSE DTAVYYCTSEKFGSNYYFDYWGQGTLVTVS S	88
100388_D4_HC2	EVQLVQSGAEVKKPGASVKVSCKASGYTFT DYEMHWVRQAPGQGLEWMGIIDPGTGGTA YNQKFQGRVTMTADKSTNTVYMELSSLRSE DTAVYYCTSEKFGSNYYFDYWGQGTLVTVS S	89
100388_D4_HC3	EVQLVQSGAEVKKPGASVKVSCKASGYTFT DYEMHWVRQAPGQRLEWMGVIDPGTGGTA YNQKFQGRVTITADKSASTAYMELSSLRSED TAVYYCTSEKFGSNYYFDYWGQGTLVTVSS	90

[00183] Table 8. Humanized light chain variable region sequences

LCVR designation	Amino Acid Sequence	SEQ ID NO.
100245_C8_LC1	EIVLTQSPATLSLSPGERATLSCRASSSVSSN YLYWYQQKPGQAPRLLIYSTSNRATGIPARF SGSGSGTDYTLTISLEPEDFAVYYCHQWSS YPPTFGQGKLEIK	91
100245_C8_LC2	DIVLTQSPATLSLSPGERATLSCRASSSVSSN YLYWYQQKPGQAPRLLIYSTSNLATGIPARF SGSGSGTDYTLTISLEPEDFAVYFCHQWSS YPPTFGQGKLEIK	92

LCVR designation	Amino Acid Sequence	SEQ ID NO.
100245 C8 LC3	DIVLTQSPGTLSPGEEKVTLSCRASSSVSSN YLYWYQQKPGQAPRLVIYSTSNLATGIPDRF SGSGSGTDYTLTISRLEPEDFAVYFCHQWSS YPPTFGQGTKVEIK	93
100389 D4 LC1	DVVMTQSPSLPVTLGQPASISCRSSQTIVHS DGNTYLEWYQQRPGQSPRLLIYKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYC FQGSHVPLTFGQGTKLEIK	94
100389 D4 LC2	DIVMTQSPSLPVTLGQPASISCRSSQTIVHS DGNTYLEWYQQRPGQSPKLLIYKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYC FQGSHVPLTFGQGTKLEIK	95
100389 D4 LC3	DIVMTQTPLSSPVTLGQPASISCRSSQTIVHS DGNTYLEWYQQRPGQPPRLLIYKVSNRFSG VPDRFSGSGAGTDFTLKISRVEAEDVGVYY CFQGSHVPLTFGQGTKLEIK	96

[00184] Table 9. HCVR and LCVR pairings for humanized antibodies

Humanized Antibody Designation	Heavy Chain Variable Region	Light Chain Variable Region
244C8-1	100244 C8 HC1 (SEQ ID NO: 85)	100245 C8 LC1 (SEQ ID NO: 91)
244C8-2	100244 C8 HC1 (SEQ ID NO: 85)	100245 C8 LC3 (SEQ ID NO: 93)
244C8-3	100244 C8 HC2 (SEQ ID NO: 86)	100245 C8 LC1 (SEQ ID NO: 91)
388D4-1	100388 D4 HC3 (SEQ ID NO: 90)	100389 D4 LC1 (SEQ ID NO: 94)
388D4-2	100388 D4 HC3 (SEQ ID NO: 90)	100389 D4 LC3 (SEQ ID NO: 96)
388D4-3	100388 D4 HC1 (SEQ ID NO: 88)	100389 D4 LC3 (SEQ ID NO: 96)

[00185] EXAMPLE 4. Antibody-Ligand Competition for Binding to PD-1

[00186] A. Competitive Binding Assays

[00187] It was found that while some of the anti-PD-1 antibodies disclosed herein competitively inhibit binding of PD-1 ligands, others do not. For example, in surface plasmon resonance-based competitive binding assays and flow cytometry-based competitive binding assays, it was found that antibody 388D4 competitively inhibits binding of PD-L1 to PD-1, but does not inhibit binding of PD-L2. In contrast, it was found that antibody 244C8 does not competitively inhibit binding of PD-L1 or PD-L2.

[00188] Competitive Binding Analysis was performed on humanized IgG₄ antibodies 244C8-2 and 388D4-2, using ForteBio Biolayer Interferometry (BLI). Humanized IgG antibody was immobilized to AHC biosensors by loading 3 µg/mL IgG to a target level of 1.0 nm over a 160 second load time. A single concentration (100 nM) of active PD-1, plus an

appropriate negative control to correct for drift, was bound the immobilized IgG. A pH of 7.4 was used for association and dissociation. The bound PD-1 was then exposed to seven concentrations of PD-L1 (9, 3, 1, 0.333, 0.111, 0.037, and 0 μ M) or PD-L2 (2000, 666.7, 222.2, 74.1, 24.7, 8.2, and 0 nM). The association/dissociation of PD-L1 and PD-L2 to the mAb/PD-1 complex immobilized on biosensor tips was evaluated.

[00189] Materials used in these assays were as follows: PD1 (His Tag): ABCAM, Cat# ab174035, Lot# GR199119-1, 100 mg; PD-L1 (His Tag): Sino Biological, Cat# 10084-H08H, Lot# LC098E0901, 200 mg; PD-L2 (His Tag): Sino Biological, Cat# 10292-H08H, Lot# LC07DE3022, 100 mg; PD1-Fc: R&D Systems, Cat# 1086-PD, Lot# FVQ0413051, 50 mg; Anti-Human IgG-Fc Capture (AHC) Biosensors: ForteBio, Cat# 18-5060, Lot# 1501211; 1X Kinetic Buffer: 20 mM Phosphate, 150 mM NaCl, 0.02 % Tween-20, 0.05% Sodium Azide, 0.1 mg/ml BSA, pH 7.4; Test Samples: Humanized IgG₄ - 244C8-2 (3.04 mg/mL), Humanized IgG₄ - 388D4-2 (2.89 mg/mL).

[00190] The flow cytometry assays were conducted essentially as follows. HEK293 cells expressing PD-1 were incubated with 10 μ g/ml of an isotype antibody (negative control), commercially available antibody EH12.2H7 (positive control), antibody 388D4, or antibody 244C8. Cells were washed and stained with soluble PD-L1-Ig protein fluorescently labeled with Alexa-488. Cells were washed again, and PD-L1 binding (by displacing previously bound antibody) was assessed by fluorescence activated cell sorting (FACS) analysis.

Representative results are shown in FIGS. 11A-11D.

[00191] EXAMPLE 5. Human Cell Based Assays

[00192] A. Human Cells

[00193] Human tumor tissue procurement and tumor dissociation were as follows. Fresh tumor samples from NSCLC patients undergoing surgical resection of tumors were obtained from the Cooperative Human Tissue Network, National Cancer Institute. Analysis was performed using single-cell suspensions of tumor cells from these tumor samples.

[00194] Solid tumor biopsy samples were mechanically disrupted into single-cell suspensions using a gentleMACS Dissociator (Miltenyi Biotec) with enzymes A, H and R. The single-cell suspensions were then prepared for cell counting and initial FACS analysis.

[00195] B. FACS Analysis

[00196] For FACS analysis, anti-CD45-PerCP-Cy5.5 (clone 2D1), anti-CD4-PE-Cy7 (SK3), anti-CD8-FITC (SK1), anti-BTLA-Biotin (MIH26), anti-CTLA-4-PE (14D3), and anti-LAG-3-APC (3DS223H) were purchased from eBioscience. Anti-CD25-BV605 (2A3), anti-PD-1-BV605 (EH121), and Streptavidin-BV711 were purchased from BD Bioscience. Anti-CD45RABV421 (HI100), anti-CCR7 AlexaFluor647 (G043H7), and anti-Tim-3-BV421 (F38-2E2) were purchased from Biolegend. Heterogeneous cell suspensions prepared from dissociated primary tumors (as described above) were washed, resuspended in PBS, and blocked with a commercial Fc blocking reagent (BD Biosciences). Viable cells were identified by lack of dead cell staining positivity, and by negativity for EpCAM expression. CD45-positive cells were gated for CD4 or CD8 expression, and then the cells were assessed for expression of PD-1, TIM3, LAG3 or TIGIT. These FACS data are shown below, in Table 10, with results expressed as percentage of positive cells.

[00197] Table 10. Results of FACS analysis

Tumor	% EpCAM -CD45+	%CD3+	%CD4 ⁺ PD1 ⁺	%CD8 ⁺ PD1 ⁺	%CD4 ⁺ TIM3 ⁺	%CD8 ⁺ TIM3 ⁺	%CD4 ⁺ LAG3 ⁺	%CD8 ⁺ LAG3 ⁺	%CD4 ⁺ TIGIT ⁺	%CD8 ⁺ TIGIT ⁺
WD-36444	25.7	14	55	75	5.5	13	<1	<1	6	6.3
WD-36571	10.3	6.3	47	64	1.8	<1%	0	0	12.6	21
WD-36686	21.6	17	55	84	6	16	0	0	20	15
WD-36790	16.8	10.4	38	68	1	5.2	<1	<1	33	47
WD-36904	12.8	7	63	72	9.5	24.5	<1	<1	41	25.6
M115801 A2	3.4	2.9	79	84	22	16	1.4	1.6	56	35
WD-36923	1.6	0.9	53	51	27				24.5	
WD-36988	8.9	7	58	93	22	62	0	0	25	52
M415095 2	5.4	3	78	79	26	15	0	0	32	23

[00198] The T cell surface marker expression data in Table 10 provide a comparison of the immunomodulatory receptor profiles of tumor infiltrating lymphocytes (TILS) from various

human NSCLC tumor biopsy samples. Such data provided a useful biological context for the assays performed using the tumor samples.

[00199] C. Stimulation of Tumor infiltrating lymphocytes (TILs)

[00200] To establish polyclonal stimulation of TILs among the dissociated tumor cells, a 96-well assay plate was coated with 0.5 µg/mL anti-CD3 (OKT3) in coupling buffer, overnight at 4°C. The antibody coating solution was removed, and the plate was washed. Tumor suspensions were resuspended to a density of approximately 1.5×10^6 cells per mL. Then 200 µL of this was added to each experimental well, together with 2 µg/mL anti-CD28 (clone 28.2, eBioscience). At specific time points, supernatants were used for ELISA analysis or cells were used for FACS analysis or single cell analysis on microwell array devices.

[00201] D. Enzyme-linked immunosorbent assay

[00202] Supernatants from cultured tumor digests containing tumor cells, stromal cells and immune cells were collected at fixed time points after experimental treatment, and cytokine production was assessed by ELISA. To begin the ELISA, 96-well plates were coated with capture antibody, blocked with assay diluent buffer, and washed, prior to incubation with serial dilutions of supernatants from the cultured tumor-derived cells. The samples were incubated for one hour, and then the ELISA plates were washed. The detection antibody-HRP, in assay diluent, was then added, the assay plate was washed, and substrate solution was added to the wells in the assay plate. After the enzyme reaction was stopped, colorimetric density at 450 nm was measured in a conventional plate reader. Measurements of IFNγ secretion, normalized to internal standards included on each plate, was used as the experimental readout for T cell effector function.

[00203] E. TIL Function Increased by PD-1 Blockade

[00204] FIG. 12 summarizes results from an experiment showing restoration of T cell function by PD-1 blockade with nivolumab or different humanized forms of antibodies 388D4 and 244C8, *i.e.*, 388D4-2, 388D4-3, 244C8-1, 244C8-2, and 244C8-3. A population of 3×10^5 cells, which included 17% lymphocytes (activated as described above) was incubated for 24 hours with anti-PD-1 antibodies at a concentration of 20 µg/mL. IFNγ was measured by ELISA, and the data were expressed in terms of fold-activation relative to treatment with the isotype control antibody. As shown in FIG. 12, each of the anti-PD-1 antibodies restored T cell function, increasing IFNγ secretion approximately 7- to 8-fold,

relative to the isotype control. The data illustrated in FIG. 12 are presented in Table 11 below.

[00205] Table 11. Restoration of T cell function by PD-1 blockade

	Fold Induction IFN- γ	Standard Deviation
Isotype Control	1.00	0.39
nivolumab	7.07	0.15
388D4-2	7.58	0.23
388D4-3	7.20	0.23
244C8-1	8.04	0.33
244C8-2	7.76	0.11
244C8-3	7.79	0.42

[00206] FIG. 13 summarizes results from an experiment to measure the increase in T cell effector function, as indicated by IFN γ secretion, in response to treatment with antibody 244C8-2 alone versus treatment with 244C8-2 plus 388D4-2, with results normalized relative to the response to treatment with nivolumab. A population of 3×10^5 cells, which included 7.5% lymphocytes sub-optimally activated as described above, was incubated for 24 hours with anti-PD-1 antibodies at a total antibody concentration of 20 $\mu\text{g/mL}$. As shown in FIG. 13, treatment with 244C8-2 alone increased IFN γ 1.77-fold (± 0.19 sd), while treatment with 244C8-2 in combination with 388D4-2 increased IFN γ secretion 2.11-fold (± 0.21 sd). These unexpected results obtained in response to treatment with the combination of a competitive inhibitory antibody and a non-competitive inhibitory antibody indicate that combining these two different mechanisms of action to inhibit PD-1 produces an enhanced response, despite the fact that both antibodies are directed against the same target. The data illustrated in FIG. 13 are presented in Table 12 below.

[00207] Table 12. Increase in IFN- γ induction by combination of anti-PD-1 antibodies

	Fold Induction IFN- γ	Standard Deviation
Nivolumab	1.00	0.12
244C8-2	1.77	0.19
388D4-2 + 244C8-2	2.11	0.21

[00208] FIG. 14 shows results from an experiment similar to the experiment yielding the results presented in FIG. 12, except that different humanized forms of antibodies 388D4 and

244C8 were tested, and nivolumab was tested in combination with 244C8. In each treatment, a population of 3×10^5 cells, which included 9% lymphocytes (sub-optimally activated as described above) was incubated for 24 hours with anti-PD-1 antibodies at a total concentration of 20 $\mu\text{g/mL}$. Following PD-1 blockade, cells and supernatants were collected for FACS analysis, analysis by microengraving in a microwell array device (Varadarajan, supra), or ELISA detection of cytokines. FIG. 14 shows IFN γ secretion, as measured by ELISA, with data expressed in terms of fold-induction of IFN γ secretion, relative to treatment with the isotype control antibody. The data used to create FIG. 14 are shown in Table 13 below.

[00209] Table 13. Increase in IFN- γ induction by combination of anti-PD-1 antibodies

	Fold Induction IFN- γ	Standard Deviation
anti-CD3+anti-CD28 only	1.00	0.25
Nivolumab (10 $\mu\text{g/mL}$)	1.89	0.07
388D4-2 (5 $\mu\text{g/mL}$)	2.06	0.12
388D4-2 (10 $\mu\text{g/mL}$)	1.82	0.38
244C8-2 (5 $\mu\text{g/mL}$)	2.66	0.49
244C8-2 (10 $\mu\text{g/mL}$)	2.81	0.42
388D4-2 (5 $\mu\text{g/mL}$) +244C8-2 (5 $\mu\text{g/mL}$)	2.85	0.55
Nivolumab (5 $\mu\text{g/mL}$) +244C8-2 (5 $\mu\text{g/mL}$)	3.76	0.08

[00210] The data summarized in FIG. 14 show significantly increased response to treatment of TILS from NSCLC biopsies with the combination of nivolumab, which is a competitive inhibitory anti-PD-1 antibody, and 244C8-2, which is a non-competitive inhibitory anti-PD-1 antibody. Individual antibody treatment with each of two different anti-PD-1 antibodies that compete with PD-L1 for binding to PD-L1, *i.e.*, nivolumab and 388D4-2, increased T cell effector function approximately 2-fold. Individual antibody treatment with an anti-PD-1 antibody that does not compete with PD-L1 for binding to PD-L1, *i.e.*, 244C8-2, increased T cell effector function between 2.5-fold and 3-fold. Treatment with nivolumab plus 244C8-2 increased T cell effector function between 3.5-fold and 4-fold.

[00211] When experiments such as these are performed on human patient tumor biopsy samples, the magnitude of increase in T cell effector function observed in response to the

same antibody treatment can vary from experiment to experiment as a function of patient-to-patient variation. In spite of such patient-to-patient variation, these results indicate that the addition of a non-competitive inhibitory anti-PD-1 antibody treatment to a competitive inhibitory anti-PD-1 antibody treatment can yield a greater increase in effector function of TILs, as compared to treatment with the competitive inhibitory anti-PD-1 antibody alone.

[00212] F. Antibody 244C8 and Cytokine Secretion in MLR Assays

[00213] Increased secretion of various cytokines was observed in response to antibody 244C8 in mixed lymphocyte reaction (MLR) assays. FIGS. 15A-15F summarize the results of an MLR assay performed on human PBMCs treated with anti-PD-1 antibodies. The MLR assay was performed using commercially available monocyte-derived dendritic cells as stimulator cells and purified CD4⁺ T lymphocytes as responder cells from a different healthy blood donor. Supernatants were collected at 2.5 days after beginning the assay. Cytokine secretion was measured in a multiplex capture sandwich immunoassay using MagPlex® microspheres (Luminex, Austin, TX) and ProCartaPlex® Human TH1/TH2 Chemokine Panel (Luminex), according to the vendor's instructions. Fluorescence of the various labels was detected using a MagPix® fluorescence detection system (Luminex). The data in FIGS. 15A-15F indicate that treatment with antibody 244C8-1 resulted in increased secretion of cytokines IL-6, IL-12, IL-18, TNF- α , GM-CSF, and IL-1 β , in comparison with antibody 388D4-2 or the IgG4 isotype control (Biolegend). Similar results (data not shown) were observed with cells from two other blood donors, at two time points.

[00214] G. Antibody 244C8 and Cytokine Secretion by TILs

[00215] Increased secretion of various cytokines by TILs was observed in response to antibody 244C8. FIGS. 16A-16F show alteration of tumor infiltrating lymphocyte (TIL) function by PD-1 blockade with antibodies 388D4-2, and 244C8-2. In this experiment, a population of 3×10^5 dissociated and suspended human cells from a non-small cell lung cancer (NSCLC) biopsy, which included 7% lymphocytes that had been activated as described in Example 5C (above) was incubated for 24 hours with an anti-PD-1 antibody or IgG4 isotype control, at a concentration of 10 μ g/mL. Cytokine secretion was measured in a multiplex capture sandwich immunoassay using MagPlex® microspheres (Luminex) and ProCartaPlex® Human TH1/TH2 Chemokine Panel (Luminex), according to the vendor's instructions. Fluorescence of the various labels was detected using a MagPix® fluorescence detection system (Luminex). These data indicate that treatment with antibody 244C8-2

resulted in increased secretion of cytokines IL-6, IL-12, IL-18, TNF- α , GM-CSF, and IL-1 β , in comparison with antibody 388D4-2 or the IgG4 isotype control (Biolegend). Similar results (not shown) were obtained by employing this protocol with three other human tumor samples.

[00216] EXAMPLE 6. Animal Models

[00217] A. Patient-Derived Xenografts in Humanized Mice

[00218] Antibodies 244C8 and 388D4 displayed anti-tumor activity in patient-derived xenograft (PDX) tumor growth in humanized mice. FIG. 17A shows results from an in vivo efficacy experiment involving a human lung tumor PDX-derived from a metastatic stage IV non-small cell lung cancer (NSCLC) patient (lung tumor LG1306; Jackson Labs) implanted in mice engineered to have a human immune system (hu-CD34 NSGTM mice; Jackson Labs). The five treatment groups were: vehicle control, antibody 388D4-3, antibody 244C8-2, pembrolizumab, or a combination of antibody 244C8-2 and pembrolizumab. Mice that had been engrafted with human CD34+ cells and had >25% human CD45+ cells in the peripheral blood at twelve weeks post-engraftment were implanted subcutaneously on the right flank with tumor fragments from PDX model LG1306. Mice were randomized into five treatment groups (n=10), based on tumor volume, when volume reached 60-120 mm³. Animals received a total of six intra-peritoneal 5 mg/kg doses at five-day intervals (Q5D x 6). All doses were delivered in PBS as vehicle. In the treatment groups that received 388D4-3, 244C8-2, or pembrolizumab, the first dose of the antibody was given as a 10 mg/kg dose, followed by the additional doses at the 5 mg/kg dose. The combination treatment group (244C8-2 + pembrolizumab) received a dose each of 5 mg/kg pembrolizumab and 5 mg/kg of 244C8-2 at each dosing time point. Tumor volumes were measured twice weekly (Day 3, 6, 10, 13, 17, 20, 24 and 28) using a digital caliper to determine length and width of the tumors. Error bars represent the 95% confidence interval.

[00219] All treatment groups showed significant tumor growth inhibition compared to the vehicle control group. In this experiment, no significant difference in tumor growth inhibition was observed among treatment with antibody 388D4-3, antibody 244C8-2, pembrolizumab, or the combination of antibody 244C8-2 with pembrolizumab. As shown in FIG. 17B, at day 28 (end of study), the tumor volume for each of the treatment groups was significantly smaller than the vehicle group. The Student T-test p values between each treatment group and the vehicle group were: pembrolizumab = 0.00167; 388D4 = 0.00105;

244C8 = 0.00277; and pembrolizumab and 244C8 in combination = 0.00275. FIG. 17C shows percentage tumor volume of each treatment group relative to vehicle on day 28. The calculated percent tumor growth inhibition (%TGI) for each treatment is shown above each bar in FIG. 17C. Percent tumor growth inhibition was calculated according to the following formula:

$$\% \text{ TGI on Day X} = (1 - (T_{\text{DayX}} - T_{\text{Day-1}}) / (C_{\text{DayX}} - C_{\text{Day-1}})) * 100$$

where:

T = the average tumor volume for a treatment group; and

C = the average tumor volume for the control group.

[00220] In this experiment, it was surprisingly discovered that in the combination treatment, a combined antibody dose totaling 10mg/kg was well tolerated by the animals, for the duration of the study.

INCORPORATION BY REFERENCE

[00221] The relevant teachings of all patents, published applications, and references cited herein are incorporated by reference in their entirety.

EQUIVALENTS

[00222] While this invention has been particularly shown and described with references to examples of embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. An isolated antibody that binds to programmed cell death protein 1 (PD-1), comprising a heavy chain variable region (HCVR) having complementarity determining regions (CDRs) selected from the group consisting of: CDRs 1-3 of SEQ ID NO: 1; CDRs 1-3 of SEQ ID NO: 2; CDRs 1-3 of SEQ ID NO: 3; CDRs 1-3 of SEQ ID NO: 4; CDRs 1-3 of SEQ ID NO: 5; CDRs 1-3 of SEQ ID NO: 6; CDRs 1-3 of SEQ ID NO: 7; CDRs 1-3 of SEQ ID NO: 8; CDRs 1-3 of SEQ ID NO: 9; CDRs 1-3 of SEQ ID NO: 10; CDRs 1-3 of SEQ ID NO: 11; CDRs 1-3 of SEQ ID NO: 12; CDRs 1-3 of SEQ ID NO: 13; CDRs 1-3 of SEQ ID NO: 14; CDRs 1-3 of SEQ ID NO: 15; CDRs 1-3 of SEQ ID NO: 16; CDRs 1-3 of SEQ ID NO: 17; CDRs 1-3 of SEQ ID NO: 18; CDRs 1-3 of SEQ ID NO: 19; CDRs 1-3 of SEQ ID NO: 20; CDRs 1-3 of SEQ ID NO: 21; CDRs 1-3 of SEQ ID NO: 22; CDRs 1-3 of SEQ ID NO: 23; CDRs 1-3 of SEQ ID NO: 24; CDRs 1-3 of SEQ ID NO: 25; and CDRs 1-3 of SEQ ID NO: 26.
2. An isolated antibody that binds to PD-1, comprising a heavy chain variable region (HCVR) selected from the group consisting of SEQ ID NOs: 1-26.
3. An isolated antibody that binds to PD-1, comprising a light chain variable region (LCVR) having CDRs selected from the group consisting of: CDRs 1-3 of SEQ ID NO: 27; CDRs 1-3 of SEQ ID NO: 28; CDRs 1-3 of SEQ ID NO: 29; CDRs 1-3 of SEQ ID NO: 30; CDRs 1-3 of SEQ ID NO: 31; CDRs 1-3 of SEQ ID NO: 32; CDRs 1-3 of SEQ ID NO: 33; CDRs 1-3 of SEQ ID NO: 34; CDRs 1-3 of SEQ ID NO: 35; CDRs 1-3 of SEQ ID NO: 36; CDRs 1-3 of SEQ ID NO: 37; CDRs 1-3 of SEQ ID NO: 38; CDRs 1-3 of SEQ ID NO: 39; CDRs 1-3 of SEQ ID NO: 40; CDRs 1-3 of SEQ ID NO: 41; CDRs 1-3 of SEQ ID NO: 42; CDRs 1-3 of SEQ ID NO: 43; CDRs 1-3 of SEQ ID NO: 44; CDRs 1-3 of SEQ ID NO: 45; CDRs 1-3 of SEQ ID NO: 46; CDRs 1-3 of SEQ ID NO: 47; CDRs 1-3 of SEQ ID NO: 48; CDRs 1-3 of SEQ ID NO: 49; CDRs 1-3 of SEQ ID NO: 50; CDRs 1-3 of SEQ ID NO: 51; CDRs 1-3 of SEQ ID NO: 52; and CDRs 1-3 of SEQ ID NO: 53.

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4. An isolated antibody that binds to PD-1, comprising a LCVR selected from the group consisting of SEQ ID NOs: 27-53.
5. An isolated antibody that binds to PD-1, comprising a HCVR selected from the group consisting of SEQ ID NOs: 1-26 and a LCVR selected from the group consisting of SEQ ID NOs: 27-53.
6. The antibody according to any one of claims 1-5, wherein the antibody comprises a HCVR and LCVR pair selected from the group consisting of: a HCVR having the sequence set forth in SEQ ID NO: 4 and a LCVR having the sequence set forth in SEQ ID NO: 28 (244C7); a HCVR having the sequence set forth in SEQ ID NO: 4 and a LCVR having the sequence set forth in SEQ ID NO: 27 (244C7m1); a HCVR having the sequence set forth in SEQ ID NO: 1 and a LCVR having the sequence set forth in SEQ ID NO: 28 (244C8); a HCVR having the sequence set forth in SEQ ID NO: 1 and a LCVR having the sequence set forth in SEQ ID NO: 27 (244C8m1); a HCVR having the sequence set forth in SEQ ID NO: 3 and a LCVR having the sequence set forth in SEQ ID NO: 31 (246F7); a HCVR having the sequence set forth in SEQ ID NO: 5 and a LCVR having the sequence set forth in SEQ ID NO: 44 (258C1); a HCVR having the sequence set forth in SEQ ID NO: 2 and a LCVR having the sequence set forth in SEQ ID NO: 30 (258F6); a HCVR having the sequence set forth in SEQ ID NO: 2 and a LCVR having the sequence set forth in SEQ ID NO: 29 (258F6m); a HCVR having the sequence set forth in SEQ ID NO: 6 and a LCVR having the sequence set forth in SEQ ID NO: 34 (392C4); a HCVR having the sequence set forth in SEQ ID NO: 7 and a LCVR having the sequence set forth in SEQ ID NO: 41 (394D5); a HCVR having the sequence set forth in SEQ ID NO: 8 and a LCVR having the sequence set forth in SEQ ID NO: 35 (394G1); a HCVR having the sequence set forth in SEQ ID NO: 12 and a LCVR having the sequence set forth in SEQ ID NO: 39 (388C12A); a HCVR having the sequence set forth in SEQ ID NO: 12 and a LCVR having the sequence set forth in SEQ ID NO: 32 (388C12B); a HCVR having the sequence set forth in SEQ ID NO: 13 and a LCVR having the sequence set forth in SEQ ID NO: 39 (388C16A); a HCVR having the sequence set forth in SEQ ID NO: 13 and a LCVR having the sequence set forth in SEQ ID NO: 32 (388C16B); a HCVR having the sequence set forth in SEQ ID NO: 9

and a LCVR having the sequence set forth in SEQ ID NO: 38 (392C5A); a HCVR having the sequence set forth in SEQ ID NO: 9 and a LCVR having the sequence set forth in SEQ ID NO: 37 (392C5B); a HCVR having the sequence set forth in SEQ ID NO: 17 and a LCVR having the sequence set forth in SEQ ID NO: 40 (392D2); a HCVR having the sequence set forth in SEQ ID NO: 16 and a LCVR having the sequence set forth in SEQ ID NO: 43 (392H4); a HCVR having the sequence set forth in SEQ ID NO: 20 and a LCVR having the sequence set forth in SEQ ID NO: 53 (246A10); a HCVR having the sequence set forth in SEQ ID NO: 18 and a LCVR having the sequence set forth in SEQ ID NO: 47 (388D4); a HCVR having the sequence set forth in SEQ ID NO: 19 and a LCVR having the sequence set forth in SEQ ID NO: 48 (392A6); a HCVR having the sequence set forth in SEQ ID NO: 21 and a LCVR having the sequence set forth in SEQ ID NO: 52 (411C2); a HCVR having the sequence set forth in SEQ ID NO: 22 and a LCVR having the sequence set forth in SEQ ID NO: 51 (413D2); and a HCVR having the sequence set forth in SEQ ID NO: 25 and a LCVR having the sequence set forth in SEQ ID NO: 45 (413E1).

7. The antibody according to any one of claims 1-6, comprising a HCVR having the sequence set forth in SEQ ID NO: 20 and a LCVR having the sequence set forth in SEQ ID NO: 53 (246A10); a HCVR having the sequence set forth in SEQ ID NO: 25 and a LCVR having the sequence set forth in SEQ ID NO: 45 (413E1); a HCVR having the sequence set forth in SEQ ID NO: 22 and a LCVR having the sequence set forth in SEQ ID NO: 51 (413D2); a HCVR having the sequence set forth in SEQ ID NO: 18 and a LCVR having the sequence set forth in SEQ ID NO: 47 (388D4); a HCVR having the sequence set forth in SEQ ID NO: 1 and a LCVR having the sequence set forth in SEQ ID NO: 28 (244C8); or a HCVR having the sequence set forth in SEQ ID NO: 9 and a LCVR having the sequence set forth in SEQ ID NO: 38 (392C5A).
8. The antibody according to claim 1 or 3 comprising a humanized or human framework region.
9. An isolated antibody that binds to PD-1, comprising a heavy chain variable region (HCVR) selected from the group consisting of SEQ ID NOs: 85-90.

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10. An isolated antibody that binds to PD-1, comprising a light chain variable region (LCVR) selected from the group consisting of SEQ ID NOs: 91-96.
11. An isolated antibody that binds to PD-1, comprising a HCVR selected from the group consisting of SEQ ID NOs: 85-90 and a LCVR selected from the group consisting of SEQ ID NOs: 91-96.
12. The antibody according to any one of claims 9-11, comprising a HCVR having the sequence set forth in SEQ ID NO: 90 and a LCVR having the sequence set forth in SEQ ID NO: 94; a HCVR having the sequence set forth in SEQ ID NO: 88 and a LCVR having the sequence set forth in SEQ ID NO: 96; a HCVR having the sequence set forth in SEQ ID NO: 90 and a LCVR having the sequence set forth in SEQ ID NO: 96; a HCVR having the sequence set forth in SEQ ID NO: 85 and a LCVR having the sequence set forth in SEQ ID NO: 91; a HCVR having the sequence set forth in SEQ ID NO: 85 and a LCVR having the sequence set forth in SEQ ID NO: 93; or a HCVR having the sequence set forth in SEQ ID NO: 86 and a LCVR having the sequence set forth in SEQ ID NO: 91.
13. An isolated antibody that binds to PD-1, wherein the antibody binds to a sequence in PD-1 selected from the group consisting of SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, SEQ ID NO: 66, SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, and SEQ ID NO: 84.
14. The antibody according to any one of claims 1-13, wherein the antibody is monoclonal.
15. The antibody according to any one of claims 1-14, wherein the antibody is a PD-1 agonist.
16. The antibody according to any one of claims 1-14, wherein the antibody is a PD-1 antagonist.

17. An isolated antibody that binds to PD-1 and competitively inhibits the binding of the antibody according to any one of claims 1-16 to human PD-1.
18. The antibody of claim 17, wherein said antibody binds to a sequence in PD-1 selected from the group consisting of SEQ ID NOs: 54-84.
19. The antibody of claim 17, wherein said antibody competitively inhibits the binding of an antibody of claim 6.
20. An isolated nucleic acid comprising a nucleotide sequence encoding the HCVR of claim 1 or 9.
21. An expression vector comprising the nucleic acid of claim 20.
22. A host cell transformed with an expression vector of claim 21.
23. An isolated nucleic acid comprising a nucleotide sequence encoding the LCVR of claim 3 or 10.
24. An expression vector comprising the nucleic acid of claim 23.
25. A host cell transformed with an expression vector of claim 24.
26. A method of producing an antibody comprising a HCVR, a LCVR, or a combination thereof, the method comprising:
 - (a) growing the host cell of claim 22 or 25, under conditions such that the host cell expresses the antibody comprising the HCVR, the LCVR, or a combination thereof; and
 - (b) isolating the antibody comprising the HCVR, the LCVR, or combination thereof.
27. A method of treating a PD-1-mediated disease or disorder in a mammal, comprising administering an effective amount of the antibody according to any one of claims 1-19 to a mammal in need thereof.
28. The method of claim 27, wherein the mammal has an immune disorder or cancer.

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29. A method of diagnosing a PD-1-mediated adaptive immune resistance in a patient who has cancer, comprising:
- contacting a tumor microenvironment in the patient with the antibody according to any one of claims 1-19 labeled with a detectable moiety; and detecting expression of PD-1 on CD8⁺ T cells within the tumor microenvironment.
30. The method of claim 29, further comprising detecting expression of PD-L1 in the tumor microenvironment.
31. A method for increasing T cell effector function, comprising contacting a T cell with a combination of: (a) an effective amount of an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell; and (b) an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.
32. The method of claim 31, wherein the T cell is contacted with the combination *in vivo*.
33. The method of claim 32, wherein the T cell is contacted with the combination in a human patient in need of increased T cell effector function.
34. A method of treating cancer in a mammal, comprising contacting a T cell in a mammal in need thereof with a combination of: (a) an effective amount of an anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell; and (b) an effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.
35. The method of claim 34, wherein the cancer is selected from the group consisting of: melanoma, renal cancer, prostate cancer, pancreatic adenocarcinoma, breast cancer, colon cancer, lung cancer, esophageal cancer, squamous cell carcinoma of the head and neck, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, and lymphoma.
36. The method of claim 31 or 34, wherein the anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell is

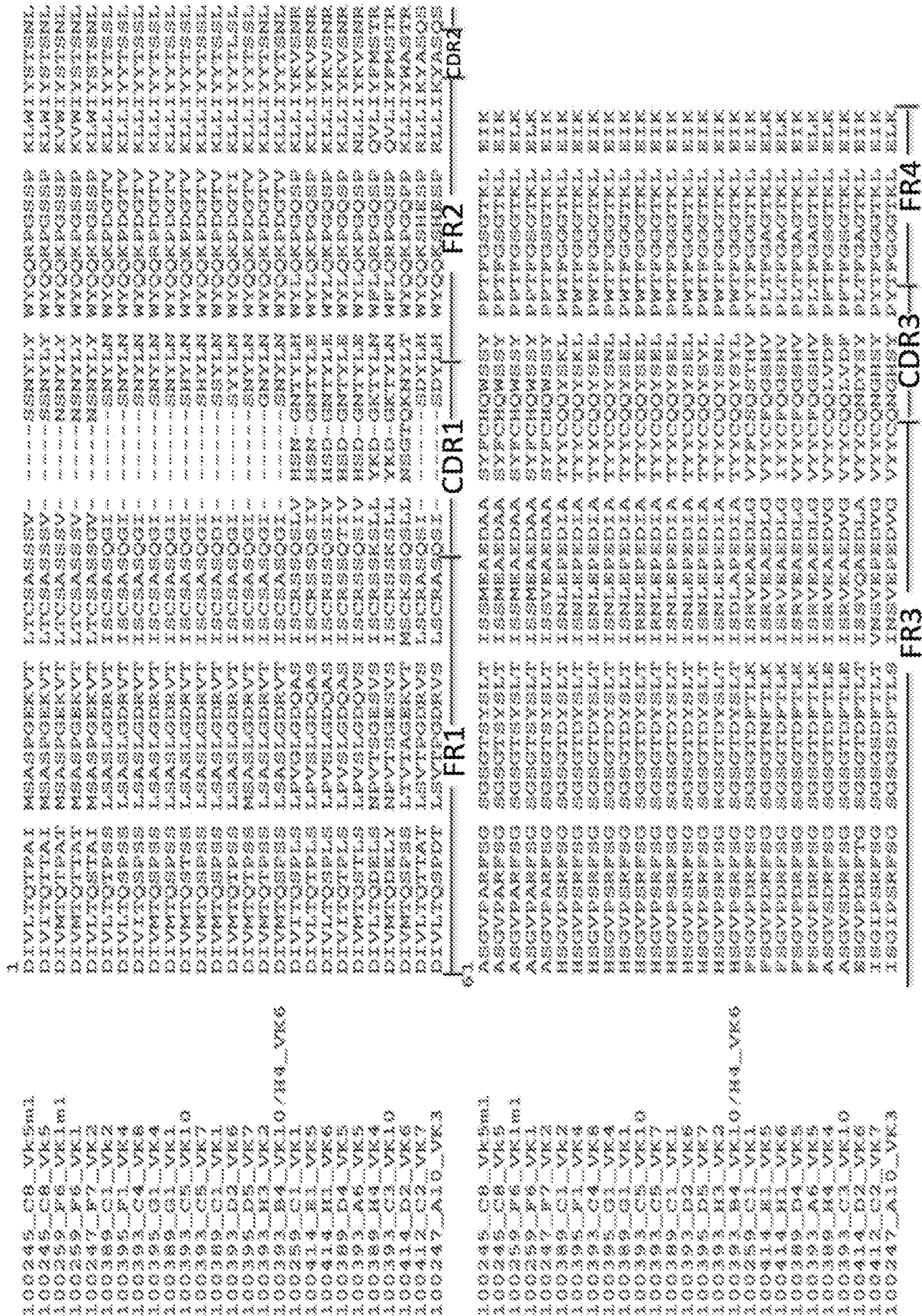
selected from the group consisting of: 388D4, nivolumab, pembrolizumab, EH12.2H7 and J105.

37. The method of claim 36, wherein the anti-PD-1 antibody that competitively inhibits binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell is 388D4.
38. The method of claim 1 or 4, wherein the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell is 244C8.
39. The method of claim 31 or 34, wherein the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell binds to one or more of the following amino acid sequences: SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 83 and SEQ ID NO: 84.
40. The method of claim 39, wherein the anti-PD-1 antibody binds to all of the following amino acid sequences: SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 83 and SEQ ID NO: 84.
41. The method of claim 31 or 34, wherein the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell binds to a PD-1 epitope bound by 244C8.
42. The method of claim 31 or 34, wherein the anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell competes with 244C8 for binding to PD-1.
43. A method for increasing T cell effector function, comprising contacting a T cell with an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of the T cell.
44. A method for increasing lymphocyte secretion of a cytokine selected from the group consisting of IL-6, IL-12, IL-18, TNF- α , IL-1 β and GM-CSF in a human patient in need of increased T cell effector function, comprising administering to the patient a therapeutically effective amount of an anti-PD-1 antibody that does not competitively inhibit binding of PD-L1 or PD-L2 to PD-1 expressed on the surface of a T cell.

45. The method of claim 43 or 44, wherein the antibody comprises a heavy chain variable region having complementarity determining regions (CDRs) selected from the group consisting of CDRs 1-3 of SEQ ID NO:85, CDRs 1-3 of SEQ ID NO:86, and CDRs 1-3 of SEQ ID NO:87; and a light chain variable region having CDRs selected from the group consisting of CDRs 1-3 of SEQ ID NO:91, CDRs 1-3 of SEQ ID NO:92, and CDRs 1-3 of SEQ ID NO:93.
46. The method of claim 45, wherein the antibody comprises a heavy chain variable region selected from the group consisting of SEQ ID NOS: 85, 86 and 87 and a light chain variable region selected from the group consisting of SEQ ID NOS: 91, 92 and 93.
47. The method of claim 45 or 46, wherein the antibody is selected from the group consisting of antibody 244C8-1, antibody 244C8-2 and antibody 244C8-3.

1	100244	C8	VH3	EVQLOESSGP	LVRPGASVKM	SCRASGYTFT	--SYWHHWVYKQ	RPGQGLEWIG	MIDPNSSETS	LNQKFKDKAT
	100258	F6	VH4	EVQLOESSGP	LVRPGASVKM	SCRASGYTFT	--SYWHHWVYKQ	RPGQGLEWIG	MIDPNSSETS	LNQKFKDKAT
	100246	F7	VH3	EVQLOESSGP	LVRPGASVKM	SCRASGYTFT	--SYWHHWVYKQ	RPGQGLEWIG	MIDPNSSETS	LNQKFKDKAT
	100244	C7	VH10	EVQLOESSGP	LVRPGASVKM	SCRASGYTFT	--SYWHHWVYKQ	RPGQGLEWIG	MIDPNSSETS	LNQKFKDKAT
	100258	C1	VH4	EVQLOESSGP	LVRPGASVKM	SCRASGYTFT	--SYWHHWVYKQ	RPGQGLEWIG	MIDPNSSETS	LNQKFKDKAT
	100394	G1	VH1	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100392	C4	VH5	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100394	D5	VH9	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100392	C5	VH6	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100392	H3	VH6	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100394	F1	VH8	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100388	C1	VH2	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100388	C1	VH6	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100388	G1	VH3	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100392	H4	VH10	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100392	D2	VH7	EVQLOESSGP	LVRPGASVKI	PCRASGYTFT	--DYNMDWVYKQ	NHGKSLRWIG	DIDPNSGTTI	YHQKFKDKAT
	100388	D4	VH3	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100392	A6	VH8	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100411	C2	VH3	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100413	D2	VH9	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100388	H4	VH1	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100392	C3	VH7	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100413	E1	VH9	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100413	H1	VH3	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100244	C8	VH3	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100258	F6	VH4	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100246	F7	VH3	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100244	C7	VH10	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100258	C1	VH4	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100394	G1	VH1	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100392	C4	VH5	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100394	D5	VH9	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100392	C5	VH6	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100392	H3	VH6	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100394	F1	VH8	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100388	C1	VH2	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100388	C1	VH6	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNVYKQ	TPVHGLRWIG	VIDPNTGCTA	YHQKFKDKAT
	100388	C1	VH3	EVQLOESSGP	LVRPGASVTL	SCRASGYTFT	--DYEIHNV			

FIG. 1



1.	100244	C8_VH3_C8_VK5m1
2.	100244	C8_VH3_C8_VK5
3.	100258	F6_VH4_F6_VK1m1
4.	100258	F6_VH4_F6_VK1
5.	100246	F7_VH3_F7_VK2
6.	100244	C7_VH10_C8_VK5m1
7.	100244	C7_VH10_C8_VK5
8.	100258	C1_VH4_C1_VK1
9.	100392	C4_VH5_C4_VK8
10.	100394	D5_VH9_D5_VK7
11.	100394	G1_VH1_G1_VK4
12.	100392	C5_VH8_C5_VK10
13.	100392	C5_VH6_C5_VK7
14.	100392	H3_VH8_H3_VK2
15.	100394	F1_VH8_F1_VK4
16.	100388	C1_VH2_C1_VK1
17.	100388	C1_VH2_C1_VK2
18.	100388	C1_VH6_C1_VK1
19.	100388	C1_VH6_C1_VK2
20.	100388	G1_VH5_G1_VK1
21.	100392	B4_VH10_B4_VK10
22.	100392	H4_VH9_H4_VK6
23.	100392	D2_VH7_D2_VK6
24.	100388	D4_VH3_D4_VK5
25.	100392	A6_VH8_A6_VK5
26.	100246	A10_VH1_A10_VK3
27.	100411	C2_VH3_C2_VK7
28.	100413	D2_VH9_D2_VK6
29.	100388	H4_VH1_H4_VK4
30.	100392	C3_VH7_C3_VK10
31.	100413	E1_VH9_E1_VK5
32.	100413	H1_VH3_H1_VK6

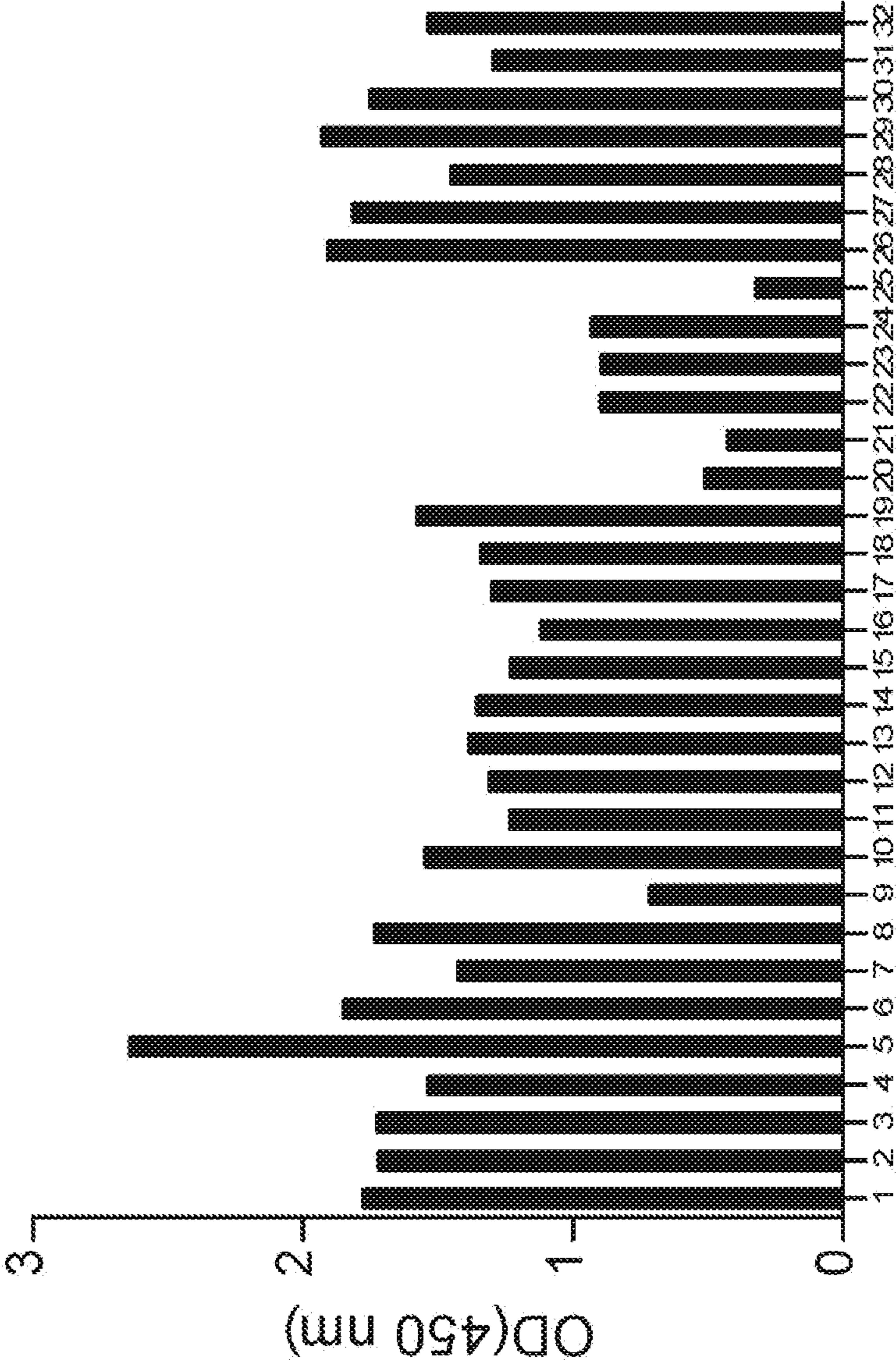


FIG. 3

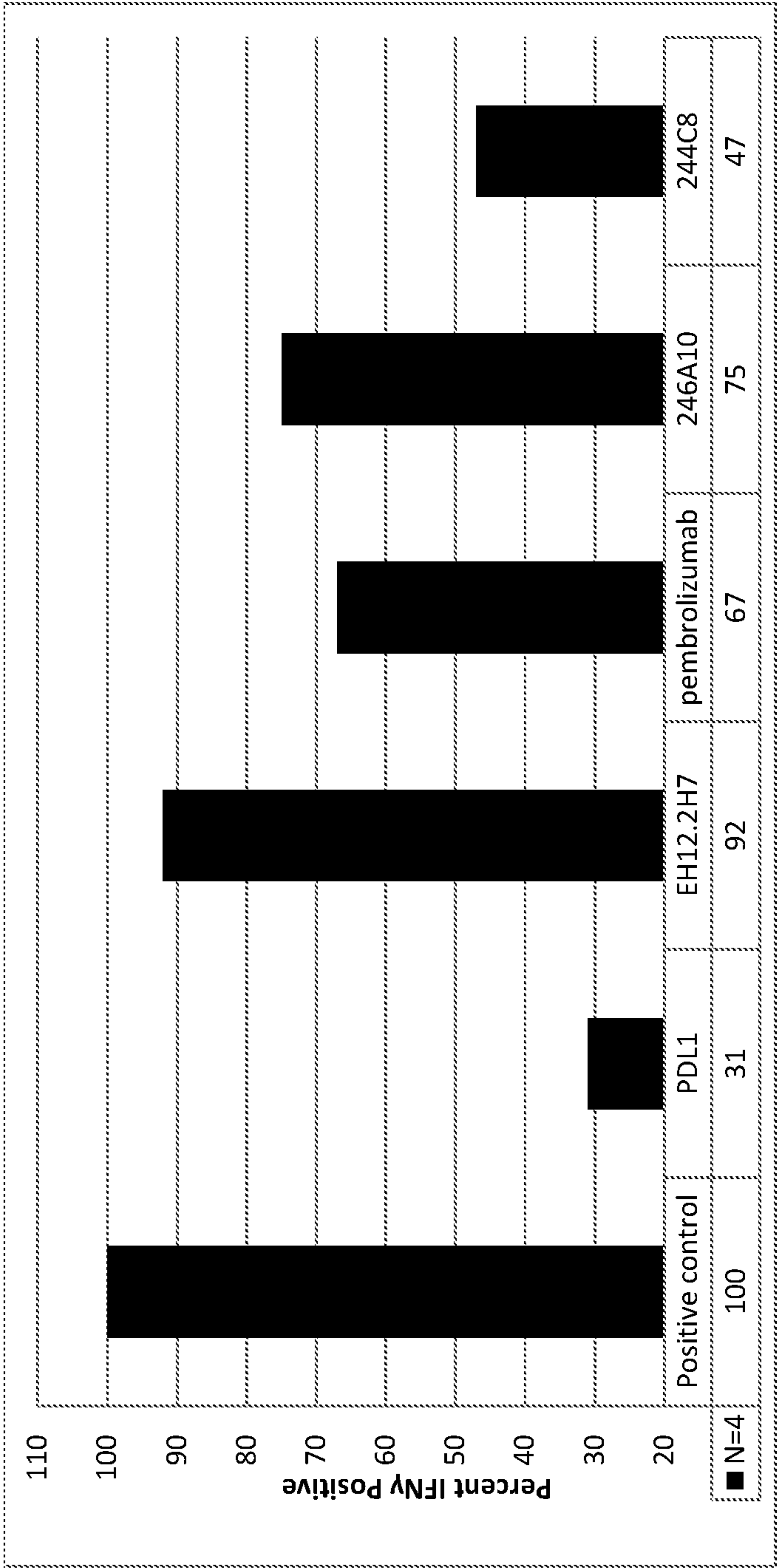


FIG. 4

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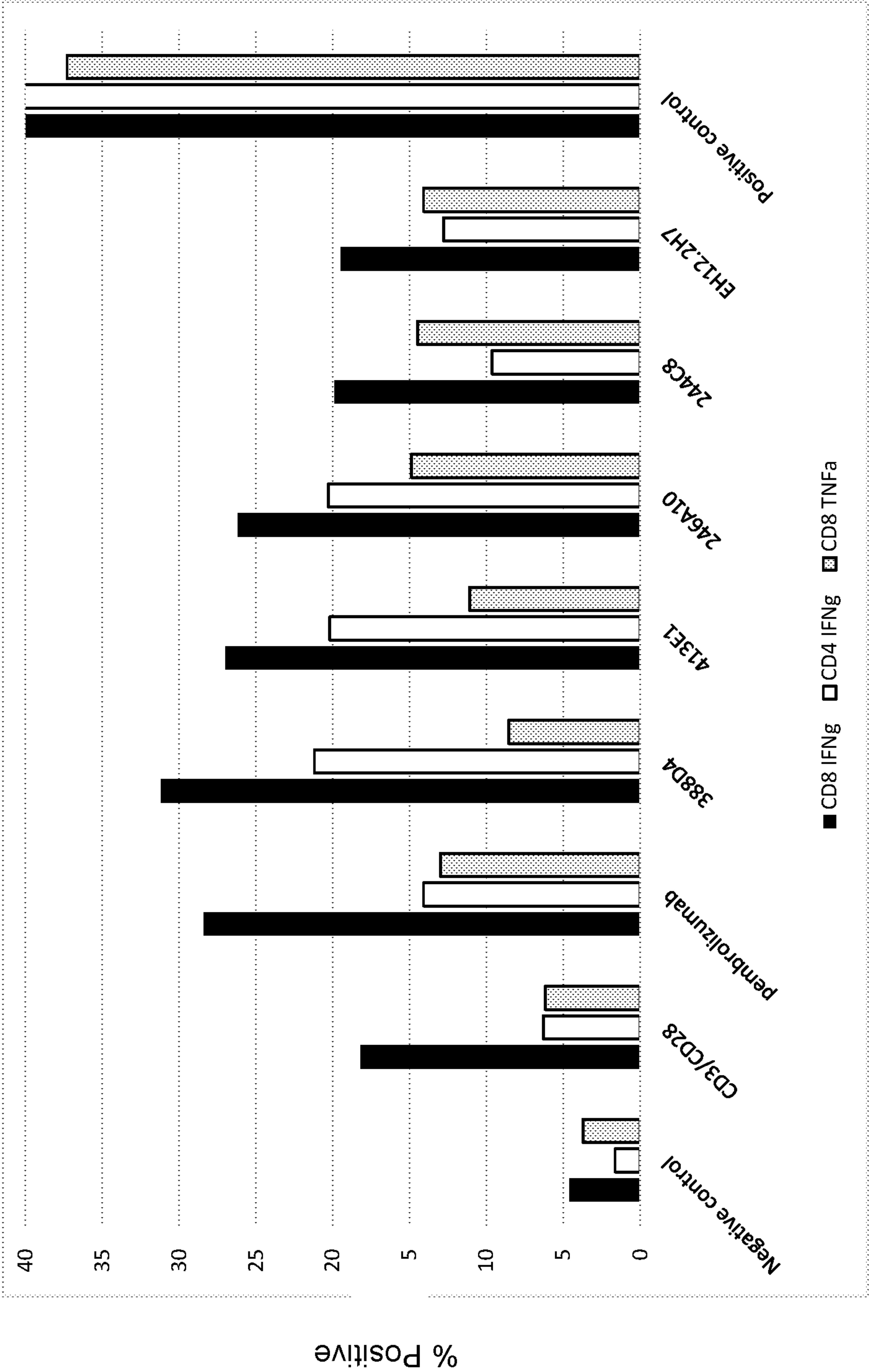


FIG. 5

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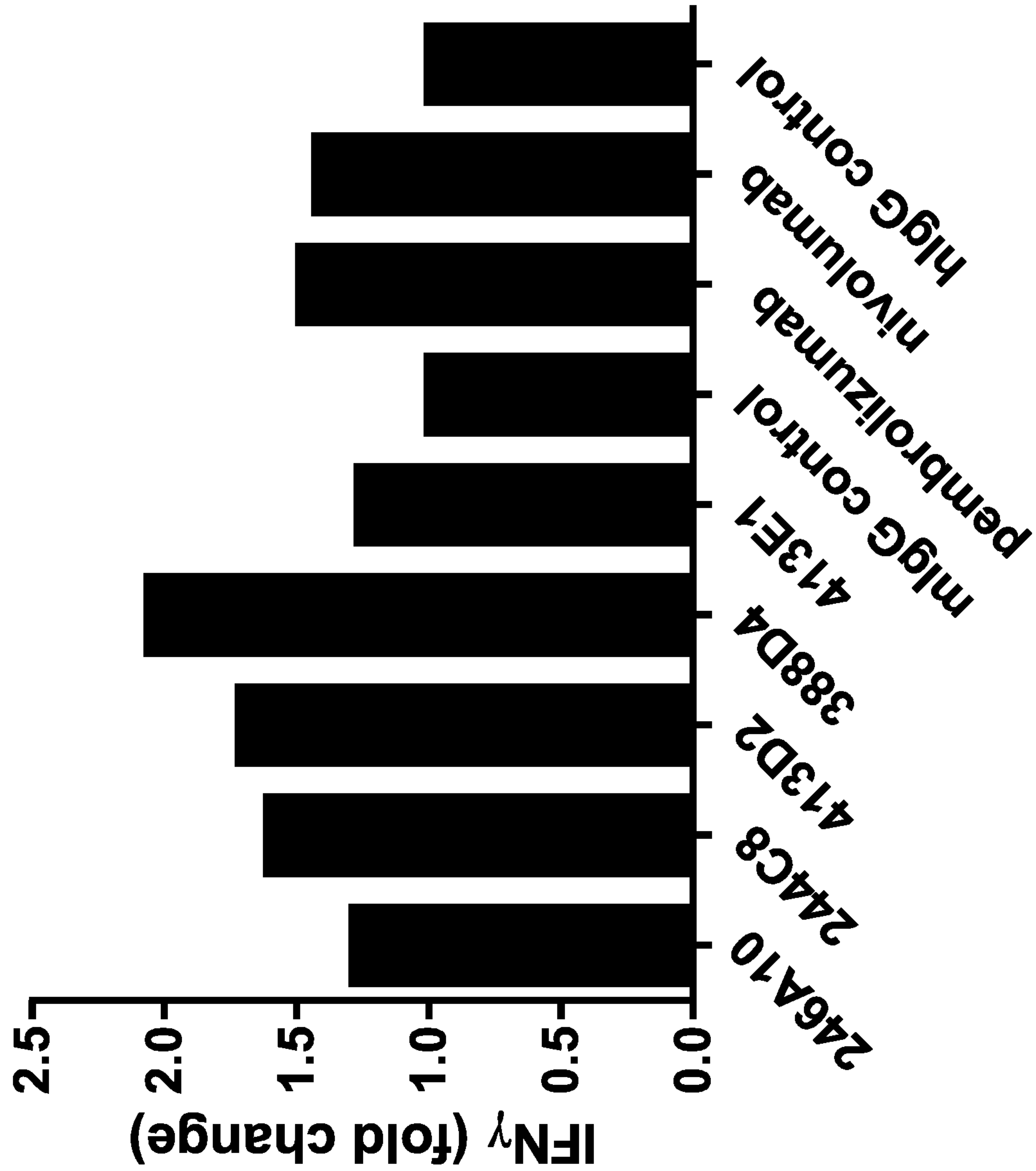


FIG. 6

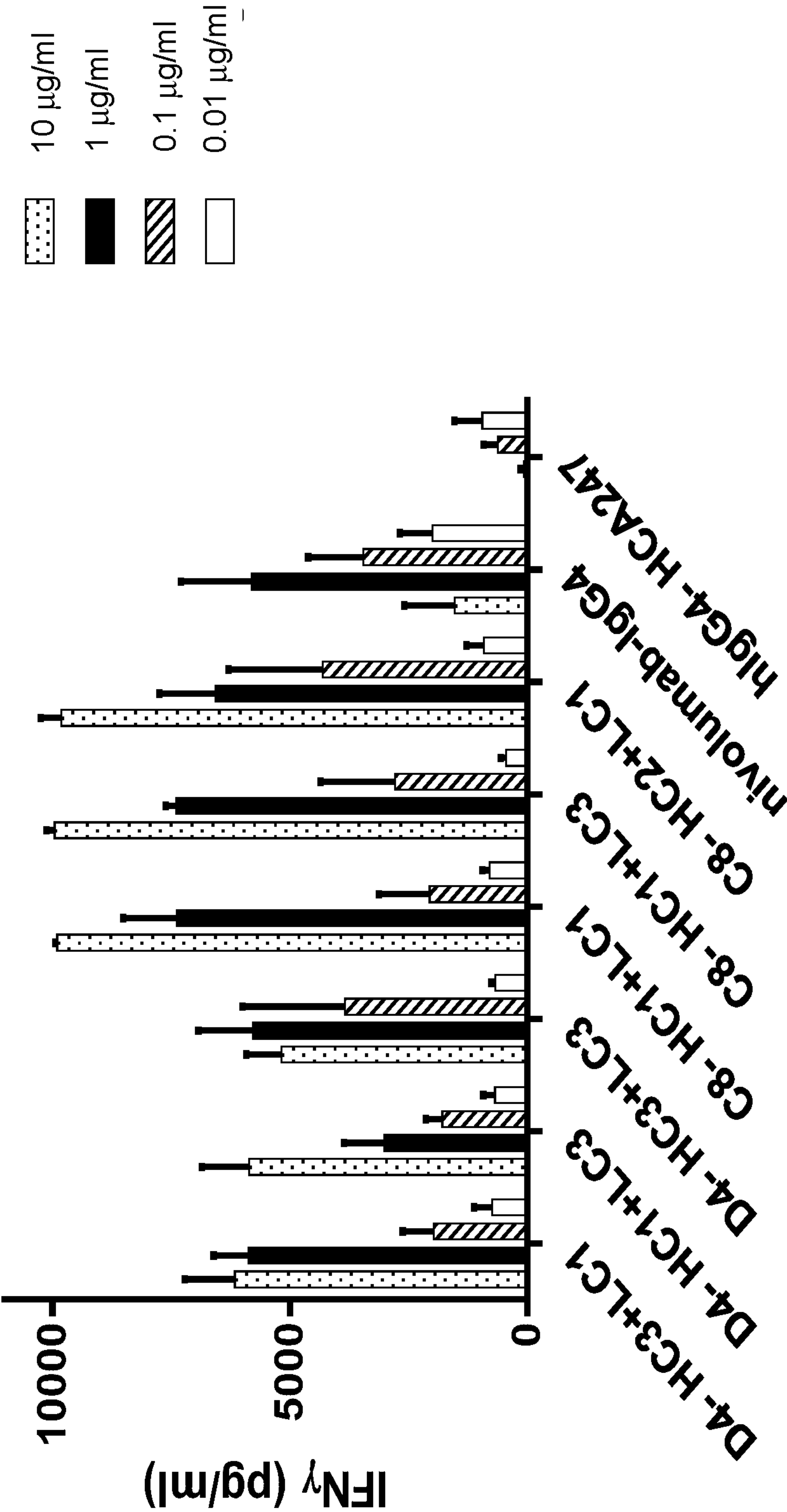


FIG. 7A

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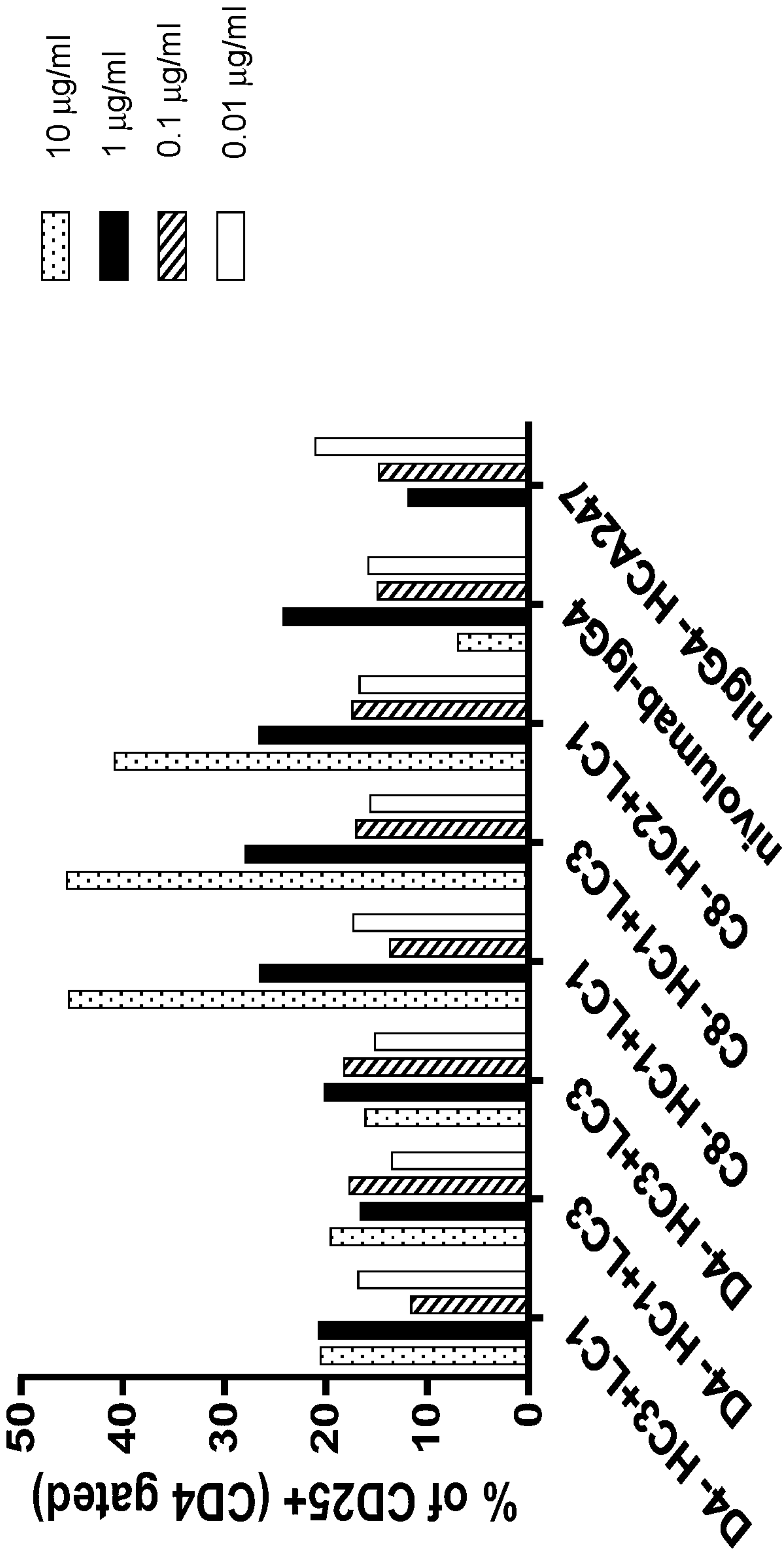


FIG. 7B

246A10
PDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSEFVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHM
SVVRARRNDSGTYLCCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPPRPAGGFQT (SEQ ID NO: 97)

244C8
PDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSEFVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHM
SVVRARRNDSGTYLCCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPPRPAGGFQT (SEQ ID NO: 97)

388D4
PDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSEFVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDF
HMSVVRARRNDSGTYLCCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPPRPAGGFQT (SEQ ID NO: 97)

413D2
PDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSEFVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDF
HMSVVRARRNDSGTYLCCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPPRPAGGFQT (SEQ ID NO: 97)

413E1
PDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSEFVLNWYRMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHM
SVVRARRNDSGTYLCCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPSPPRPAGGFQT (SEQ ID NO: 97)

FIG. 8

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FIG. 9A

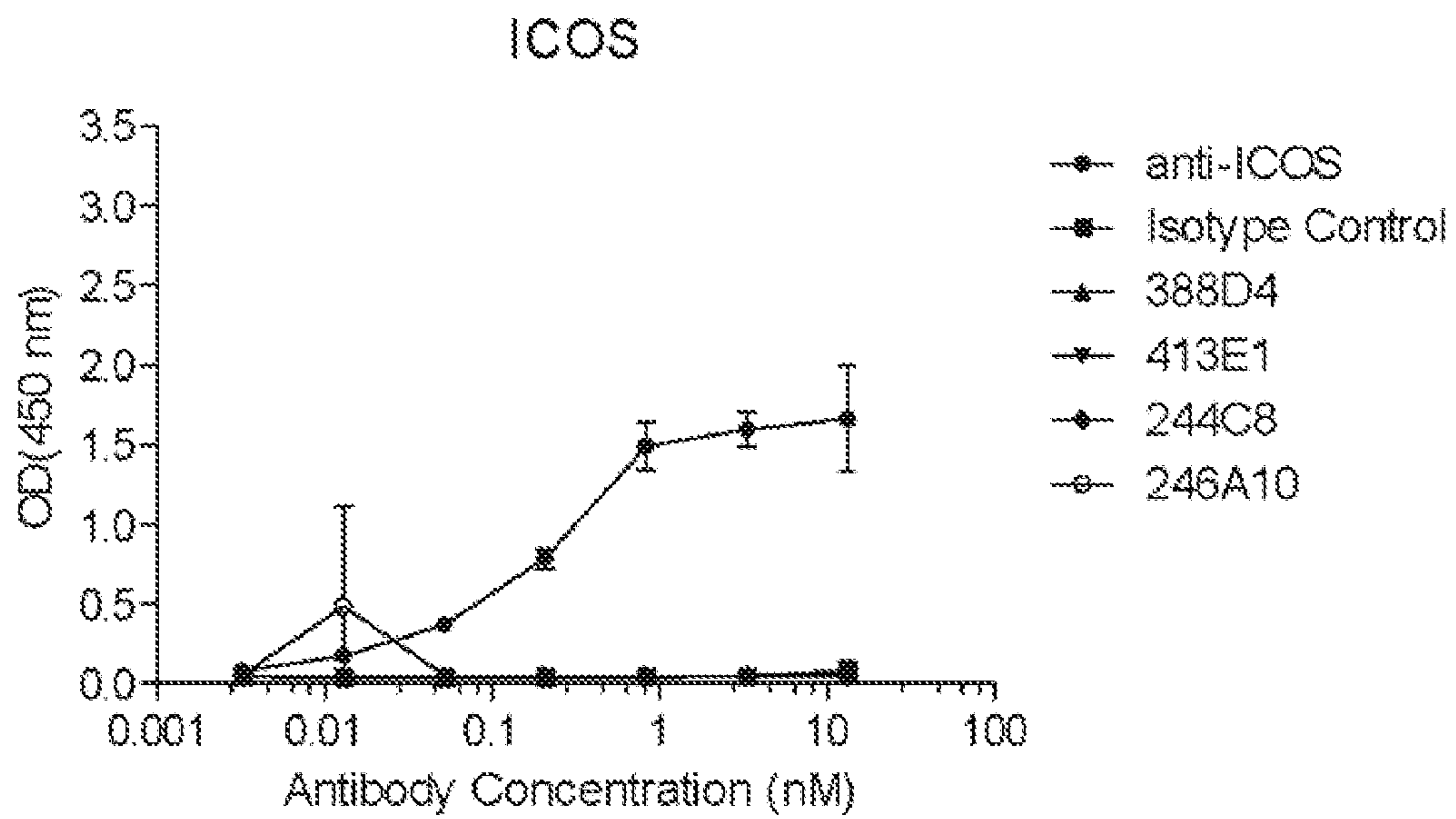
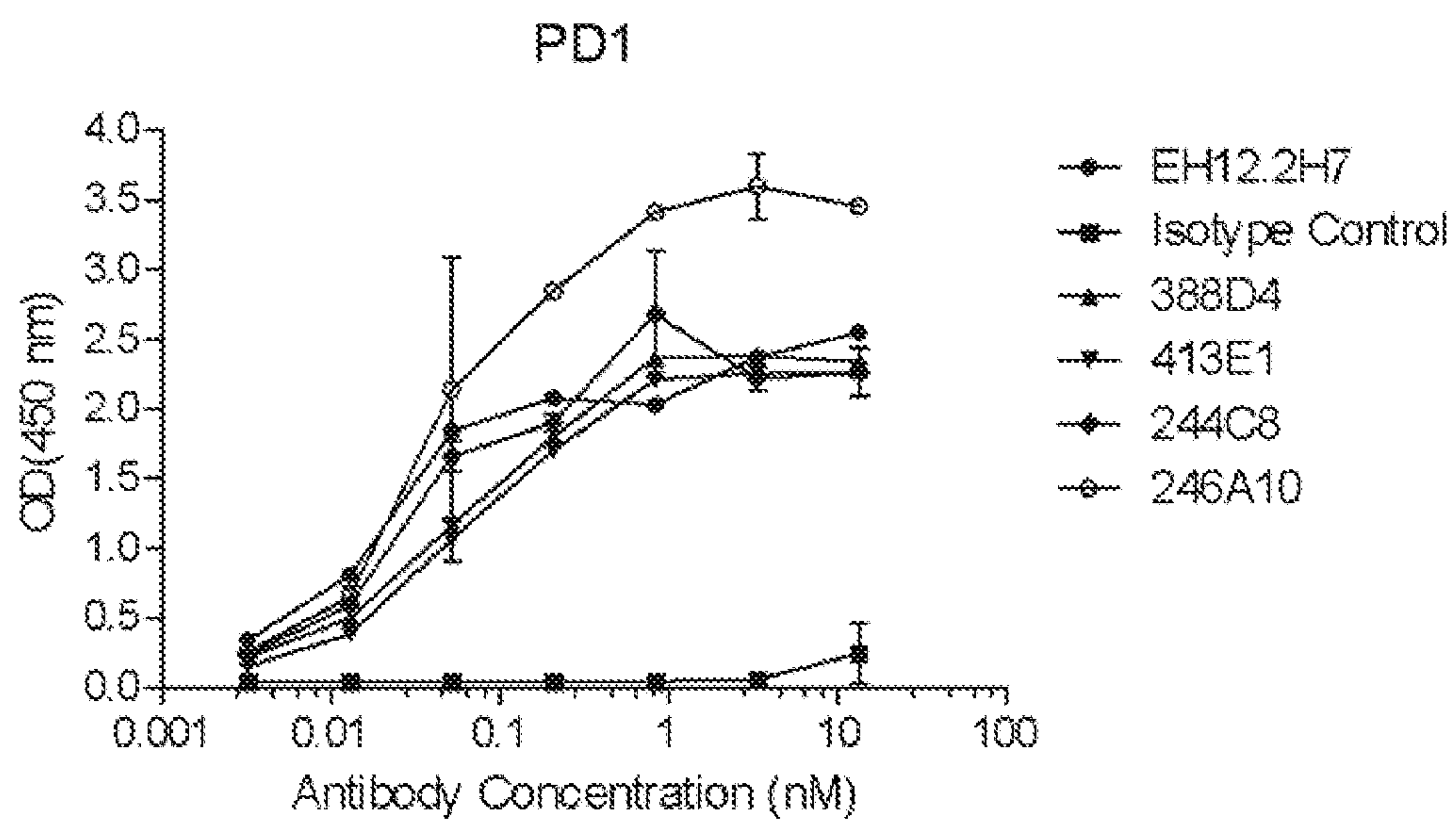


FIG. 9B



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FIG. 9C

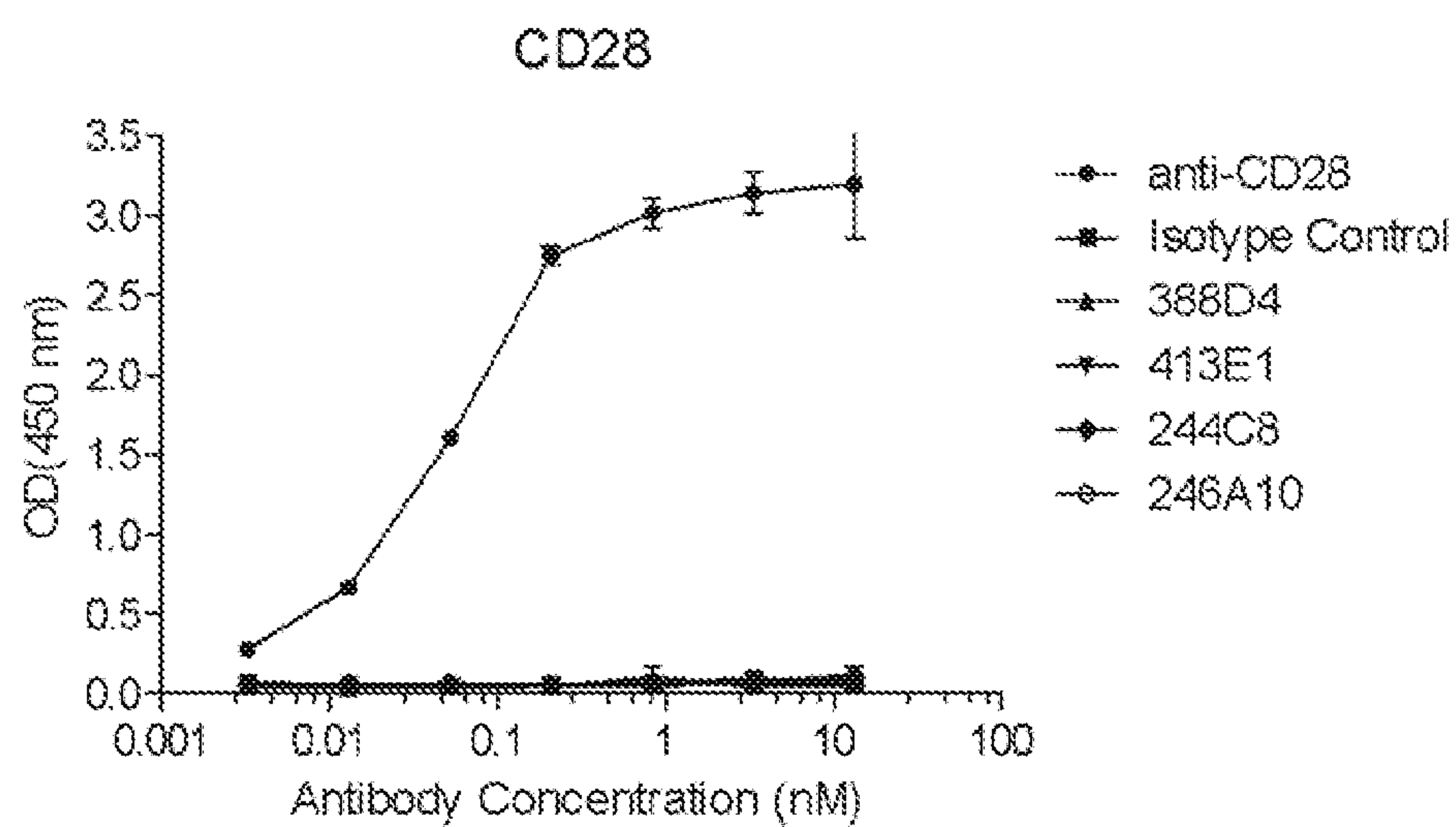
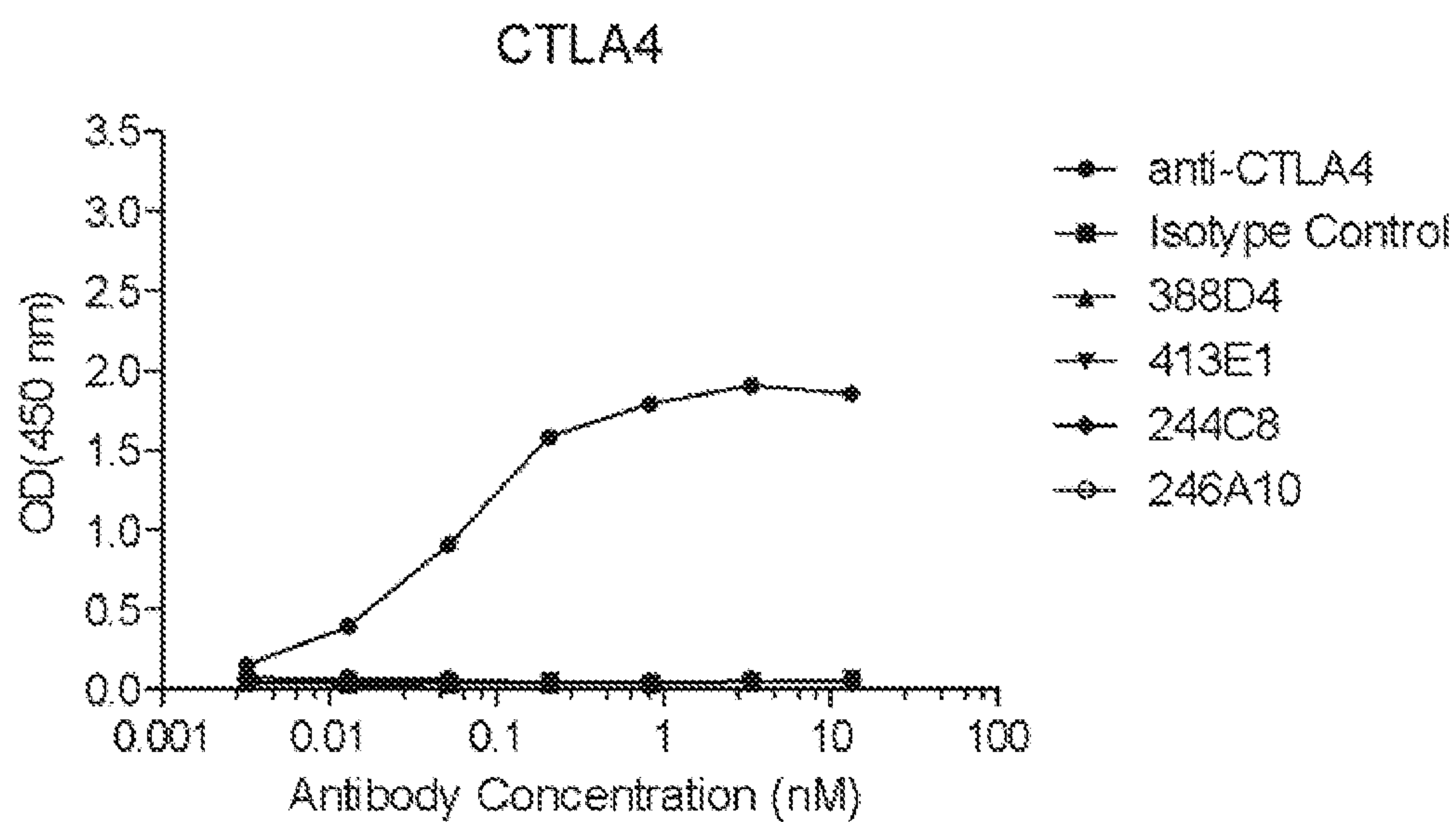


FIG. 9D



Heavy Chain

	10	20	30	40	50	60
100388_D4_VH3		EVQLQESGAELVRPGASVTLSCKASGYTFTDYEMHWVKQTPVHGLEWIGVIDPGTGGTAY				
100388_D4_HC1	...VQ...VKK...KV.....R.A.GQ...M.I.....					
100388_D4_HC2	...VQ...VKK...KV.....R.A.GQ...M.I.....					
100388_D4_HC3	...VQ...VKK...KV.....R.A.GQR...M.....					

100388_D4_VH3
100388_D4_HC1
100388_D4_HC2
100388_D4_HC3

. . . | . . . | . . . | . . . | . . . | . . . | . . . | . . . | . . . |
70 80 90 100 110 120
NQKFVKALLTADKSSNTAYMELRSLTSEDSAVYYCTSEKFGSNYYFDYWGGTTLLTVSS

.. QGRVTM .. TS V .. S R T LV ...

.. QGRVTM .. T V .. S R T LV ...

.. QGRVTI .. AS .. S R T LV ...

Light chain

	10	20	30	40	50	60
100389_D4_VK5		DIVITQTPLSLPVSGLDQASISCRSSQTI VHSDGNTYLEWYLQKPGQSPKLLIYKVSNRF				
100389_D4_LC1	V.M.S T.QP Q.R R					
100389_D4_LC2	. . M.S T.QP Q.R					
100389_D4_LC3	. . M . . . S.T.QP Q.R P.R					

	70	80	90	100	110
100389_D4_VK5		S G V P D R F S G S G T D F T L K I S R V E A E D L G V Y Y C F Q G S H V P L T F G A G T K L E I K			
100389_D4_LC1	.	.	V	.	Q
100389_D4_LC2	.	.	V	.	Q
100389_D4_LC3	A.	.	V	.	Q

FIG. 10A

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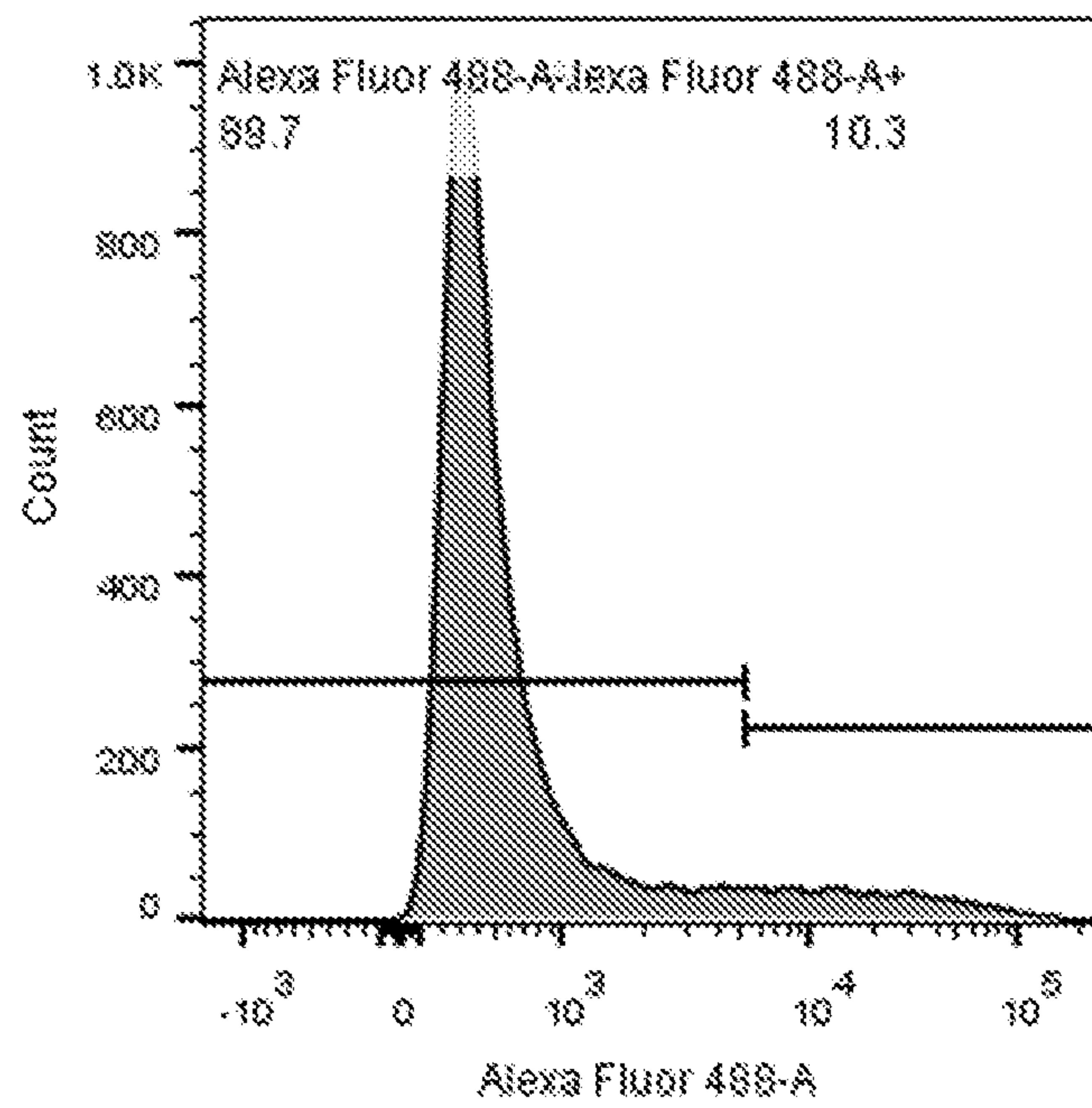


FIG. 11A

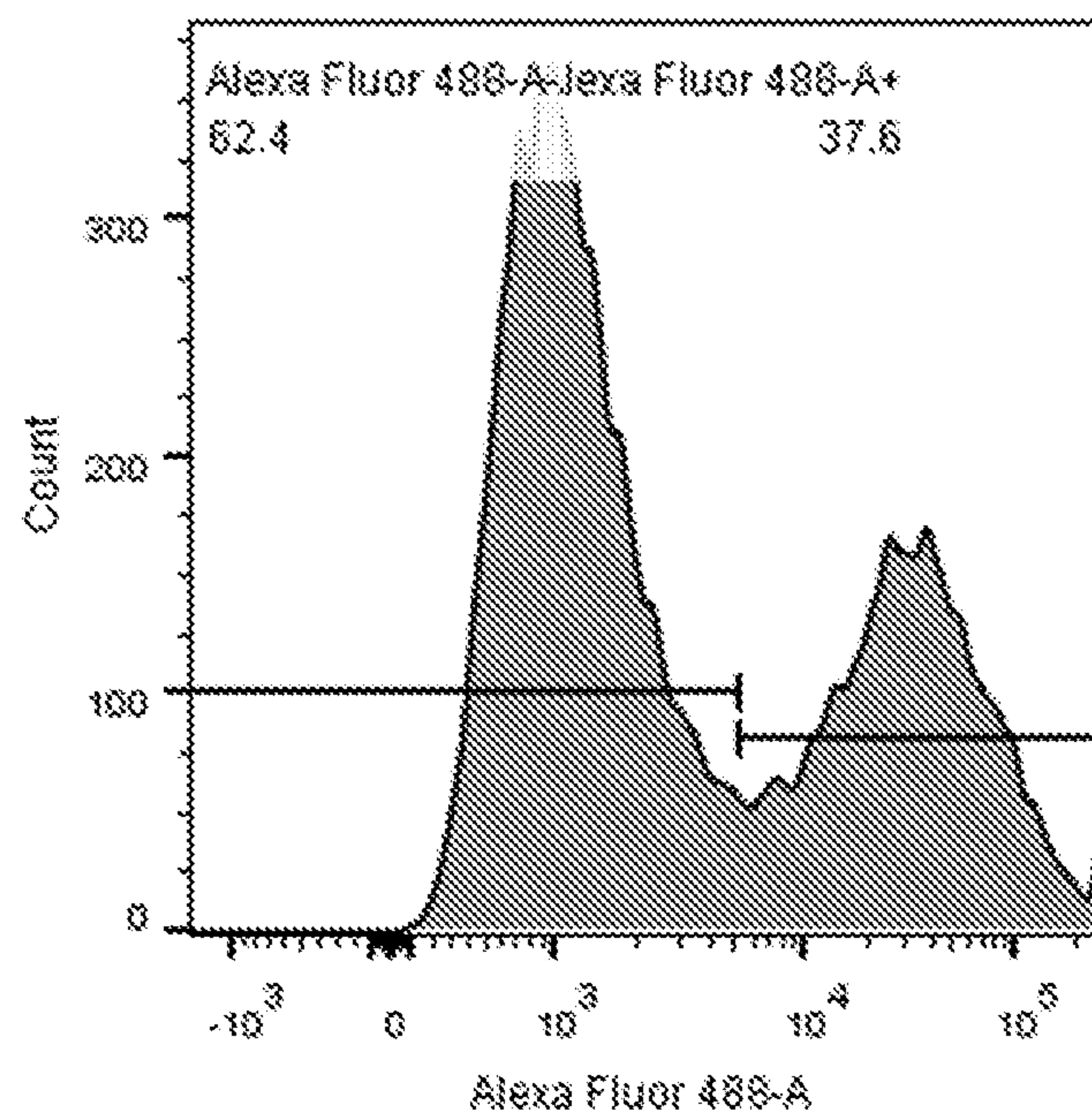


FIG. 11B

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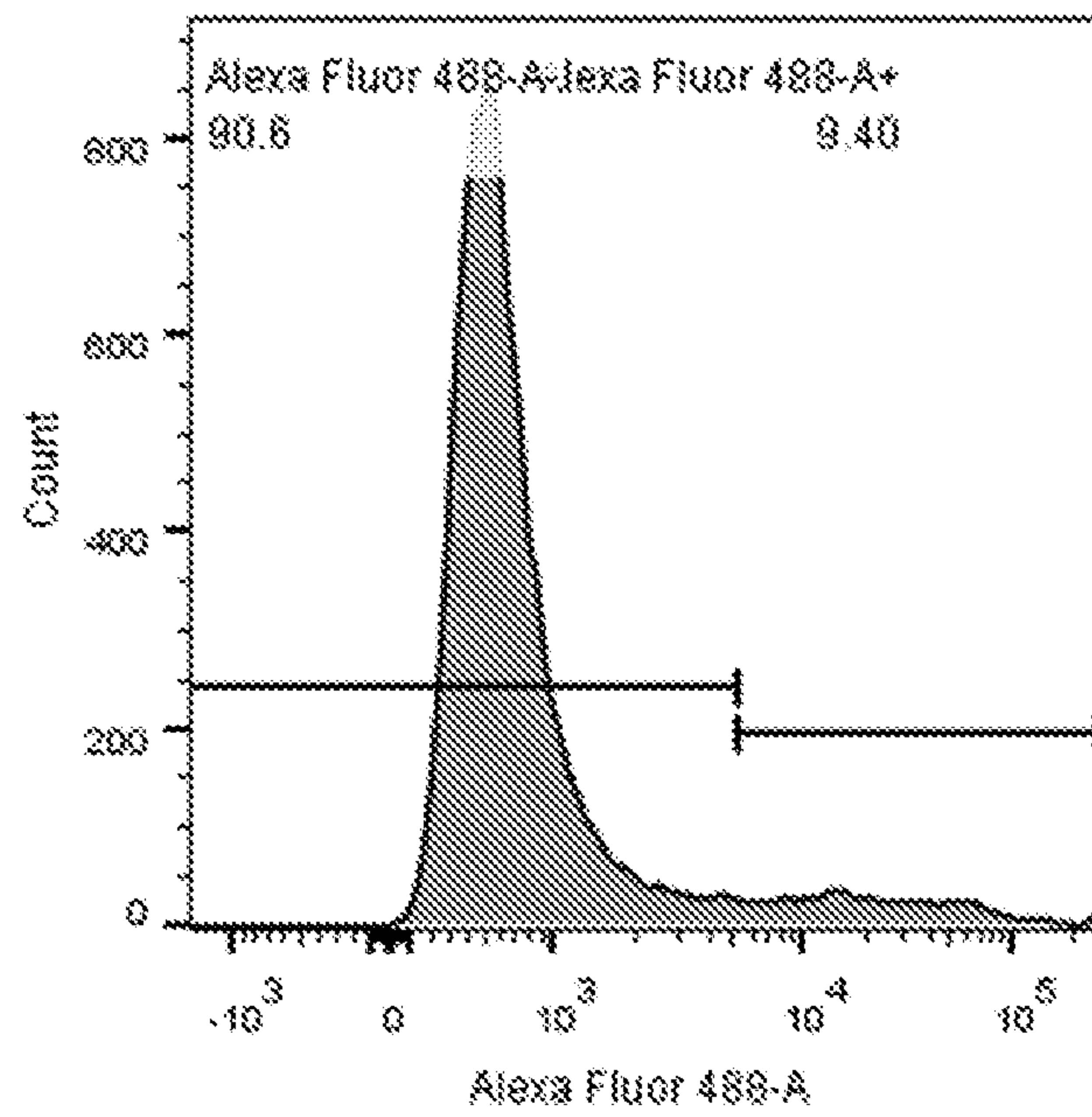


FIG. 11C

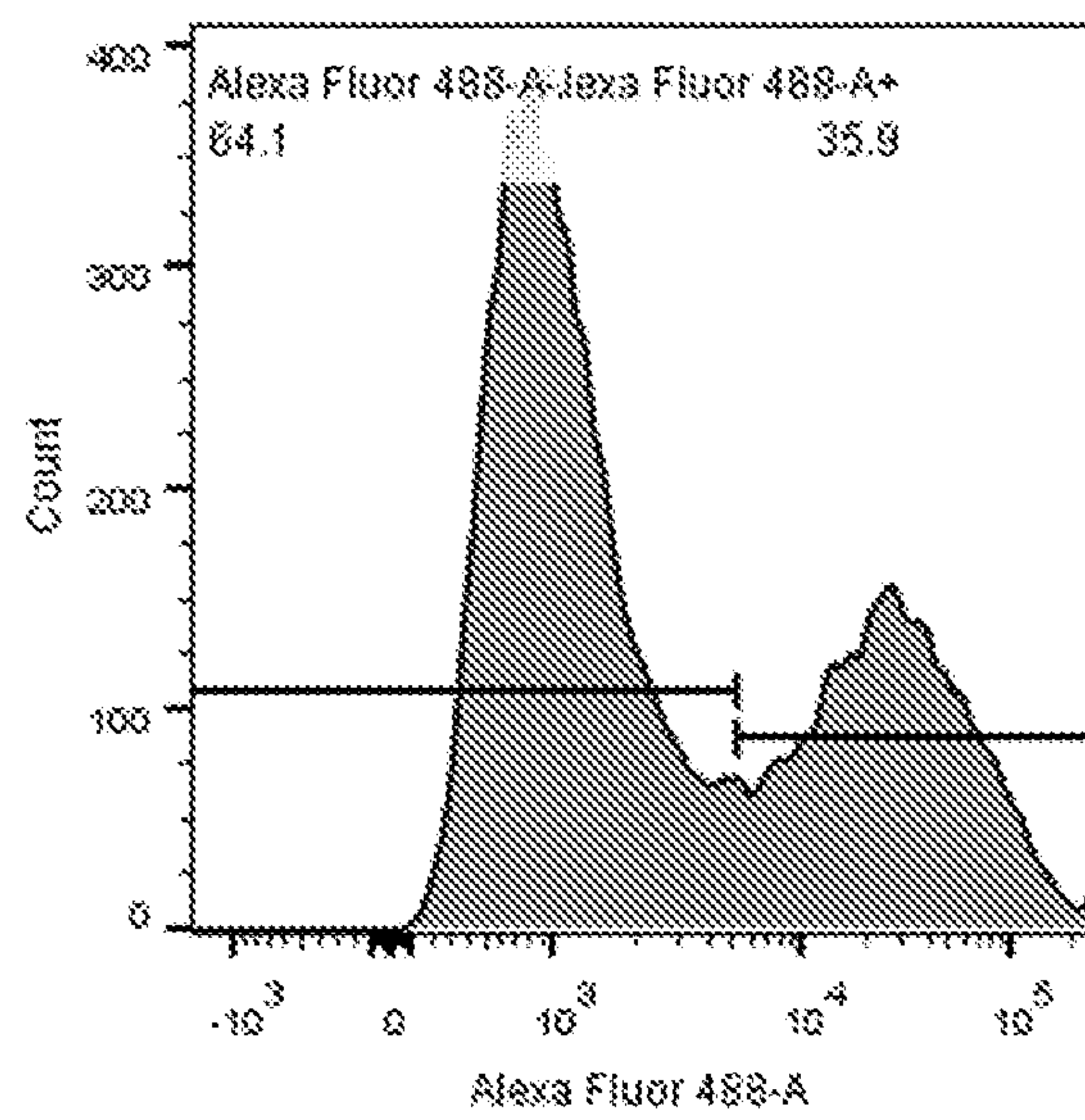


FIG. 11D

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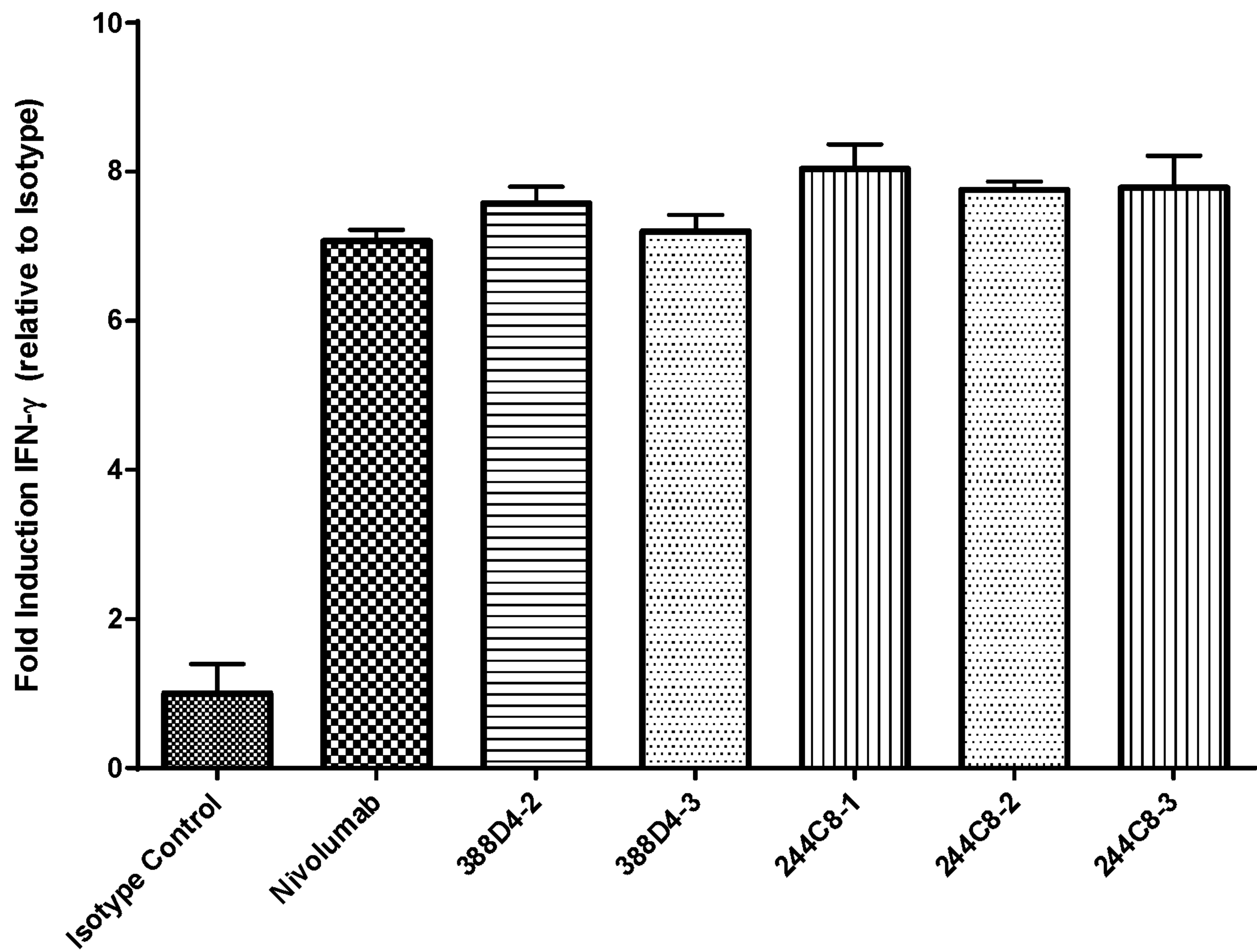


FIG. 12

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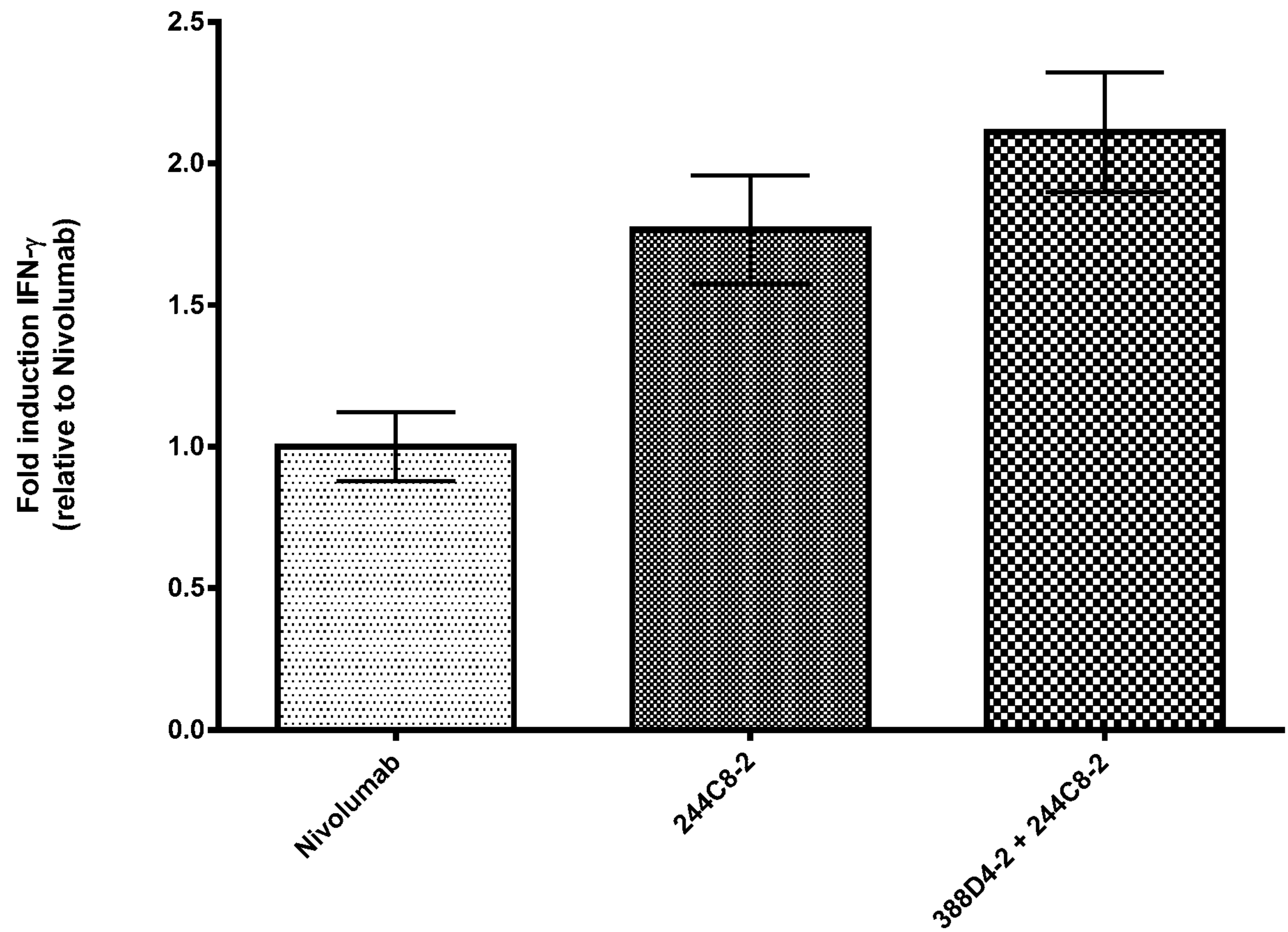


FIG. 13

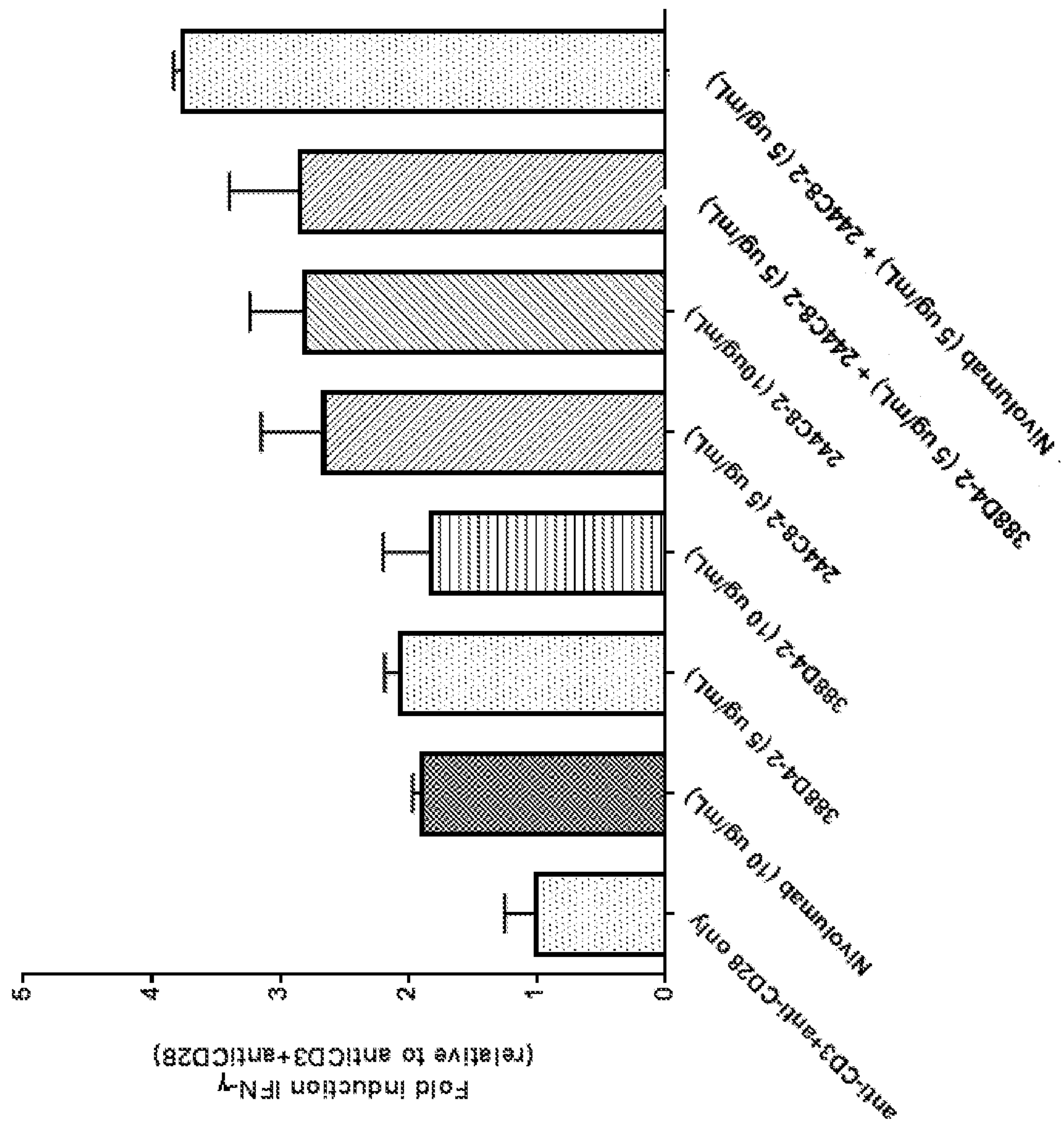


FIG. 14

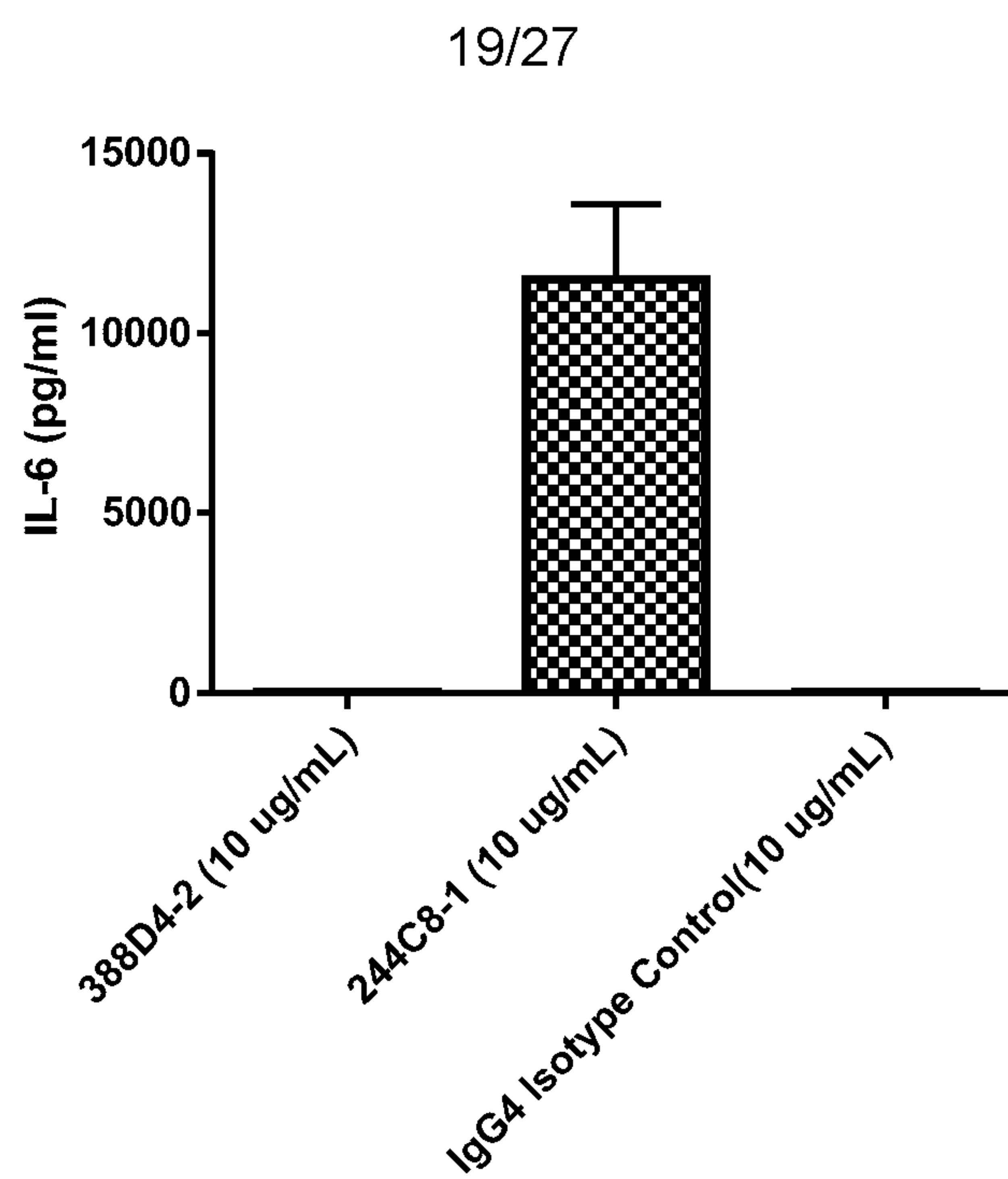


FIG. 15A

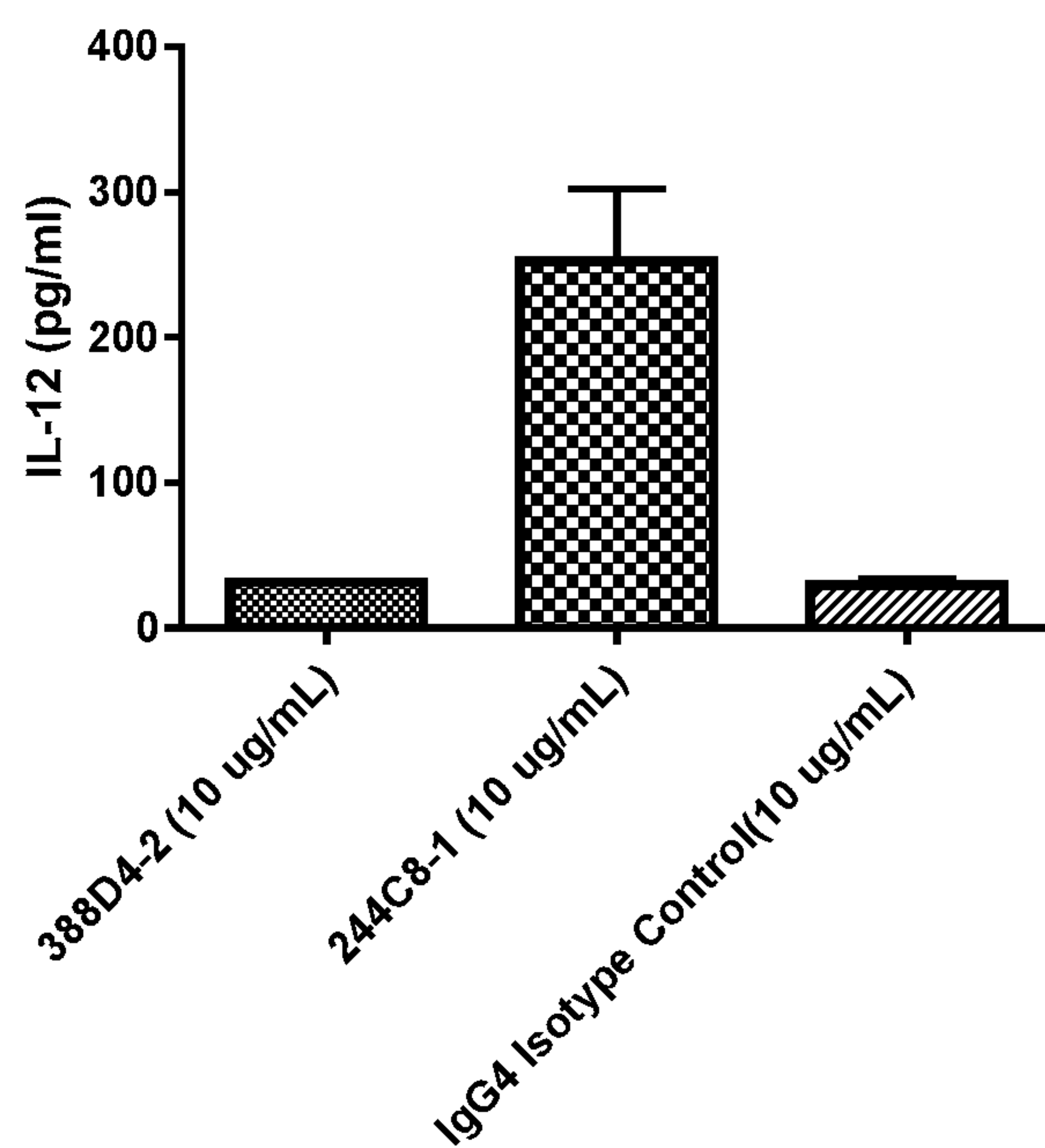


FIG. 15B

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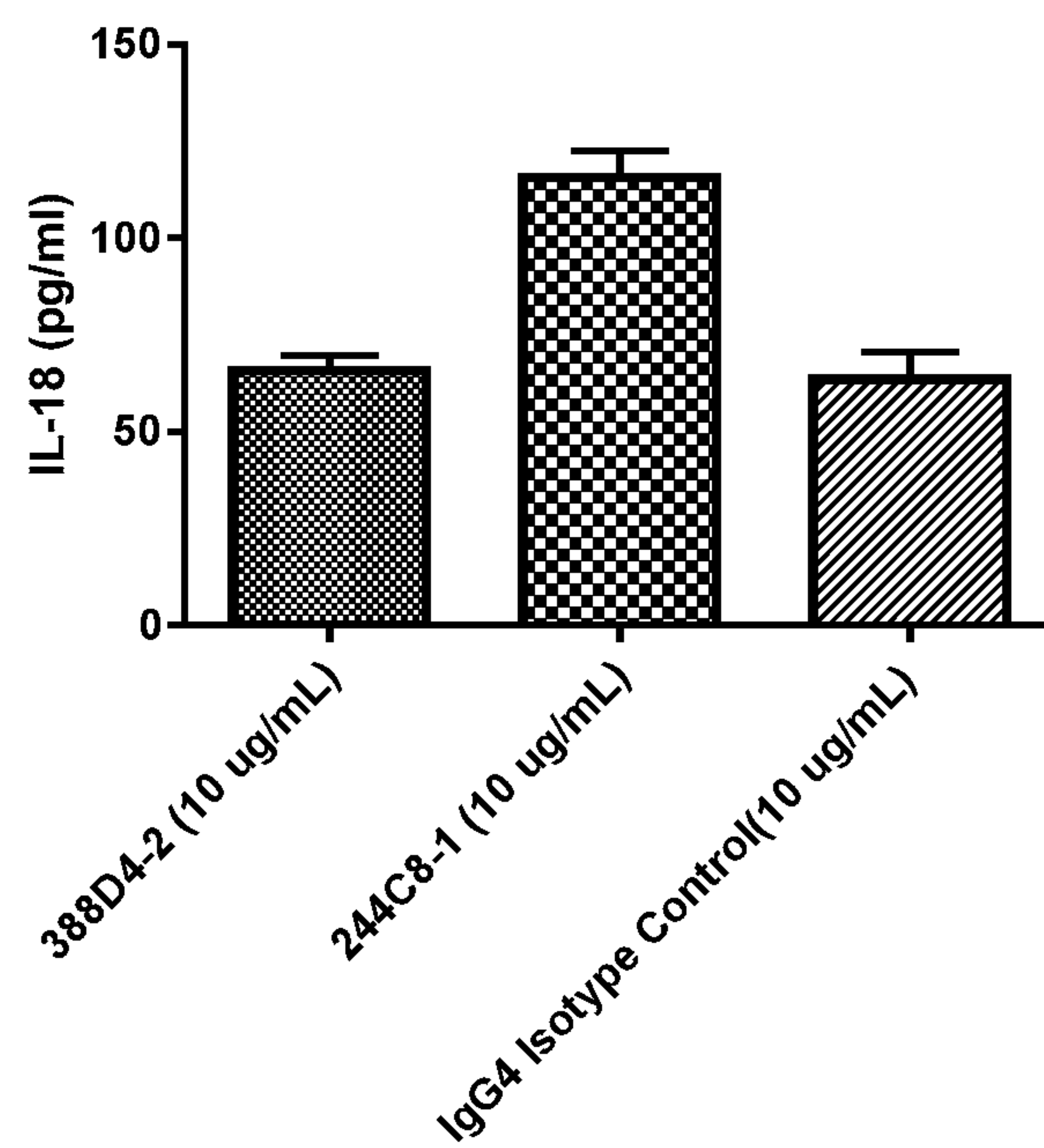


FIG. 15C

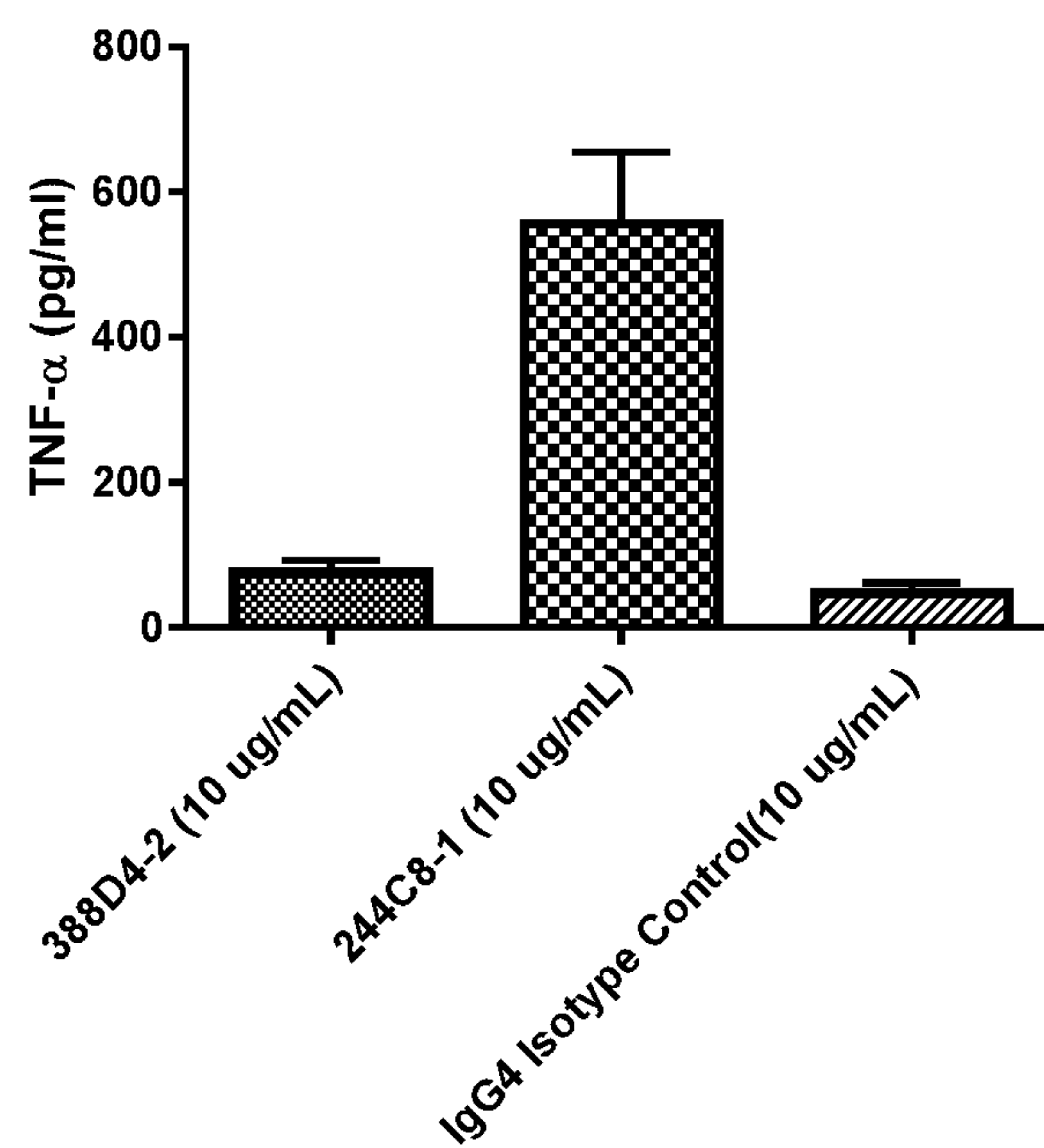


FIG. 15D

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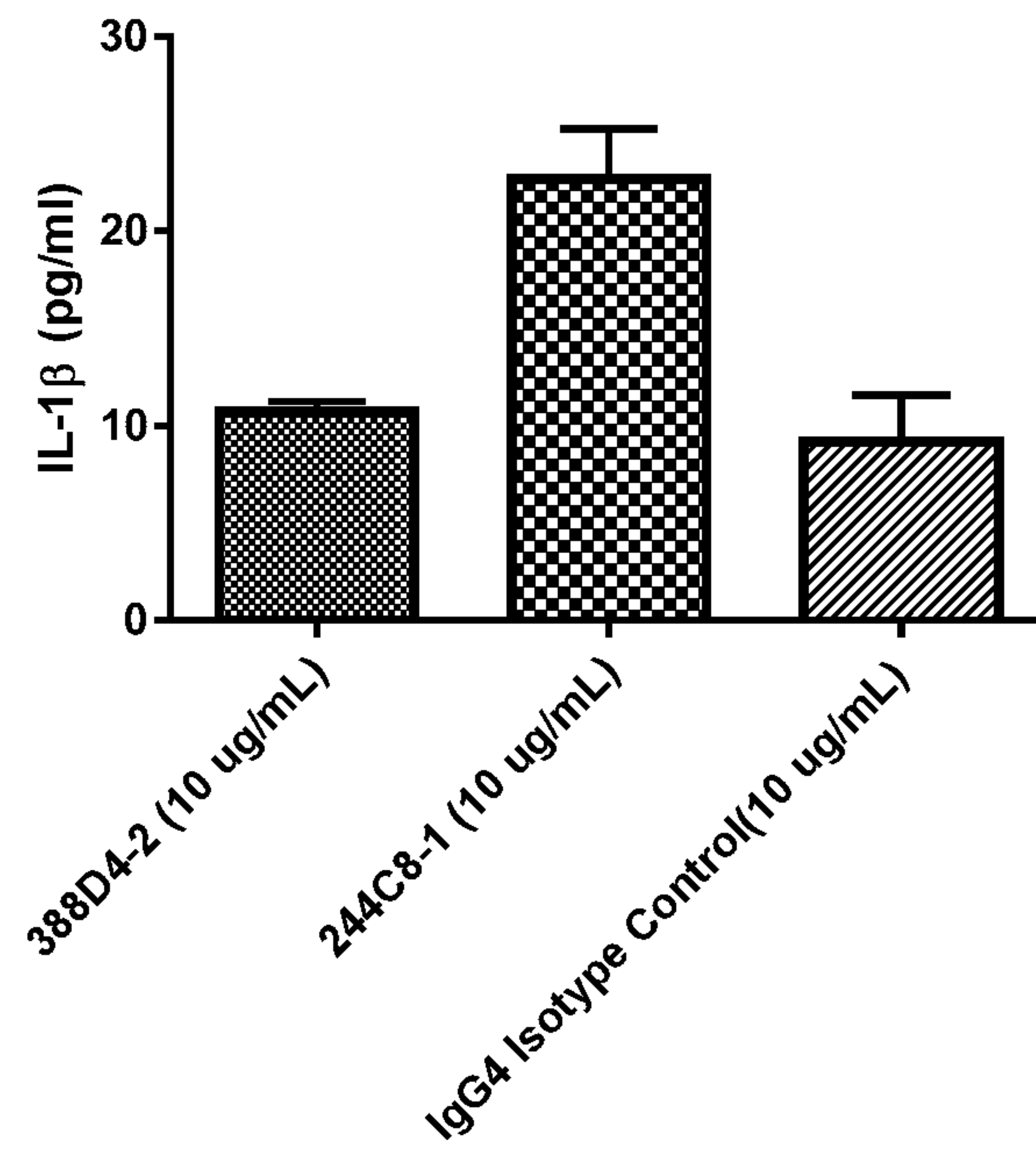


FIG. 15E

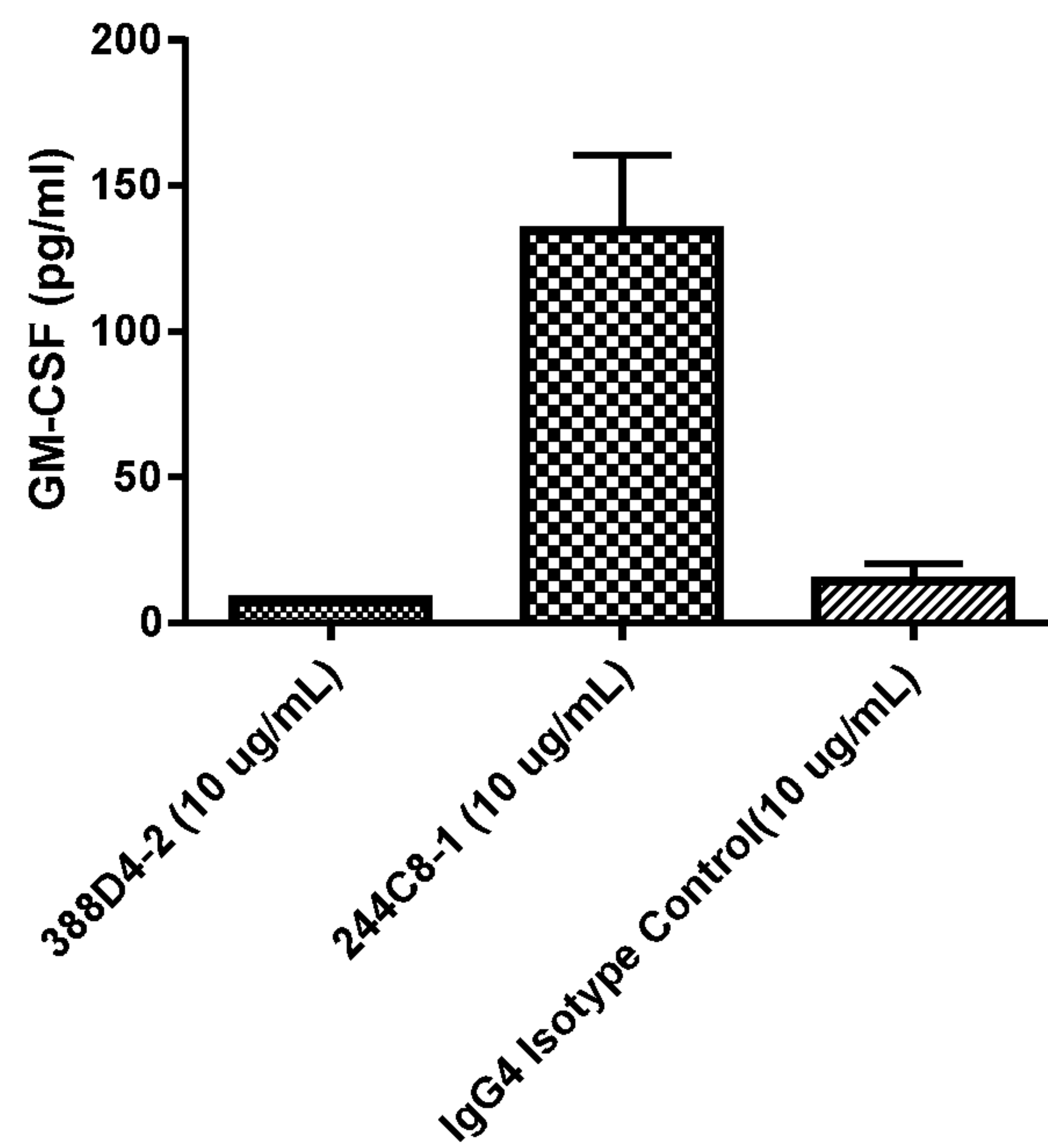


FIG. 15F

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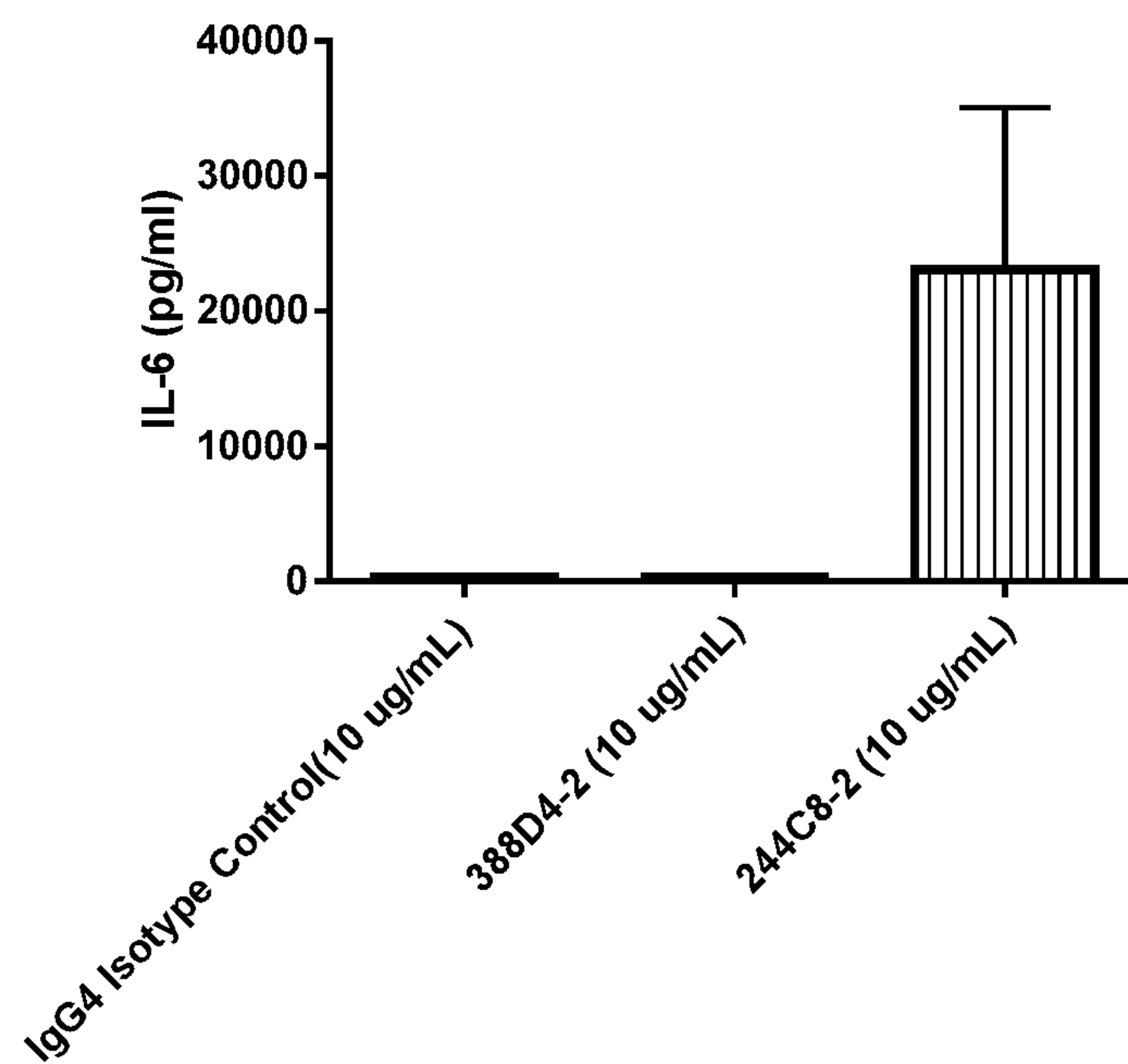


FIG. 16A

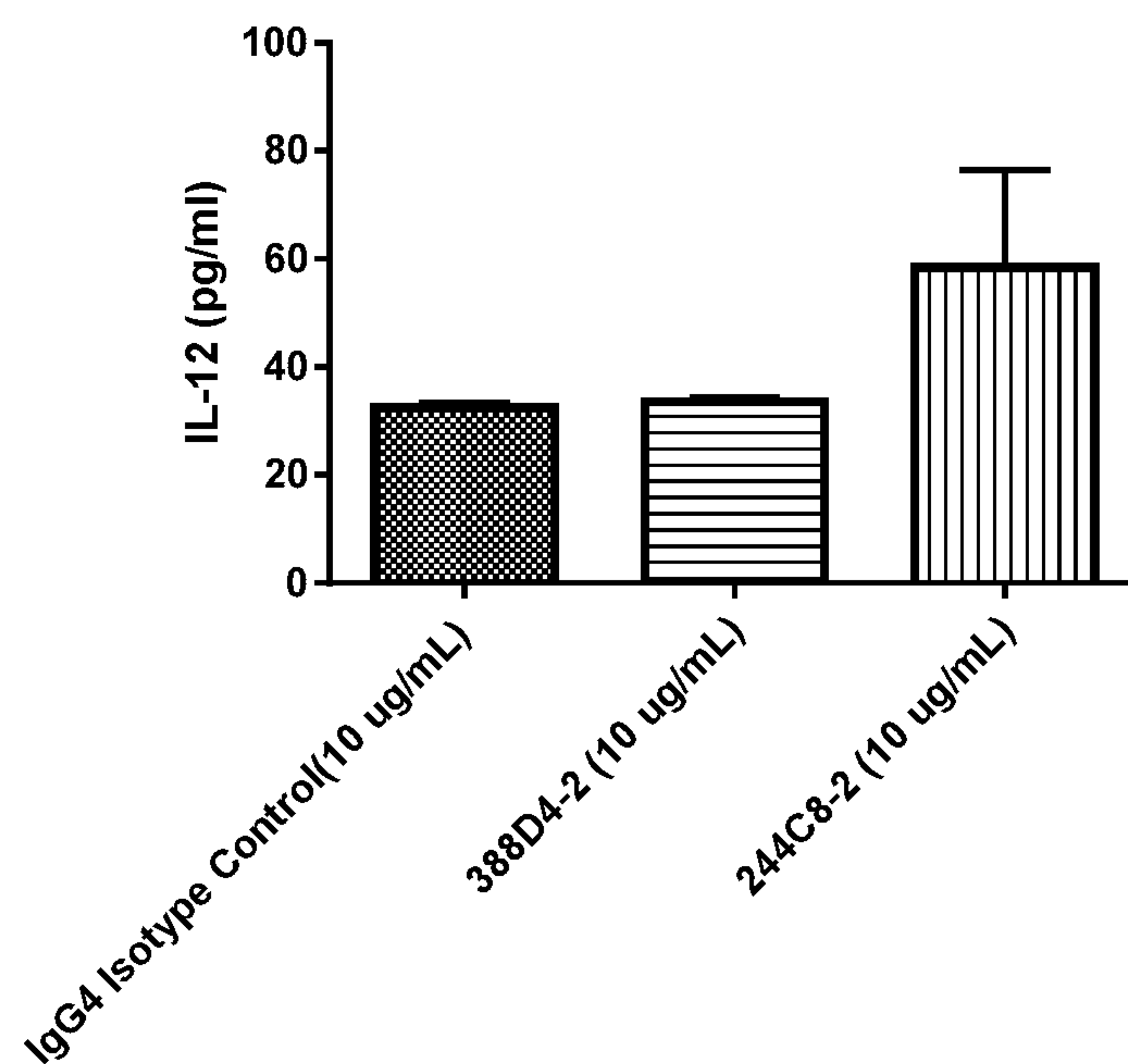


FIG. 16B

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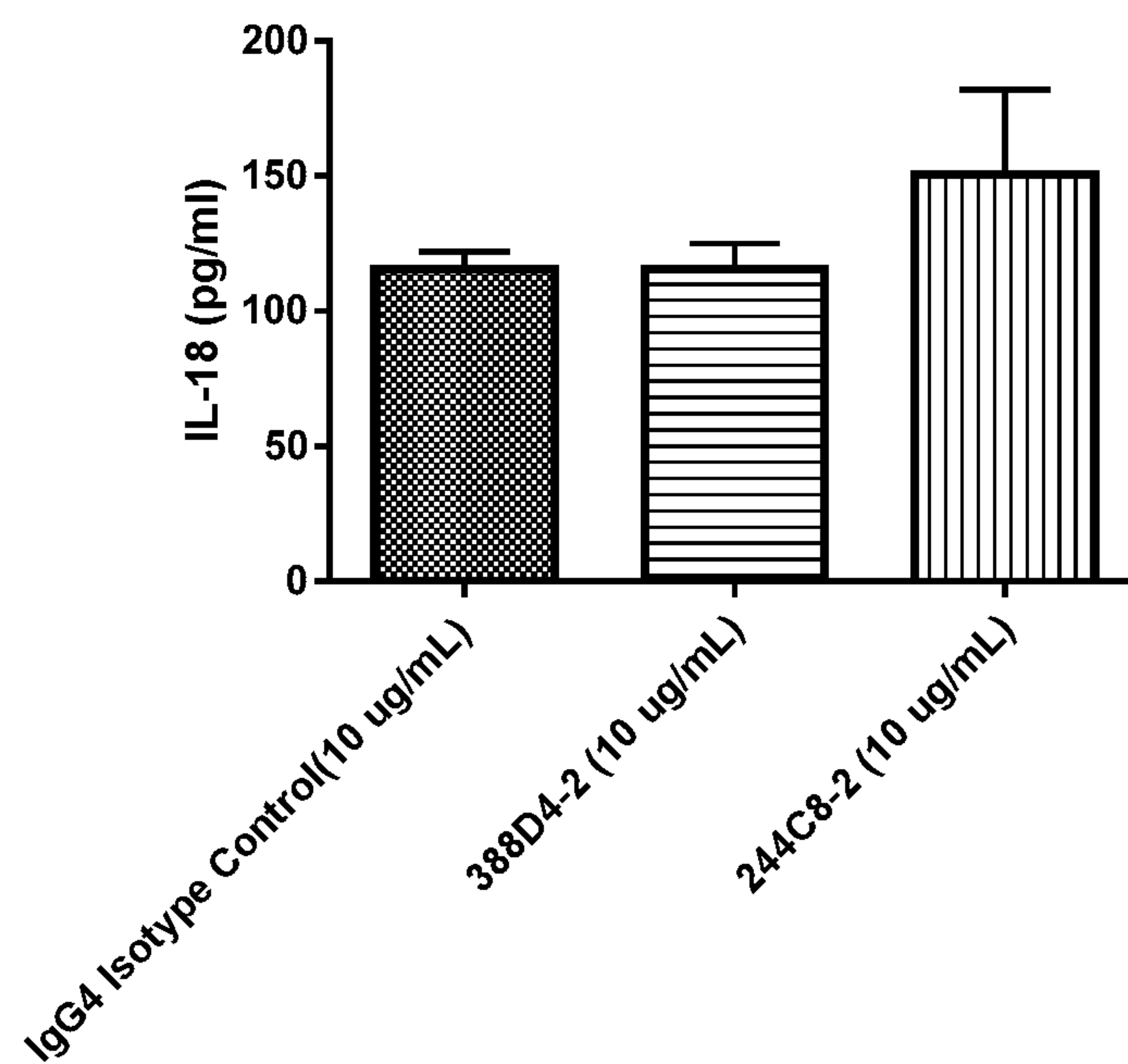


FIG. 16C

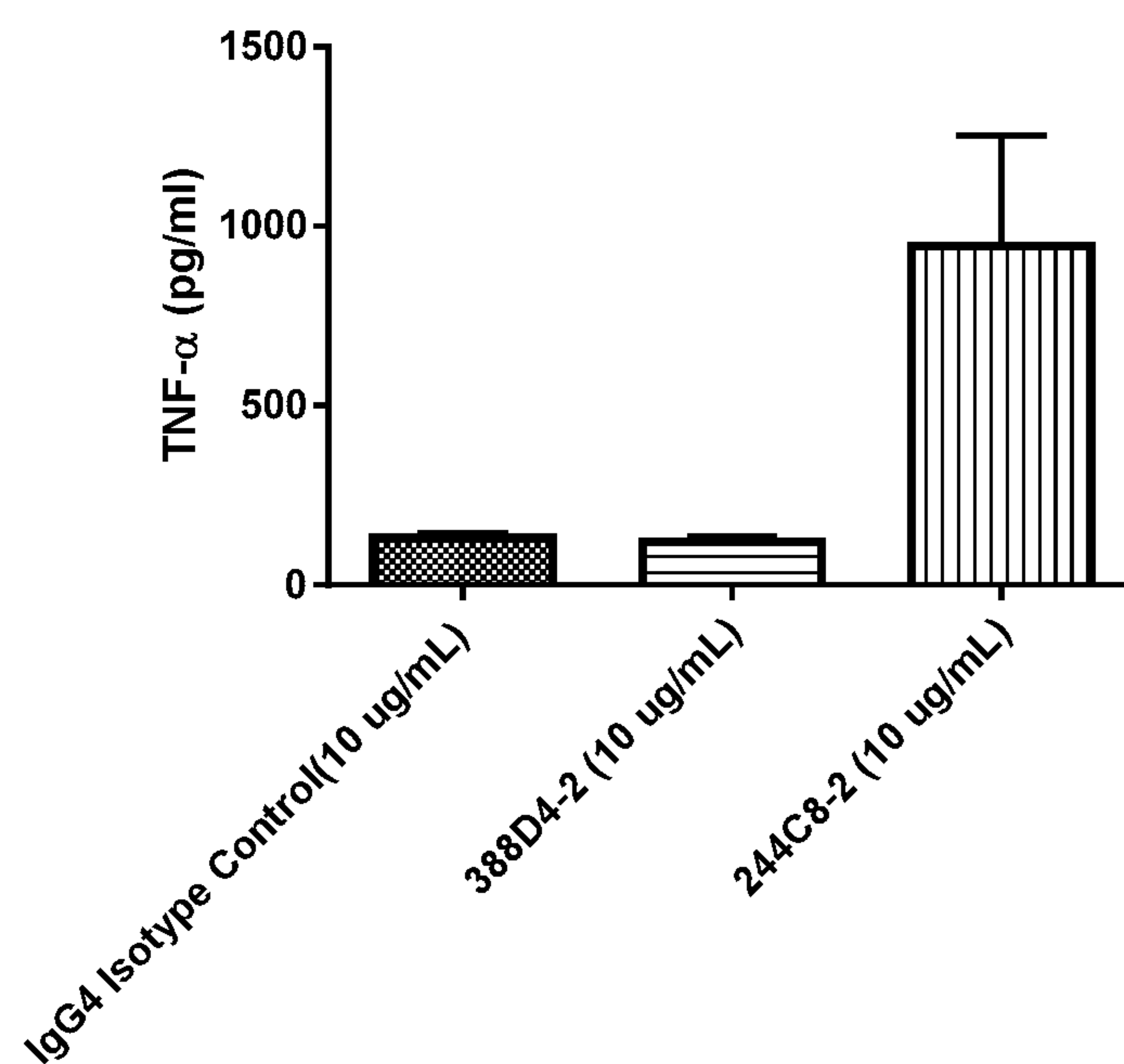


FIG. 16D

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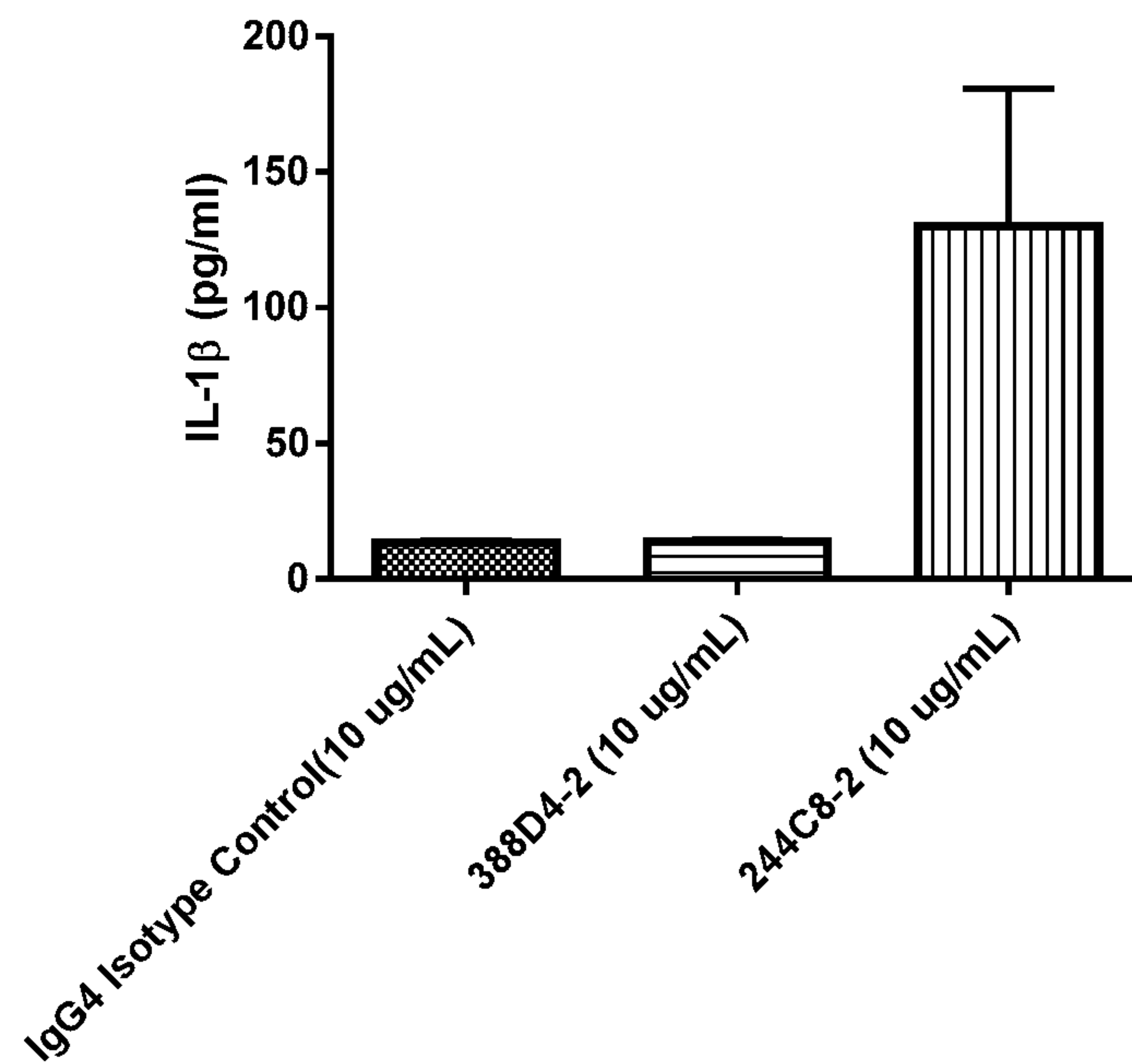


FIG. 16E

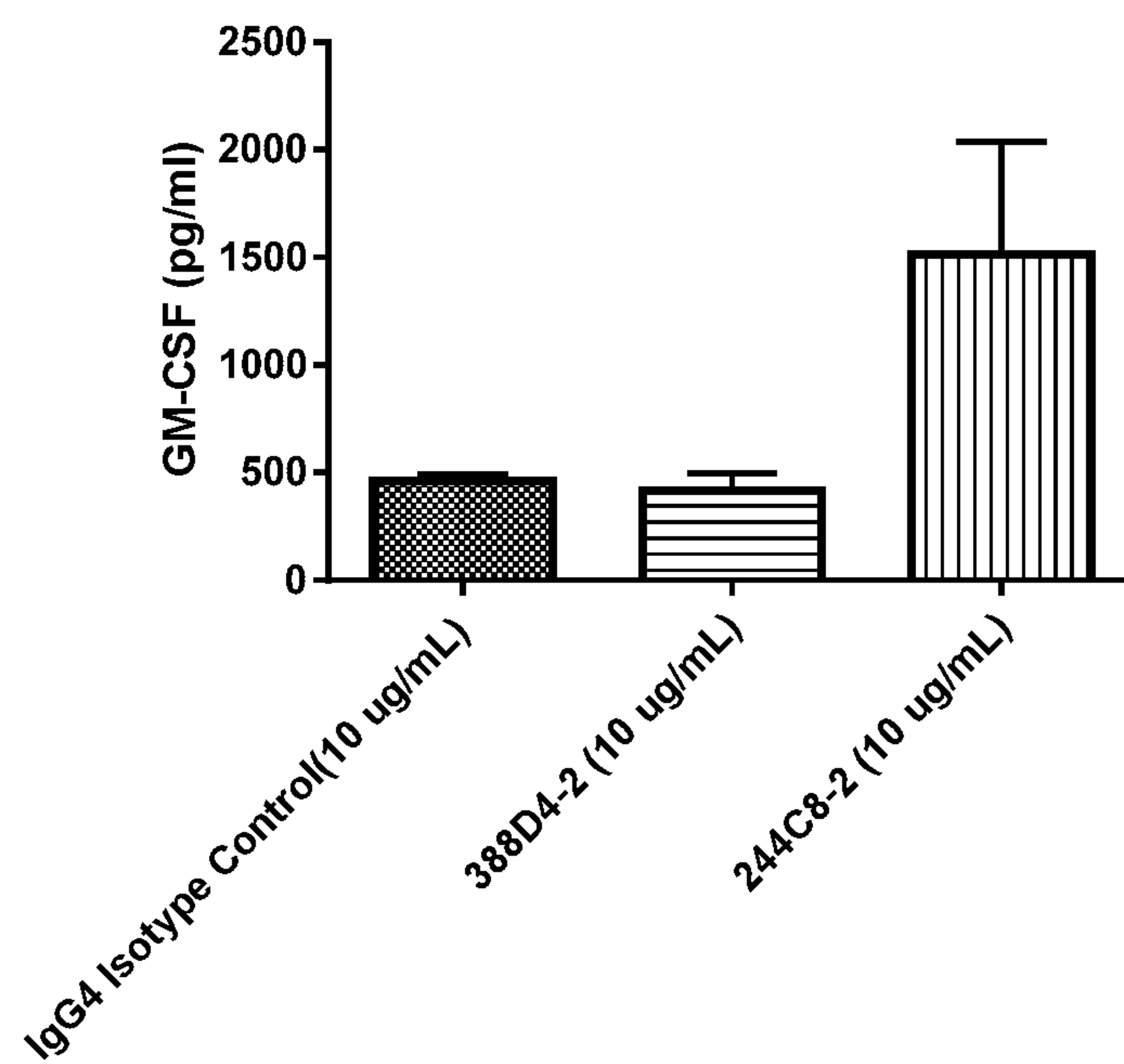


FIG. 16F

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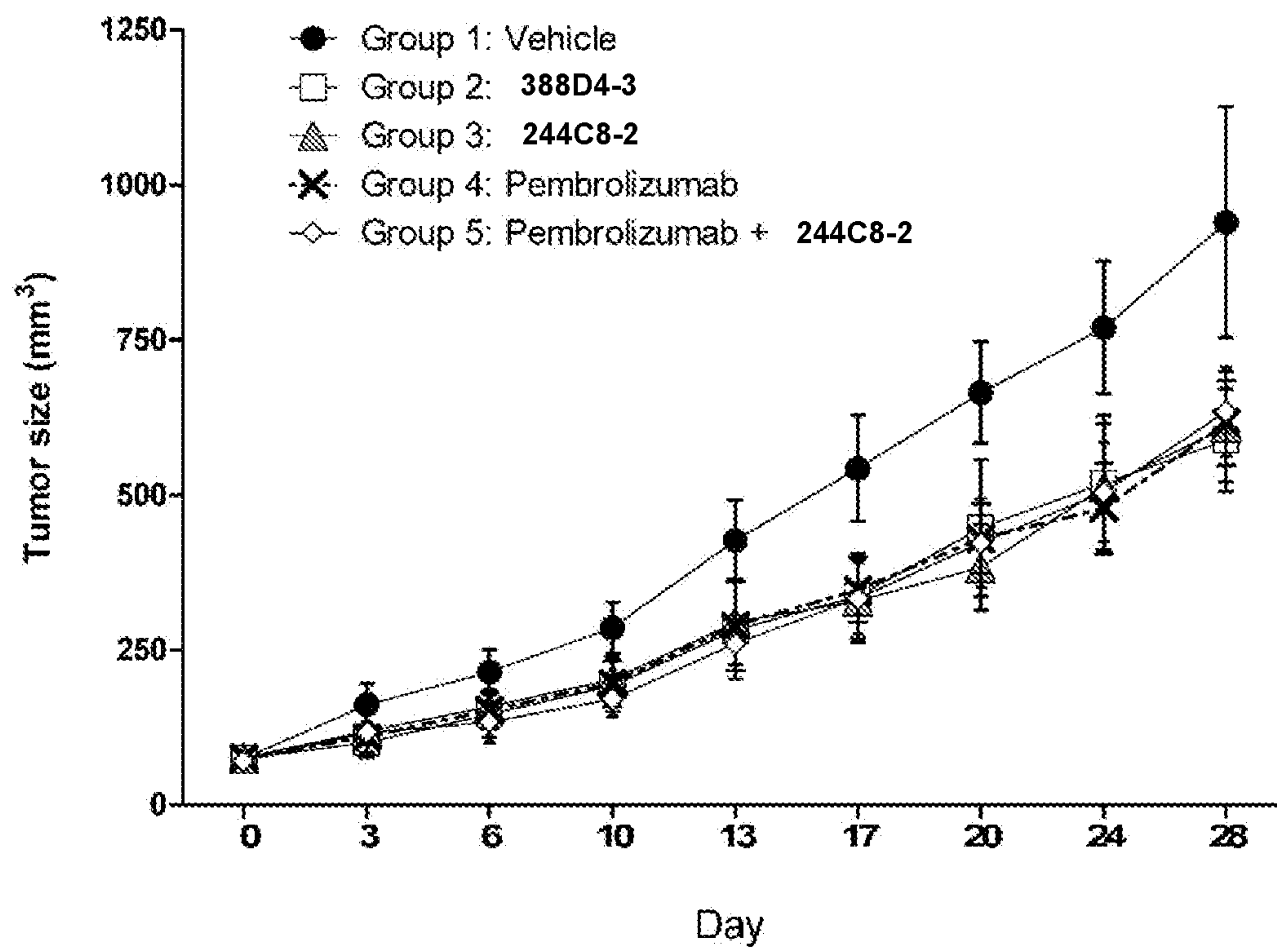


FIG. 17A

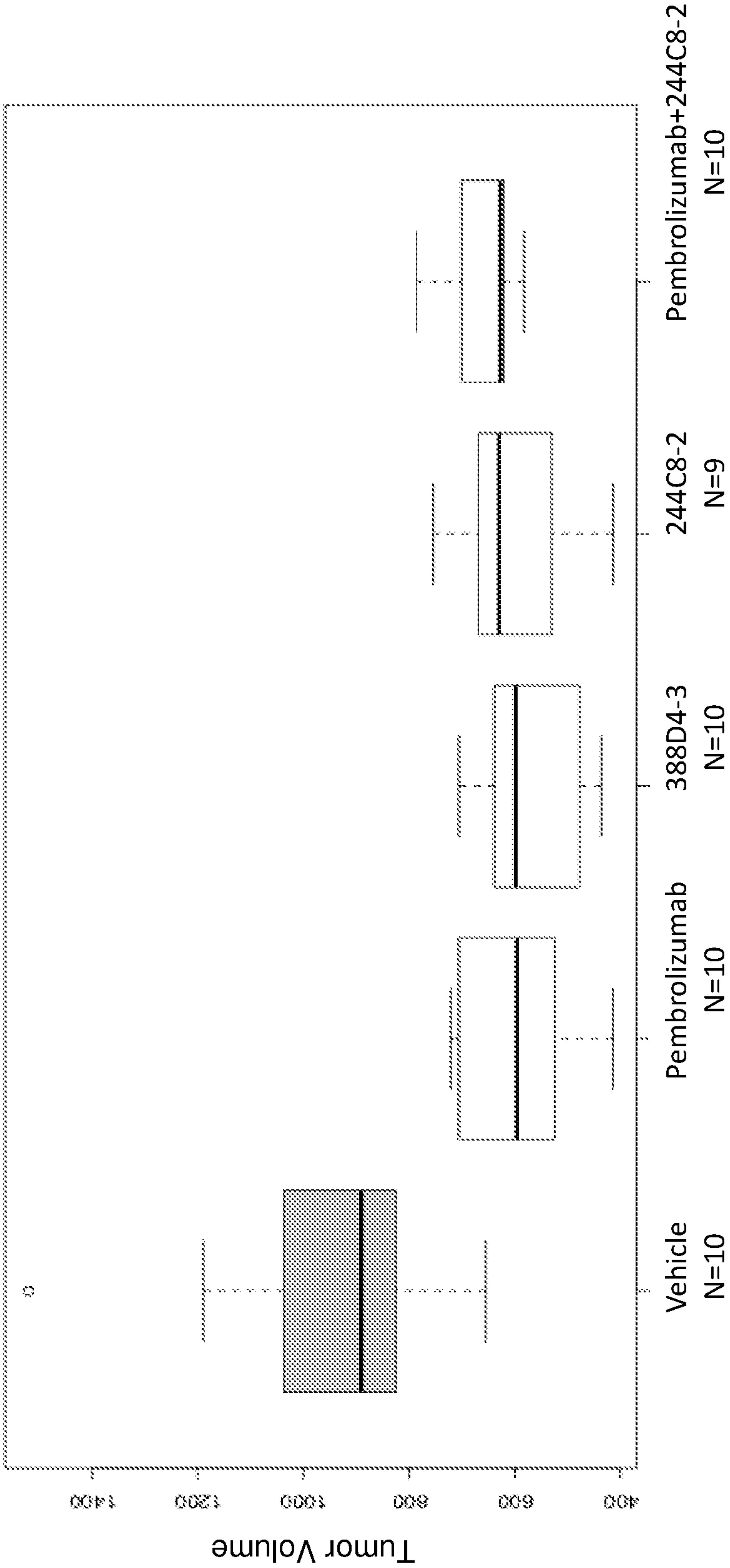


FIG. 17B

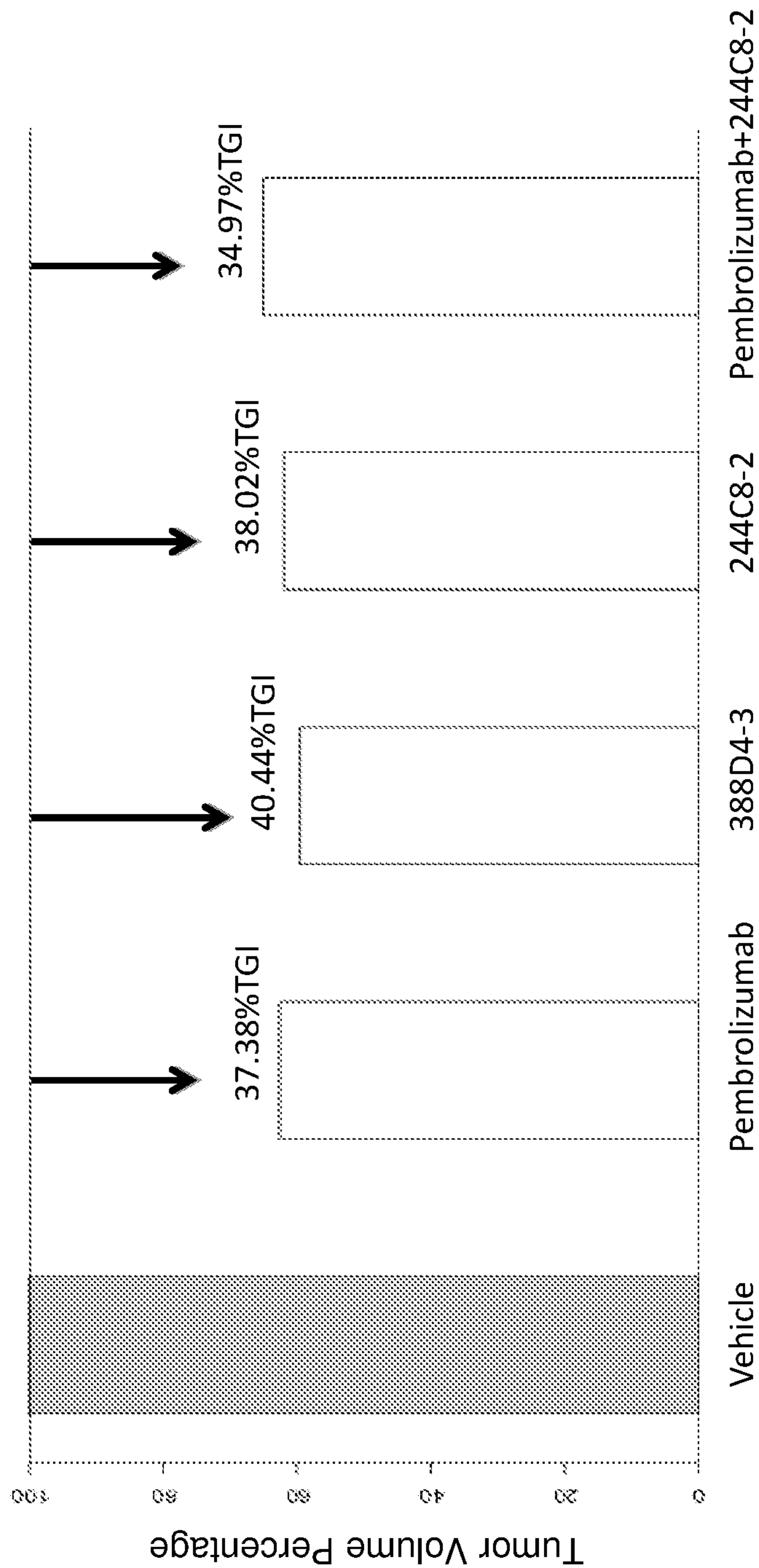


FIG. 17C