

HUMANIZED ANTI-OX40 ANTIBODIES AND USES THEREOF

BACKGROUND

[0001] OX40 (CD134; TNFRSF4) is a tumor necrosis factor receptor found primarily on activated CD4⁺ and CD8⁺ T cells, regulatory T (Treg) cells and natural killer (NK) cells (Croft *et al.*, 2009, *Immunol Rev.* 229:173-91). OX40 has one known endogenous ligand, OX40 ligand (OX40L; CD152; TNFSF4), which exists in a trimeric form and can cluster OX40, resulting in potent cell signaling events within T cells. *Id.* Signaling through OX40 on activated CD4⁺ and CD8⁺ T cells leads to enhanced cytokine production, granzyme and perforin release, and expansion of effector and memory T cell pools (Jensen *et al.*, 2010, *Semin Oncol.* 37:524-32). In addition, OX40 signaling on Treg cells inhibits expansion of Tregs, shuts down the induction of Tregs and blocks Treg-suppressive function (Voo *et al.*, 2013, *J Immunol.* 191:3641-50; Vu *et al.*, 2007, *Blood.* 110:2501-10).

[0002] Immunohistochemistry studies and early flow cytometry analyses showed that OX40 is expressed on T cells infiltrating a broad range of human cancers (Baruah *et al.*, 2011, *Immunobiology* 217:668-675; Curti *et al.*, 2013, *Cancer Res.* 73:7189-98; Ladanyi *et al.*, 2004, *Clin Cancer Res.* 10:521-30; Petty *et al.*, 2002, *Am J Surg.* 183:512-8; Ramstad *et al.*, 2000, *Am J Surg.* 179:400-6; Sarff *et al.*, 2008, *Am J Surg.* 195:621-5; discussion 625; Vetto *et al.*, 1997, *Am J Surg.* 174:258-65). While not wishing to be bound by theory, OX40 expression on tumor-infiltrating lymphocytes correlates with longer survival in several human cancers, suggesting that OX40 signals can play a role in establishing an antitumor immune response (Ladanyi *et al.*, 2004, *Clin Cancer Res.* 10:521-30; Petty *et al.*, 2002, *Am J Surg.* 183:512-8).

[0003] In a variety of nonclinical mouse tumor models, agonists of OX40, including antibodies and OX40 ligand fusion proteins, have been used successfully with promising results (Kjaergaard *et al.*, 2000, *Cancer Res.* 60:5514-21; Ndhlovu *et al.*, 2001, *J Immunol.* 167:2991-9; Weinberg *et al.*, 2000, *J Immunol.* 164:2160-9). Co-stimulating T cells through OX40 promoted anti-tumor activity

that in some cases was durable, providing long-lasting protection against subsequent tumor challenge (Weinberg *et al.*, 2000, *J Immunol.* 164:2160-9). Treg- cell inhibition and co-stimulation of effector T cells were shown to be necessary for tumor growth inhibition of OX40 agonists (Piconese *et al.*, 2008, *J Exp Med.* 205:825-39). Many strategies and technologies have been explored to enhance the anti-tumor effect of OX40 agonist therapy through combinations with vaccines, chemotherapy, radiotherapy, and immunotherapy (Jensen *et al.*, 2010, *Semin Oncol.* 37:524-32; Melero *et al.*, 2013, *Clin Cancer Res.* 19:997-1008).

SUMMARY

[0004] This disclosure provides antibodies that bind to OX40, *e.g.*, human OX40. In certain aspects the antibodies provided are humanized antibodies. For example, this disclosure provides an antibody or antigen-binding fragment thereof that includes a humanized heavy chain variable region (VH) and a humanized light chain variable region (VL), where the VH includes an amino acid sequence with the formula:

HFW1-HCDR1-HFW2-HCDR2-HFW3-HCDR3-HFW4,

where HFW1 is SEQ ID NO: 6 or SEQ ID NO: 7, HCDR1 is SEQ ID NO: 8, HFW2 is SEQ ID NO: 9 (WIRX₃₉HPGKGLX₄₇X₄₈G; where X₃₉ is Q or K, X₄₇ is W or Y, and X₄₈ is I or M), HCDR2 is SEQ ID NO: 14, SEQ ID NO: 15 or SEQ ID NO: 16, HFW3 is SEQ ID NO: 17 (RITINX₇₁DTSKNQX₇₈SLQLNSVTPEDTAVYX₉₁CAR; where X₇₁ is P or R, X₇₈ is F or Y, and X₉₁ is Y or F, HCDR3 is SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27), and HFW4 is SEQ ID NO: 28, where the VL includes the amino acid sequence SEQ ID NO: 29 or SEQ ID NO: 32; and where the antibody or fragment thereof can specifically bind to human OX40. In certain aspects, the amino acid sequence of HFW2 is SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 1, and in certain aspects the amino acid sequence of HFW3 is SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0005] In certain aspects, the VH of the provided antibody or fragment thereof includes the amino acid sequence SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID

NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, or SEQ ID NO: 67. In certain aspects, the VL of the provided antibody or fragment thereof includes the amino acid sequence SEQ ID NO: 29 and the VH includes the amino acid sequence SEQ ID NO: 59.

[0006] In certain aspects, the provided antibody or fragment thereof further includes a light chain constant region or fragment thereof fused to the C-terminus of the VL, *e.g.*, a human kappa constant region or a human lambda constant region. In certain aspects, the provided antibody or fragment thereof further includes a heavy chain constant region or fragment thereof fused to the C-terminus of the VH, *e.g.*, a human IgG1 constant region, a human IgG4P constant region, a human IgG1TM constant region or a murine IgG1 constant region. In certain aspects the heavy chain constant region is a human IgG1 constant region. In certain aspects, the provided antibody or fragment thereof includes the heavy chain amino acid sequence SEQ ID NO: 71 and the light chain amino acid sequence SEQ ID NO: 30. An antigen-binding fragment of an antibody as provided by this disclosure can be, *e.g.*, an Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, or an sc(Fv)₂ fragment, or any combination thereof.

[0007] In certain aspects, the provided antibody or fragment thereof can specifically bind to human, cynomolgus monkey, or rhesus monkey OX40, *e.g.*, can specifically bind to OX40 as expressed on Jurkat cells, primary activated CD4⁺ or CD8⁺ T cells from human, cynomolgus monkey, rhesus monkey, or any combination thereof. In certain aspects, the provided antibody or fragment thereof does not bind to murine or rat OX40. In certain aspects, the provided antibody or fragment thereof does not cross react with related TNFRSF proteins.

[0008] In certain aspects, the provided antibody or fragment thereof can have a binding affinity for human OX40 expressed on primary activated human CD4⁺ T cells of about 250 pM to about 370 pM as measured by flow cytometry, *e.g.*, about 312 pM. In certain aspects, the provided antibody or fragment thereof can achieve 20% receptor occupancy on primary activated human CD4⁺ T cells (EC₂₀) at about 63 to about 93 pM, 50% receptor occupancy on primary activated human CD4⁺ T cells (EC₅₀) at about 250 to about 370 pM, and 90% receptor occupancy on primary activated human CD4⁺ T cells (EC₉₀) at about 2290 to about 3330 pM as measured by flow

cytometry. For example, in certain aspects, EC₂₀ is about 78 pM, EC₅₀ is about 312 pM, and EC₉₀ is about 2810 pM.

[0009] In certain aspects, the provided antibody or fragment thereof can have a binding affinity for human OX40 expressed on OX40-overexpressing Jurkat cells of about 250 pM to about 600 pM as measured by flow cytometry, *e.g.*, about 424 pM. In certain aspects, the provided antibody or fragment thereof can achieve EC₂₀ on OX40-overexpressing Jurkat cells at about 60 to about 150 pM, EC₅₀ on OX40-overexpressing Jurkat cells at about 250 to about 600 pM, and EC₉₀ on OX40-overexpressing Jurkat cells at about 2260 to about 4380 pM as measured by flow cytometry. For example, in certain aspects, EC₂₀ is about 106 pM, EC₅₀ is about 424 pM, and EC₉₀ is about 3820 pM.

[0010] In certain aspects, the provided antibody or fragment thereof can have a binding affinity for cynomolgus monkey OX40 expressed on primary activated cynomolgus monkey CD4⁺ T cells of about 340 pM to about 820 pM as measured by flow cytometry, *e.g.*, about 580 pM. In certain aspects, the provided antibody or fragment thereof can have a binding affinity for rhesus monkey OX40 expressed on primary activated rhesus monkey CD4⁺ T cells of about 130 pM to about 600 pM as measured by flow cytometry, *e.g.*, about 370 pM.

[0011] In certain aspects, the provided antibody or fragment thereof can induce dose-dependent proliferation of activated CD4⁺ T cells and dose-dependent cytokine release in primary activated CD4⁺ T cells in a plate-based assay. For example, in certain aspects, a 20% maximal proliferation response (EC₂₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 14 pM to about 28 pM, a 50% maximal proliferation response (EC₅₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 0.3 pM to about 130 pM, and a 90% maximal proliferation response (EC₉₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 50 pM to about 90 pM, all as measured by flow cytometry. In certain aspects, EC₂₀ is about 21 pM, EC₅₀ is about 28 pM, and EC₉₀ is about 72 pM. The released cytokine in primary activated human CD4⁺ T cells can be one, two, three or more of, without limitation, IFN γ , TNF α , IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1 β , or any combination thereof, for example, IFN γ , TNF α , IL-5, IL-10, IL-13, or any combination thereof. In certain aspects, the provided antibody or fragment thereof can

achieve CD4⁺ T cell proliferation and cytokine release in primary activated cynomolgus monkey CD4⁺ T cells and in primary activated rhesus monkey CD4⁺ T cells.

[0012] In certain aspects, the provided antibody or fragment thereof can activate the NFκB pathway in OX40 expressing T cells in the presence of FcγR-expressing cells. For example, the OX40-expressing T cells can be OX40-overexpressing Jurkat NFκB-luciferase reporter cells that produce luciferase in response to stimulation of the NFκB signaling pathway. In certain aspects, the provided antibody or fragment thereof can trigger complement-dependent or antibody-dependent cellular cytotoxicity against OX40-expressing cells. In certain aspects, the provided antibody or fragment thereof can bind to human C1q and trigger NK-mediated antibody-dependent cellular cytotoxicity against the OX40-expressing cells.

[0013] In certain aspects, administration of an effective dose of the provided antibody or fragment thereof to a subject in need of cancer treatment can inhibit tumor growth in the subject. For example, the tumor growth inhibition can be achieved in the presence of T cells. In certain aspects, tumor growth is inhibited by at least 10%, at least 20%, at least 30%, at least 40%, and least 50%, at least 60%, or at least 70% compared to administration of an isotype-matched control antibody or fragment thereof.

[0014] This disclosure further provides a composition including the antibody or fragment thereof as described above, and a carrier.

[0015] This disclosure further provides a polynucleotide that includes a nucleic acid that encodes the provided antibody or fragment thereof, or encodes a polypeptide subunit of the provided antibody or fragment thereof. In certain aspects, the provided polynucleotide includes the nucleic acid of SEQ ID NO: 60, the nucleic acid of SEQ ID NO: 31, the nucleic acid of SEQ ID NO: 72, or any combination thereof. This disclosure further provides a vector that includes the provided polynucleotide and a host cell that includes the provided polynucleotide or the provided vector. In another aspect, the disclosure provides a method of producing an antibody or fragment thereof, where the method includes culturing the provided host cell under conditions in which the antibody or fragment thereof encoded by the polynucleotide is expressed, and recovering the antibody or fragment thereof.

[0016] In additional aspects, the disclosure provides a method to promote survival or proliferation of activated T cells, where the method includes contacting activated T cells with the provided

antibody or fragment thereof, and where the antibody or fragment thereof can specifically bind to OX40 on the surface of the T cells.

[0017] In additional aspects, the disclosure provides a method of inducing cytokine release from activated T cells, where the method includes contacting activated T cells with the provided antibody or fragment thereof, and where the antibody or fragment thereof can specifically bind to OX40 on the surface of the T cells. In certain aspects the released cytokine can be one, two, three or more of, without limitation, $\text{IFN}\gamma$, $\text{TNF}\alpha$, IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1 β , or any combination thereof, *e.g.*, $\text{IFN}\gamma$, $\text{TNF}\alpha$, IL-5, IL-10, IL-13, or any combination thereof. In certain aspects, the activated T cells are activated CD4^+ T cells, activated CD8^+ T cells, or a combination thereof. In certain aspects, the activated CD4^+ T cells are human CD4^+ T cells, cynomolgus monkey CD4^+ T cells, rhesus monkey CD4^+ T cells, or a combination thereof.

[0018] In additional aspects, the disclosure provides a method of promoting T cell activation, where the method includes contacting T cells with the provided antibody or fragment thereof, where the antibody or fragment thereof can specifically bind to OX40 on the surface of the T cells. In certain aspects, T cell activation can be measured through stimulation of the $\text{NF}\kappa\text{B}$ signal transduction pathway. In certain aspects, the T cells are activated CD4^+ T cells, activated CD8^+ T cells, or a combination thereof. In certain aspects, the activated CD4^+ T cells are human CD4^+ T cells, cynomolgus monkey CD4^+ T cells, rhesus monkey CD4^+ T cells, or a combination thereof. In certain aspects, the contacting includes administering an effective amount of the antibody or fragment thereof to a subject.

[0019] In additional aspects, the disclosure provides a method of treating cancer in a subject, where the method includes administering to a subject in need of treatment an effective amount of the provided antibody or fragment thereof, or the provided composition. In certain aspects, the cancer is a solid tumor. In certain aspects, administration of the antibody or fragment thereof or composition can inhibit tumor growth, can promote tumor reduction, or both. In certain aspects, tumor growth inhibition is achieved in the presence of T cells.

[0020] In additional aspects, the disclosure provides a method of enhancing an immune response in a subject, where the method includes administering to a subject in need thereof a therapeutically effective amount of the provided antibody or fragment thereof, or the provided composition.

- [0021] In the therapeutic methods provided by this disclosure, the subject to be treated can be a human subject.
- [0022] In certain aspects, the provided antibody or fragment thereof can bind to an epitope of human OX40 that falls within amino acids 108 to 146 of SEQ ID NO: 91. In certain aspects, the epitope includes at least amino acids leucine 116 (L116) and alanine 126 (A126) of SEQ ID NO: 91. In certain aspects, the provided antibody or fragment thereof can bind to a mouse OX40 variant that has the amino acid sequence of SEQ ID NO: 92, except for a Q113L mutation and a V124A mutation.
- [0023] This disclosure further provides an isolated peptide consisting of 100 or fewer amino acids, where the peptide can be specifically bound by the provided antibody or fragment thereof. In certain aspects, the peptide includes amino acids 116 to 126 of SEQ ID NO: 91. In certain aspects, the peptide includes amino acids 108 to 146 of SEQ ID NO: 91 except for one, two, three, four, five, or six single amino acid substitutions, deletions, or insertions at any position except L116 and A126.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

- [0024] Figure 1. Mutations in humanized 9B12 VH regions: Numbers in the clonal nick name represent the position of the amino acid in VH, according to Kabat numbering. Mabs 1, 2, 5 and 8 are VH chimeric variants paired with humanized VL. Mabs 10-17 are humanized VH variants with mouse back mutations. Mabs 18-27 are variants engineered to remove potential sequence liabilities. Variable regions of Mab24 and mAb27 were grafted onto different heavy constant regions for the resulting isotype variants named mAb28-30 and mAb31-32, mAb37, respectively.
- [0025] Figure 2A-D. Binding of OX40mAb24 and 9B12 to OX40 Expressed on the Surface of Primary Activated Human CD4⁺ T cells. MFI = mean fluorescence intensity of AlexaFluor® 647 labeled secondary anti-human antibody binding to OX40mAb24 (2A-B) or AlexaFluor® 488 labeled anti-mouse secondary antibody binding to 9B12 (2C-D) on primary human CD4⁺ T cells.
- [0026] Figure 3A-F. Binding of OX40mAb24 and 9B12 to Human OX40 Expressed on Jurkat T cells. MFI = mean fluorescence intensity of AlexaFluor® 647 labeled secondary anti-human

antibody binding to OX40mAb24 (3A-C) or AlexaFluor® 488 labeled anti-mouse secondary antibody binding to 9B12 (3D-F) on Jurkat T cells.

[0027] Figure 4A-B. Binding of OX40mAb24 to TNFRSF-expressing HEK293 cells and OX40-expressing Jurkat T cells. (4A) Transient expression of TNFRSF members, as indicated to left of histograms, in HEK293 cells, and binding to TNFRSF-specific mAbs or to OX40mAb24, as indicated above histograms. Gray histogram, fluorochrome-conjugated isotype control antibody binding for TNFRSF-specific mAb, or goat anti-human AlexaFluor® 647 secondary antibody binding control for OX40mAb24; Open histogram, TNFRSF-specific mAb or OX40mAb24 binding. (4B): Binding of OX40mAb24 to OX40-expressing Jurkat as a positive control. Gray histogram, goat anti-human AlexaFluor® 647 secondary antibody binding control; Open histogram, OX40mAb24 binding.

[0028] Figure 5A-B. Binding of OX40mAb24 (OX40mAb29) (complementarity-determining regions) CDRs and 9B12 to recombinant human TNFRSF members by ELISA. Results of ELISA assays demonstrating specific binding of OX40 by OX40mAb29 (5A) and 9B12 (5B). OX40mAb29 contains the CDRs of OX40mAb24. Antibody binding of OX40 was compared to binding of other human TNFRSF proteins.

[0029] Figure 6. Schematic diagram of the OX40mAb24 plate-bound bioactivity assay. Q=OX40mAb24; Y=anti-human CD3 antibody clone OKT3.

[0030] Figure 7A-C. Human CD4⁺ T cell Proliferation in response to OX40mAb24 and 9B12. (7A) CD4 T cell proliferation of four independent donors mediated by plate-immobilized OX40mAb24 in combination with sub-mitogenic TCR stimulation (anti-CD3). Data points were normalized to the lower asymptotic value of raw data curves prior to graphing to enhance visualization of the dynamic range of each response. (7B) Representative raw data from Donor 651 demonstrating proliferation-driven by OX40mAb24 plus sub-mitogenic TCR stimulation (anti-CD3) and the relative lack of proliferation mediated by the R347 human IgG1 control mAb, soluble OX40mAb24 in the presence or absence of concomitant TCR signaling, anti-CD3 mAb alone without OX40mAb24, and by plate-immobilized OX40mAb24 in the absence of anti-CD3 mAb. (7C) CD4 T cell proliferation of four independent donors mediated by plate-immobilized 9B12 in combination with sub-mitogenic TCR stimulation. Symbols, mean values; Error bars, standard deviation of the mean; n=3 technical replicates for OX40mAb24, R347 human IgG1 control mAb,

and 9B12 all in combination with anti-CD3; n=2 technical replicates for soluble OX40mAb24, and CD3 in the absence of OX40mAb24, plate bound OX40mAb24 with no anti-CD3, and soluble OX40mAb24 with no anti-CD3.

[0031] Figure 8A-E. Human CD4⁺ T cell Cytokine Release in Response to OX40mAb24. Representative OX40mAb24 induced human CD4 T cell cytokine release for Donor 651, including (8A) IFN γ , (8B) TNF α (8C) IL-10 (8D) IL-13, and (8E) IL-5. Symbols, mean values; Error bars, standard deviation of the mean; n=3 technical replicates for OX40mAb24 and R347 human IgG1 control mAb, both in combination with anti-CD3; n=2 technical replicates for soluble OX40mAb24, soluble OX40mAb24 with no anti-CD3, and anti-CD3 in the absence of OX40mAb24.

[0032] Figure 9A-E. Human CD4⁺ T cell Cytokine Release in Response to 9B12. Representative 9B12 induced human CD4 T cell cytokine release for Donor 651, including (9A) IFN γ , (9B) TNF α (9C) IL-10 (9D) IL-13, and (9E) IL-5. Symbols, mean values; Error bars, standard deviation of the mean; n=3 technical replicates for 9B12 and mouse IgG1 control mAb, both in combination with anti-CD3; n=2 technical replicates for soluble 9B12, soluble 9B12 with no anti-CD3, and anti-CD3 in the absence of 9B12.

[0033] Figure 10 is a schematic illustrating cell systems used for measuring OX40mAb24 and 9B12 bioactivity. OX40mAb24 cross linking by Fc γ R-expressing cells mediates the clustering and activation of OX40 on the cell surface of an OX40-expressing Jurkat NF κ B-luciferase reporter cell line, resulting in the NF κ B-mediated production of luciferase that can be measured as a surrogate for OX40 activation. Fc γ R = fragment crystallizable gamma receptor; NF κ B = nuclear factor kappa B.

[0034] Figure 11A-D. Bioactivity of OX40mAb24 in OX40-expressing Jurkat NF κ B Reporter Cells With and Without Cross-linking by Fc γ R. Concentration-dependent activity (in RLU) of OX40mAb24-induced signaling through human OX40 expressed on the cell surface of a Jurkat NF κ B-luciferase reporter cell line in a 2-cell bioassay. Reporter activity after cross-linking of OX40mAb24 by (11A) CD32A-expressing HEK293 cells, and HEK293 parental cells (HEK), or in the presence of (11B) CD32B-expressing HEK293 cells, (11C) Raji B cells, or (11D) CD45⁺ cells isolated from a primary lung tumor. Data is representative of other results found in Table 5-2. mAb = monoclonal antibody, RLU = relative light units.

- [0035]** Figure 12A-D. Bioactivity of 9B12 in OX40-expressing Jurkat NFκB Reporter Cells using FcγR Cross-linking by Different Cell Types in 2-Cell Bioactivity Assays. Concentration-dependent activity (in RLU) of 9B12 induced signaling through human OX40 expressed on the cell surface of a Jurkat NFκB-luciferase reporter cell lines in a two-cell bioassay. Reporter activity after cross-linking of 9B12 by (12A) CD32A-expressing HEK293 cells, (12B) CD32B-expressing HEK293 cells, (12C) Raji B cells, or (12D) CD45⁺ cells isolated from a primary lung tumor. Data is representative of other results found in Table 5-3. mAb = monoclonal antibody; RLU = relative light units.
- [0036]** Figure 13A-B. Natural Killer Cell-mediated Antibody-Dependent Cellular Cytotoxicity of OX40mAb24, Experiment 1. (13A) Specific killing of OX40-expressing activated CD4⁺ T cells by human NK cells from an allogeneic, left, or autologous, right, NK and CD4⁺ T cell donor pairs using 10 μ/mL of 9B12, OX40mAb24, or the IgG1 triple mutant (mAb29) or human IgG4P (mAb28) versions of OX40mAb24. (13B) Lysis of Toledo B cells by NK cells from donors 350 and 351 in the presence of rituximab, but not R347 human IgG1 isotype control antibody. Technical replicates were conducted in triplicate. Error bars represent standard error of the mean. ADCC = antibody-drug-dependent-cytotoxicity; mAb = monoclonal antibody; NK = natural killer.
- [0037]** Figure 14A-B. Natural Killer Cell-mediated Antibody-Dependent Cellular Cytotoxicity of OX40mAb24, Experiment 2. (14A) Specific killing of OX40-expressing activated CD4 T cells by human NK cells from an allogeneic, left, or autologous, right, NK and CD4 T cell donor pairs using 10 μ/mL of control R347 human IgG1, 9B12, OX40mAb24, the human IgG4P (mAb28) or the IgG1 triple mutant (mAb29) versions of OX40mAb24. (14B) Lysis of Toledo B cells by NK cells from donors 558 and 589 in the presence of rituximab, but not R347 human IgG1 isotype control antibody. Technical replicates were conducted in triplicate. Error bars represent standard error of the mean. ADCC = antibody-drug-dependent-cytotoxicity; mAb = monoclonal antibody; NK = natural killer.
- [0038]** Figure 15A-D. Assessment of Natural Killer Cell-mediated Antibody-Dependent Cellular Cytotoxicity of OX40mAb24 and 9B12, Experiment 3. (15A-B) Specific killing of OX40-expressing human CD4 T cells mediated by OX40mAb24, but not by 9B12, using primary human NK cells from two separate donors as indicated. (15C-D) Lysis of Toledo B cells by NK cells from donors 363 and 504 in the presence of rituximab, but not R347 human IgG1 isotype control

antibody. Technical replicates were conducted in duplicate. Error bars represent standard error of the mean. ADCC = antibody-drug-dependent-cytotoxicity; mAb = monoclonal antibody; NK = natural killer.

[0039] Figure 16A-D. Assessment of Natural Killer Cell-mediated Antibody-Dependent Cellular Cytotoxicity of OX40mAb24, Experiment 4. (16A-B) Specific killing of OX40-expressing human CD4 T cells mediated by OX40mAb24 using primary human NK cells from two separate donors as indicated. (16C-D) Lysis of Toledo B cells by NK cells from donors 464 and 532 in the presence of rituximab, but not R347 human IgG1 isotype control antibody. Technical replicates were conducted in duplicate. Error bars represent standard error of the mean. ADCC = antibody-drug-dependent-cytotoxicity; mAb = monoclonal antibody; NK = natural killer.

[0040] Figure 17A-B. Assessment of Natural Killer Cell-mediated Antibody-Dependent Cellular Cytotoxicity of OX40mAb24, Experiment 5. Specific killing of OX40-expressing human CD4⁺ T cells mediated by OX40mAb24, using primary human NK cells from donors 601 (panel A) and 602 (panel B) as indicated. Error bars represent standard error of the mean. ADCC = antibody-drug-dependent-cytotoxicity; mAb = monoclonal antibody; NK = natural killer.

[0041] Figure 18A-B. Assessment of OX40mAb24 and 9B12 binding to purified human C1q protein. The indicated concentrations of purified human C1q protein were injected onto the biosensor chip. Blank represents injections of PBS/0.005% Tween 20 vehicle alone. Panel A: OX40mAb24 binding to purified human C1q. Panel B: 9B12 binding to purified human C1q.

[0042] Figure 19A-B. OX40mAb24 Activity in Cyno/Rhesus OX40-expressing Jurkat NFκB-luciferase Clone B2 and LCL8664 Rhesus B-Cell Bioactivity Assays. Concentration-dependent induction of NFκB activity by OX40mAb24 (in RLU) in a cyno/rhesus OX40 expressing Jurkat NFκB-luciferase reporter cell line combined with rhesus B-cell line LCL8664. Data shown for 2 independent assays with 4 replicates for each data point. Error bars represent standard error of the mean are not visible due to scale. RLU = relative light units.

[0043] Figure 20A-B. 9B12 Activity in Cyno/Rhesus OX40-expressing Jurkat NFκB-luciferase Clone B2 and LCL8664 Rhesus B-Cell Bioactivity Assays. Concentration-dependent induction of NFκB activity by 9B12 (in RLU) in a cyno/rhesus OX40 expressing Jurkat NFκB-luciferase reporter cell line combined with rhesus B-cell line LCL8664. Data shown for 2 independent assays

with 4 replicates for each data point. Error bars represent standard error of the mean. RLU = relative light units.

[0044] Figure 21A-B. OX40mAb24 and 9B12 Activity in Cyno/Rhesus OX40-expressing Jurkat NFκB-luciferase Clone B2 and Fc Gamma Receptor-Expressing Rhesus Immune Cells Bioactivity Assay. Concentration-dependent induction of NFκB activity by OX40mAb24 (Panel A) and 9B12 (Panel B) (in RLU) in a cyno/rhesus OX40 expressing Jurkat NFκB-luciferase reporter cell line combined with Fcγ receptor-expressing rhesus immune cells. Two replicates per data point. RLU = relative light units.

[0045] Figure 22A-D. OX40mAb24 and 9B12 Cause Proliferation of Primary Rhesus CD4 T Cells. OX40mAb24 (22A-B) and 9B12 (22C-D) induced cell division in primary activated rhesus CD4⁺ T cells. Data is shown for 2 independent assays with triplicate wells. Error bars represent standard error of the mean.

[0046] Figure 23A-B. Effect of OX40mAb24 and 9B12 on Growth of A375 Cells in a Mouse Xenograft Model – Experiment 1. Six NOD/SCID mice in each group were engrafted SC on Day 1 with A375 cells mixed with alloreactive human CD4⁺ and CD8⁺ T cell lines at E:T ratio 1:6. OX40mAb24 (23A) and 9B12 (23B) and isotype control (23A-B) were administered IP on Days 3, 5, 7, 10 and 12. Mean values of tumor volumes are shown. A comparison between OX40mAb24-treated (23A) or 9B12-treated (23B) and the isotype control-treated animals was made on Day 25 and 18, respectively, and intergroup differences were analyzed for statistical significance by a Mann-Whitney rank sum test. Error bars represent standard error of the mean. *: TGI > 68%, $P \leq 0.05$ as compared to the isotype-control group. E:T = effector-to-target ratio; IP intraperitoneal; NOD/SCID = non-obese diabetic/severe combined immunodeficient; SC = subcutaneous; TGI = tumor growth inhibition.

[0047] Figure 24A-B. Effect of OX40mAb24 and 9B12 on Growth of A375 Cells in a Mouse Xenograft Model – Experiment 2. Six NOD/SCID mice in each group were engrafted SC on Day 1 with A375 cells mixed with alloreactive human CD4⁺ and CD8⁺ T cell lines at E:T ratio 1:6. OX40mAb24 (24A) and 9B12 (24B) and isotype control (24A-B) were administered IP on Days 3, 5, 7, 10 and 12. Mean values of tumor volumes are shown. A comparison between OX40mAb24-treated (12A) or 9B12-treated (12B) and the isotype control-treated animals was made on Day 18, and intergroup differences were analyzed for statistical significance by a Mann-

Whitney rank sum test. Error bars represent standard error of the mean. #: TGI $\geq 75\%$, $P \leq 0.0004$ as compared to the isotype-control group. *: TGI = 53%, $P \leq 0.05$ as compared to the isotype-control group. E:T = effector-to-target ratio; IP = intraperitoneal; NOD/SCID = non-obese diabetic/severe combined immunodeficient; SC = subcutaneous; TGI = tumor growth inhibition.

[0048] Figure 25. Effect of OX40mAb24 on Growth of A375 Cells in a Mouse Xenograft Model – Experiment 3. Six NOD/SCID mice in each group were engrafted SC on Day 1 with A375 cells mixed with alloreactive human CD4⁺ and CD8⁺ T cell lines at E:T ratio 1:6. OX40mAb24 was administered IP on Days 3, 6, 8, 10 and 13. Mean values of tumor volumes are shown. A comparison between OX40mAb24-treated and the isotype control-treated animals was made on Day 28, and intergroup differences were analyzed for statistical significance by a Mann-Whitney rank sum test. Error bars represent standard error of the mean. *: TGI = 75%, $P \leq 0.05$ as compared to the isotype-control group; E:T = effector-to-target ratio; IP = intraperitoneal; NOD/SCID = non-obese diabetic/severe combined immunodeficient; SC = subcutaneous; TGI = tumor growth inhibition.

[0049] Figure 26A-B. Effect of OX86 mIgG2a on Growth of CT26 Cell Line in a Mouse Syngeneic Model. Ten BALB/c mice in each group were inoculated SC on Day 1 with CT26 cells. Control (negative control) and test article (OX86 mIgG2a) were administered IP on Days 9 and 12 (arrows in (26A)). Mean (26A) and individual (26B) values of tumor volumes are shown. A comparison between OX86 mIgG2a-treated and the negative control-treated animals was made, and intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software. Error bars represent standard error of the mean. *: TGI > 50%, $P \leq 0.0001$ as compared to the isotype-control group on Day 21. IP = intraperitoneal; SC = subcutaneous; TGI = tumor growth inhibition.

[0050] Figure 27A-B. Effect of OX86 mIgG2a on Growth of CT26 Cell Line in a Mouse Syngeneic Model. Twelve BALB/c mice in each group were inoculated SC on Day 1 with CT26 cells. Test article (OX86 mIgG2a) was administered IP on Days 13 and 16 (arrows in (27A)). Mean (27A) and individual (27B) values of tumor volumes are shown. A comparison between OX86 mIgG2a-treated and the untreated control animals was made, and intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software. Error bars

represent standard error of the mean. *: TGI > 50%, $P \leq 0.0001$ as compared to the untreated control group on Day 24. IP = intraperitoneal; SC = subcutaneous; TGI = tumor growth inhibition.

[0051] Figure 28A-B. Effect of OX86 mIgG2a on Growth of MCA205 Cell Line in a Mouse Syngeneic Model. Fourteen C57BL/6 mice in each group were inoculated SC on Day 1 with MCA205 cells. Control article (isotype control) and test article (OX86 mIgG2a) were administered IP on Days 11 and 14. Mean (28A) and individual (28B) values of tumor volumes are shown. A comparison between OX86 mIgG2a-treated and the isotype control-treated animals was made, and intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software. Error bars represent standard error of the mean. *: TGI > 50%, $P \leq 0.0001$ as compared to the isotype-control group on Day 27. IP = intraperitoneal; SC = subcutaneous; TGI = tumor growth inhibition.

[0052] Figure 29A-B. Effect of OX86 mIgG2a on Growth of 4T1 Cell Line in a Mouse Syngeneic Model. Twelve BALB/c mice in each group were inoculated S on Day 1 with 4T1 cells. Control article (isotype control) and test article (OX86 mIgG2a) were administered IP on Days 13, 16, 20, and 23. Mean (29A) and individual (29B) values of tumor volumes are shown. A comparison between OX86 mIgG2a-treated and the isotype control-treated animals was made, and intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software. Error bars represent standard error of the mean. IP = intraperitoneal; SC = subcutaneous; TGI = tumor growth inhibition.

[0053] Figure 30. Amino Acid Alignment of the Extracellular Domains of Human and Mouse OX40 molecules. Human OX40 (NCBI reference sequence NP_003318.1) shares 60% sequence identity with mouse OX40 (NCBI reference sequence NP_035789.1). The alignment was performed using the method of Clustal W. The extracellular domains of OX40 are detailed. Amino acids that differ between human and mouse in the CRD3 domain are shown with arrows. The two critical epitope residues L116 and A126 are boxed. CRD = cysteine-rich domain.

[0054] Figure 31. Nomenclature and Schematic Representation of Chimeric Human/Mouse OX40 Variants. Chimeric human/mouse OX40 variants were constructed by swapping in or out various domains or residues of mouse OX40 (open) into human (solid) (KO) or of human OX40 amino acids into mouse OX40 (KI). Mutations for individual amino acids or combinations were shown

with a red (KO) and green (KI) arrows. CRD = cysteine-rich domain; KI = knock-in; KO = knock-out; TM = transmembrane domain.

[0055] Figure 32A-C. FACS Analysis of Binding of OX40mAb24 to Chimeric Human/Mouse OX40 Variants. All variants were transiently expressed using 293F cells for binding characterization with FACS analysis using OX40mAb24 and its parental mouse mAb, 9B12. Expression levels were monitored using anti-human and mouse OX40 polyclonal antibodies. (A) Using domain-swapped chimeric variants, the CRD3 domain was identified as the epitope-containing domain. OX40mAb24 and 9B12 do not bind the KO variants encoding for mouse CRD3 domain (KO_CRD3 and KO_CRD3+4), and recognize the KI variant (KI_CRD3) encoding for human CRD3 domain. (B) Additionally, critical epitope residues were determined as L116 and A126 in CRD3 domain by mutating individual or combinations of amino acids, which differ between human and mouse in the CRD3 domain (Figure 11-1). The binding of OX40mAb24 and 9B12 was abolished when replacing human residues L116 and A126 with the mouse counterparts (KO_L116+A126). (C) The KI/gain-of-function variants confirm the importance of these two critical residues. Grafting L116, A126, or the combination to mouse OX40 led to the binding of OX40mAb24 and 9B12.

[0056] Figure 33A-D. Expression Levels of Ki67 and ICOS on Peripheral Blood and Splenic CD4⁺ T cells Following Administration of OX86 mIgG2a to Naïve Mice. Seven naïve BALB/c mice in each group were inoculated intraperitoneally on Day 1 with control articles (saline and NIP228 IgG2a isotype control) and test article (OX86 mIgG2a) at the indicated dose levels. Blood (panels A and C) was collected on the indicated Days and spleens from five groups were isolated on Day 10. Expression levels of Ki67 (panel A and B) and ICOS (panels C and D) on CD4⁺ T cells were measured by flow cytometry. Mean values of the percentage of CD4 cells in blood expressing Ki67 (panel A) and ICOS (panel C) are shown for each group. Percentage of CD4 cells in the spleen expressing Ki67 (panel B) and ICOS (panel D) were plotted for each animal of individual groups. Error bars represent the standard error of the mean. *: $P \leq 0.05$ are marked in Panels A and C; P values are listed for each group with significance in Panels B and D.

[0057] Figure 34A-B. Correlation of Ki67 and ICOS Expression on Peripheral Blood and Splenic CD4⁺ T cells Following Administration of OX86 mIgG2a to Naïve Mice. A comparison was made between the percentage of Ki67 and ICOS positive CD4 T cells isolated from peripheral blood

(panel A) and spleens (panel B) of individual animals shown in Figure 12-1 10 days following treatment with control articles (saline and NIP228 IgG2a isotype control) and test article (OX86 mIgG2a) at the indicated dose levels. Measurements for individual mice were plotted. Linear regression analysis was performed using GraphPad Prism 6.0 software on the resulting group of data sets to determine a best-fit line for the data. The coefficient of determination (r^2) and significance that the slope is non-zero (P value) are provided for each graph.

[0058] Figure 35A-D. Expression Levels of Ki67 and ICOS on Peripheral Blood and Splenic CD8⁺ T cells Following Administration of OX86 mIgG2a to Naïve Mice. Seven naïve BALB/c mice in each group were inoculated intraperitoneally on Day 1 with control articles (saline and NIP228 IgG2a isotype control) and test article (OX86 mIgG2a) at the indicated dose levels. Blood (panels A and C) was collected on the indicated Days and spleens from five groups were isolated on Day 10. Expression levels of Ki67 (panel A and B) and ICOS (panels C and D) on CD8⁺ T cells were measured by flow cytometry. Mean values of the percentage of CD8 cells in blood expressing Ki67 (panel A) and ICOS (panel C) are shown for each group. Percentage of CD8 cells in the spleen expressing Ki67 (panel B) and ICOS (panel D) were plotted for each animal of individual groups. Error bars represent the standard error of the mean. *: $P \leq 0.05$ are marked in Panels A and C; P values are listed for each group with significance in Panels B and D.

[0059] Figure 36A-B. Correlation of Ki67 and ICOS Expression on Peripheral Blood and Splenic CD8⁺ T cells Following Administration of OX86 mIgG2a to Naïve Mice. A comparison was made between the percentage of Ki67 and ICOS positive CD8⁺ T cells isolated from peripheral blood (panel A) and spleens (panel B) of individual animals shown in Figure 12-1 10 days following treatment with control articles (saline and NIP228 IgG2a isotype control) and test article (OX86 mIgG2a) at the indicated dose levels. Measurements for individual mice were plotted. Linear regression analysis was performed using GraphPad Prism 6.0 software on the resulting group of data sets to determine a best-fit line for the data. The coefficient of determination (r^2) and significance that the slope is non-zero (P value) are provided for each graph.

[0060] Figure 37A-D. Effect of Mouse OX86 IgG2a on Growth of the CT26 Cell Line in Syngeneic Mouse Model Lacking Inhibitory (Fcgr2b^{-/-}) or Activating (Fcer1g^{-/-}) Fc Gamma Receptors. Groups of eight Balb/c mice genetically engineered to lack the inhibitory Fcγ receptor IIb (Fcgr2b^{-/-}; panels A and B) or the activating Fcγ receptors (Fcer1g^{-/-}; panels C and D) were inoculated SC

on Day 1 with CT26 cells. Control articles (saline/untreated and OX86 mIgG1 D265A mutant/isotype control) and test article (OX86 mIgG2a) were administered IP on Days 4 and 7. Mean (panel A and C) and individual (panel B and D) tumor volumes are shown. A comparison between OX86 mIgG2a-treated and the untreated and isotype control-treated animals was made, and intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software. Error bars represent standard error of the mean. SC = subcutaneous; IP = intraperitoneal; TGI = tumor growth inhibition

[0061] Figure 38A-D. Effect of Mouse OX86 IgG2a on Growth of the MCA205 Cell Line in a Syngeneic Mouse Model Lacking Inhibitory (Fcgr2b^{-/-}) or Activating (Fcer1g^{-/-}) Fc Gamma Receptors. Groups of eight C57BL/6 mice genetically mutated to lack the inhibitory Fcγ receptor IIb (Fcgr2b^{-/-}; panels A and B) or the activating Fcγ receptors (Fcer1g^{-/-}; panels C and D) were inoculated SC on Day 1 with MCA205 cells. Control articles (saline/untreated and OX86 mIgG1 D265A mutant/isotype control) and test article (mOX40L FP) were administered IP on Days 4 and 7. Mean (panel A and C) and individual (panel B and D) tumor volumes are shown. A comparison between mOX40L FP-treated and the untreated and isotype control-treated animals was made, and intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software. Error bars represent standard error of the mean. *: TGI ≥ 95%, $P \leq 0.023$ as compared to the untreated and isotype-control groups on study day 20. SC = subcutaneous; IP = intraperitoneal; TGI = tumor growth inhibition

[0062] Figure 39A-B. Expression Levels of Ki67 in CD4⁺ T cells Isolated from Peripheral Blood, Spleen and CT26 Tumor Following Administration of OX86 Mouse IgG2a to Mice Lacking Inhibitory (Fcgr2b^{-/-}) or Activating (Fcer1g^{-/-}) Fc Gamma Receptor. Groups of four Balb/c mice genetically engineered to lack the inhibitory Fcγ receptor IIb (Fcgr2b^{-/-}; panels A) or the activating Fcγ receptors (Fcer1g^{-/-}; panels B) were inoculated SC on Day 1 with CT26 cells; this study is independent of, but was conducted similarly to, the study presented in Figure 12-5. Control articles (saline/untreated and OX86 mIgG1 D265A mutant/isotype control) and test article (OX86 mIgG2a) were administered IP on Days 4 and 7. Blood, spleens and tumors were isolated on Day 14 for panel A and Day 13 for panel B. Expression levels of Ki67 in CD4⁺ T cells were measured by flow cytometry. Symbols represent the percentage of Ki67 positive CD4⁺ T cells from each tissue of individual mice; horizontal bar represents the mean values for each group.

Intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software, and indicated with a horizontal bar with the calculated P values. SC = subcutaneous; IP = intraperitoneal

[0063] Figure 40A-B. Expression Levels of Ki67 in CD8⁺ T cells Isolated from Peripheral Blood, Spleen and CT26 Tumor Following Administration of OX86 Mouse IgG2a to Mice Lacking Inhibitory (Fcgr2b^{-/-}) or Activating (Fcer1g^{-/-}) Fc Gamma Receptors. Groups of four Balb/c mice, genetically engineered to lack the inhibitory Fcγ receptor IIb (Fcgr2b^{-/-}; panels A) or the activating Fcγ receptors (Fcer1g^{-/-}; panels B), were inoculated SC on Day 1 with CT26 cells; this study is independent of but was conducted similarly to the study presented in Figure 12-5. Control articles (saline/untreated and OX86 mIgG1 D265A mutant/isotype control) and test article (OX86 mIgG2a) were administered IP on Days 4 and 7. Blood, spleens and tumor were isolated on Day 14 for panel A and Day 13 for panel B. Expression levels of Ki67 on CD8⁺ T cells were measured by flow cytometry. Symbols represent the percentage of Ki67 positive CD8⁺ T cells from each tissue of individual mice; horizontal bar represents the mean values. Intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software, and indicated with a horizontal bar with the calculated P values. SC = subcutaneous; IP = intraperitoneal

[0064] Figure 41A-B. Expression Levels of Ki67 in CD4 T cells Isolated from Draining Lymph Node, Spleen and MCA205 Tumor Following Administration of OX86 Mouse IgG2a to Mice Lacking Inhibitory (Fcgr2b^{-/-}) or Activating (Fcer1g^{-/-}) Fc Gamma Receptors. Groups of four C57BL/6 mice, genetically engineered to lack the inhibitory Fcγ receptor IIb (Fcgr2b^{-/-}; panels A) or the activating Fcγ receptors (Fcer1g^{-/-}; panels B), were inoculated SC on Day 1 with MCA205 cells; study is independent but conducted similarly to the study presented in Figure 12-6. Control articles (saline/untreated and OX86 mIgG1 D265A mutant/isotype control) and test article (OX86 mIgG2a) were administered IP on Days 4 and 7. Draining lymph nodes, spleens and tumor were isolated on Day 20 for panel B. Expression levels of Ki67 in CD4⁺ T cells were measured by flow cytometry. Symbols represent the percentage of Ki67 positive CD4⁺ T cells from each tissue of individual mice; horizontal bar represents the mean values. Intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software, and

indicated with a horizontal bar with the calculated P values. SC = subcutaneous; IP = intraperitoneal

[0065] Figure 42A-B. Expression Levels of Ki67 in CD8 T cells Isolated from Draining Lymph Node, Spleen and MCA205 Tumor Following Administration of OX86 Mouse IgG2a to Mice Lacking Inhibitory (Fcgr2b^{-/-}) or Activating (Fcer1g^{-/-}) Fc Gamma Receptors. Groups of four C57BL/6 mice, genetically engineered to lack the inhibitory Fcγ receptor IIb (Fcgr2b^{-/-}; panels A) or the activating Fcγ receptors (Fcer1g^{-/-}; panels B), were inoculated SC on Day 1 with MCA205 cells; this study is independent of but was conducted similarly to the study presented in Figure 12-6. Control articles (saline and mOX40L FP D265A mutant control) and test article (OX86 mIgG2a) were administered IP on Days 4 and 7. Draining lymph nodes, spleens and tumor were isolated on Day 20. Expression levels of Ki67 on CD8⁺ T cells were measured by flow cytometry. Symbols represent the percentage of Ki67 positive CD8⁺ T cells from each tissue of individual mice; horizontal bar represents the mean values. Intergroup differences were analyzed for statistical significance by a one-way ANOVA using GraphPad Prism 6.0 software, and indicated with a horizontal bar with the calculated P values. SC = subcutaneous; IP = intraperitoneal

[0066] Figure 43A-B. Reduction of Regulatory T Cells by OX40mAb24. Panel A: Percentages of human CD4⁺Foxp3⁺ Treg cells measured in the peripheral blood of mice engrafted with human immune cells just prior to KLH immunization and treatment with NIP228 huIgG1 control (huIgG1) or OX40mAb24. No mAb indicates no immunization and no mAb treatment. Panel B: Percentages of human CD4⁺FoxP3⁺ Treg cells in the peripheral blood of mice in the same groups after immunization and treatment with mAbs. Statistical p values are from one-way ANOVA. Hu = human; mAb = monoclonal antibody; Treg = regulatory T cells

[0067] Figure 44A-D. Expansion of Effector and Memory CD4 T Cells Relative to Regulatory T Cells after OX40mAb24 Treatment. Ratios of human CD4⁺ T cells to human Treg cells in the peripheral blood of mice engrafted with human immune cells either pre-treatment (left) or post-treatment (right) with KLH immunization followed by NIP228 huIgG1 mAb (huIgG1) or OX40mAb24, as indicated, for Total CD4⁺ (Panel A); CD4⁺ effector (Teff) (Panel B); CD4⁺ effector memory (Tem) (Panel C); and CD4⁺ central memory T cells (Tcm) (Panel D). No mAb indicates no immunization as well as no mAb treatment. Statistical p values are from one-way

ANOVA. Hu = human; mAb = monoclonal antibody; Tcm = central memory T cell; Teff = effector T cell; Tem = effector memory T cell

[0068] Figure 45A-B. Increased CD8⁺ Effector to Regulatory T Cell Ratio in Mice Treated with OX40mAb24. Ratios of human CD8⁺ effector T cells to human Treg cells in the peripheral blood of mice engrafted with human immune cells either pre-treatment (left) or post-treatment (right) with KLH immunization followed by NIP228 huIgG1 mAb (huIgG1) or OX40mAb24, as indicated. No mAb indicates no immunization as well as no mAb treatment. Statistical p value from one-way ANOVA comparing all groups is indicated. Hu = human; mAb = monoclonal antibody; Teff = effector T cell; Treg = regulatory T cell

[0069] Figure 46A-C. Increased CD25 (IL-2 Receptor) Levels on CD8⁺ T Cells in Mice Treated with OX40mAb24. Percentage of human CD25 (IL-2 receptor) positive cells among human CD8⁺ T cells in the peripheral blood of mice engrafted with human immune cells either post-treatment (left) or pre-treatment (right) with no treatment (no mAb), or KLH immunization followed by NIP228 huIgG1 mAb (huIgG1) or OX40MAB24, as indicated, for (A) Total CD8⁺, (B) CD8⁺ effector (Teff), (C) CD8⁺ effector memory (Tem) T cells. No mAb indicates no immunization as well as no mAb treatment. Statistical p values are from one-way ANOVA. Hu = human; mAb = monoclonal antibody; Teff = effector T cell; Tem = effector memory T cell.

DETAILED DESCRIPTION

[0070] Engagement of the OX40 receptor on T cells, *e.g.*, CD4⁺ T cells during, or shortly after, priming by an antigen results in an increased response of the T cells, *e.g.*, CD4⁺ T cells to the antigen. In the context of the present disclosure, the term "engagement" refers to binding to and stimulation of at least one activity mediated by the OX40 receptor. For example, engagement of the OX40 receptor on antigen specific T cells, *e.g.*, CD4⁺ T cells can result in increased T cell proliferation as compared to the response to antigen alone, and increased cytokine production. The elevated response to the antigen can be maintained for a period of time substantially longer than in the absence of OX40 receptor engagement. Thus, stimulation via the OX40 receptor enhances the antigen specific immune response by boosting T cell recognition of antigens, *e.g.*, tumor antigens.

[0071] OX40 agonists can enhance antigen specific immune responses in a subject, such as a human subject, when administered to the subject during or shortly after priming of T cells by an

antigen. OX40 agonists include OX40 ligand ("OX40L"), such as soluble OX40L fusion proteins and anti-OX40 antibodies or fragments thereof. A specific example is a humanized antibody that specifically binds to OX40, thereby triggering signaling. A collection of humanized anti-OX40 monoclonal antibodies are provided by this disclosure. Also described are nucleic acids including polynucleotide sequences that encode such antibodies. This disclosure also provides methods for enhancing an antigen specific immune response in a subject using humanized anti-OX40 monoclonal antibodies.

DEFINITIONS

[0072] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a binding molecule," is understood to represent one or more binding molecules. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0073] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following embodiments: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0074] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0075] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or aspects of the disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0076] As used herein, the term “non-naturally occurring” substance, composition, entity, and/or any combination of substances, compositions, or entities, or any grammatical variants thereof, is a conditional term that explicitly excludes, but only excludes, those forms of the substance, composition, entity, and/or any combination of substances, compositions, or entities that are, or that might be at any time, determined or interpreted by a judge or an administrative or judicial body to be, “naturally-occurring.”

[0077] As used herein, the term “polypeptide” is intended to encompass a singular “polypeptide” as well as plural “polypeptides,” and refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term “polypeptide” refers to any chain or chains of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, “protein,” “amino acid chain,” or any other term used to refer to a chain or chains of two or more amino acids are included within the definition of “polypeptide,” and the term “polypeptide” can be used instead of, or interchangeably with any of these terms. The term “polypeptide” is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, and derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide can be derived from a biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It can be generated in any manner, including by chemical synthesis.

[0078] A polypeptide as disclosed herein can be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides can have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides which do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded. As used herein, the term glycoprotein refers to a *protein* coupled to at least one carbohydrate moiety that is attached to the protein via an oxygen-containing or a nitrogen-containing side chain of an amino acid, *e.g.*, a serine or an asparagine.

[0079] By an "isolated" polypeptide or a fragment, variant, or derivative thereof is intended a polypeptide that is not in its natural milieu. No particular level of purification is required. For example, an isolated polypeptide can be removed from its native or natural environment. Recombinantly produced polypeptides and proteins expressed in host cells are considered isolated as disclosed herein, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique.

[0080] As used herein, the term "non-naturally occurring" polypeptide, or any grammatical variants thereof, is a conditional term that explicitly excludes, but only excludes, those forms of the polypeptide that are, or that might be at any time, determined or interpreted by a judge or an administrative or judicial body to be, "naturally-occurring."

[0081] Other polypeptides disclosed herein are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof. The terms "fragment," "variant," "derivative" and "analog" as disclosed herein include any polypeptides which retain at least some of the properties of the corresponding native antibody or polypeptide, for example, specifically binding to an antigen. Fragments of polypeptides include, for example, proteolytic fragments, as well as deletion fragments, in addition to specific antibody fragments discussed elsewhere herein. Variants of, *e.g.*, a polypeptide include fragments as described above, and also polypeptides with altered amino acid sequences due to amino acid substitutions, deletions, or insertions. In certain aspects, variants can be non-naturally occurring. Non-naturally occurring variants can be produced using art-known mutagenesis techniques. Variant polypeptides can comprise conservative or non-conservative amino acid substitutions, deletions or additions. Derivatives are polypeptides that have been altered so as to exhibit additional features not found on the original polypeptide. Examples include fusion proteins. Variant polypeptides can also be referred to herein as "polypeptide analogs." As used herein a "derivative" of a polypeptide can also refer to a subject polypeptide having one or more amino acids chemically derivatized by reaction of a functional side group. Also included as "derivatives" are those peptides that contain one or more derivatives of the twenty standard amino acids. For example, 4-hydroxyproline can be substituted for proline; 5-hydroxylysine can be substituted for lysine; 3-methylhistidine can be substituted for histidine; homoserine can be substituted for serine; and ornithine can be substituted for lysine.

[0082] A "conservative amino acid substitution" is one in which one amino acid is replaced with another amino acid having a similar side chain. Families of amino acids having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). For example, substitution of a phenylalanine for a tyrosine is a conservative substitution. In certain embodiments, conservative substitutions in the sequences of the polypeptides and antibodies of the present disclosure do not abrogate the binding of the polypeptide or antibody containing the amino acid sequence, to the antigen to which the binding molecule binds. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate antigen-binding are well-known in the art (see, *e.g.*, Brummell *et al.*, *Biochem.* 32: 1180-1 187 (1993); Kobayashi *et al.*, *Protein Eng.* 12(10):879-884 (1999); and Burks *et al.*, *Proc. Natl. Acad. Sci. USA* 94:412-417 (1997)).

[0083] The term "polynucleotide" is intended to encompass a singular nucleic acid as well as plural nucleic acids, and refers to an isolated nucleic acid molecule or construct, *e.g.*, messenger RNA (mRNA), cDNA, or plasmid DNA (pDNA). A polynucleotide can comprise a conventional phosphodiester bond or a non-conventional bond (*e.g.*, an amide bond, such as found in peptide nucleic acids (PNA)). The terms "nucleic acid" or "nucleic acid sequence" refer to any one or more nucleic acid segments, *e.g.*, DNA or RNA fragments, present in a polynucleotide.

[0084] By an "isolated" nucleic acid or polynucleotide is intended any form of the nucleic acid or polynucleotide that is separated from its native environment. For example, gel-purified polynucleotide, or a recombinant polynucleotide encoding a polypeptide contained in a vector would be considered to be "isolated." Also, a polynucleotide segment, *e.g.*, a PCR product, which has been engineered to have restriction sites for cloning is considered to be "isolated." Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in a non-native solution such as a buffer or saline. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of polynucleotides, where the transcript is not one that would be found in nature.

Isolated polynucleotides or nucleic acids further include such molecules produced synthetically. In addition, polynucleotide or a nucleic acid can be or can include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

[0085] As used herein, a “non-naturally occurring” polynucleotide, or any grammatical variants thereof, is a conditional definition that explicitly excludes, but only excludes, those forms of the polynucleotide that are, or that might be at any time, determined or interpreted by a judge or an administrative or judicial body to be, “naturally-occurring.”

[0086] As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it can be considered to be part of a coding region, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, *e.g.*, on a single vector, or in separate polynucleotide constructs, *e.g.*, on separate (different) vectors. Furthermore, any vector can contain a single coding region, or can comprise two or more coding regions, *e.g.*, a single vector can separately encode an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region. In addition, a vector, polynucleotide, or nucleic acid can include heterologous coding regions, either fused or unfused to another coding region. Heterologous coding regions include without limitation, those encoding specialized elements or motifs, such as a secretory signal peptide or a heterologous functional domain.

[0087] In certain embodiments, the polynucleotide or nucleic acid is DNA. In the case of DNA, a polynucleotide comprising a nucleic acid which encodes a polypeptide normally can include a promoter and/or other transcription or translation control elements operably associated with one or more coding regions. An operable association is when a coding region for a gene product, *e.g.*, a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the

expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of that nucleic acid. The promoter can be a cell-specific promoter that directs substantial transcription of the DNA in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription.

[0088] A variety of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (the immediate early promoter, in conjunction with intron-A), simian virus 40 (the early promoter), and retroviruses (such as Rous sarcoma virus). Other transcription control regions include those derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit β -globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (*e.g.*, promoters inducible by interferons or interleukins).

[0089] Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from picornaviruses (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence).

[0090] In other embodiments, a polynucleotide can be RNA, for example, in the form of messenger RNA (mRNA), transfer RNA, or ribosomal RNA.

[0091] Polynucleotide and nucleic acid coding regions can be associated with additional coding regions which encode secretory or signal peptides, which direct the secretion of a polypeptide encoded by a polynucleotide as disclosed herein. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells can have a signal peptide fused to the N-terminus of the polypeptide, which is

cleaved from the complete or "full length" polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, *e.g.*, an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, can be used. For example, the wild-type leader sequence can be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse β -glucuronidase.

[0092] Disclosed herein are certain binding molecules, or antigen-binding fragments, variants, or derivatives thereof. Unless specifically referring to full-sized antibodies, the term "binding molecule" encompasses full-sized antibodies as well as antigen-binding subunits, fragments, variants, analogs, or derivatives of such antibodies, *e.g.*, engineered antibody molecules or fragments that bind antigen in a manner similar to antibody molecules, but which use a different scaffold.

[0093] As used herein, the term "binding molecule" refers in its broadest sense to a molecule that specifically binds to a receptor, *e.g.*, an epitope or an antigenic determinant. As described further herein, a binding molecule can comprise one or more "antigen binding domains" described herein. A non-limiting example of a binding molecule is an antibody or fragment thereof that retains antigen-specific binding.

[0094] As used herein, the terms "binding domain," "receptor binding domain," or "antigen binding domain" refer to a region of a binding molecule that is necessary and sufficient to specifically bind to an epitope. For example, an "Fv," *e.g.*, a variable heavy chain and variable light chain of an antibody, either as two separate polypeptide subunits or as a single chain, is considered to be a "binding domain." Other binding domains include, without limitation, the variable heavy chain (VHH) of an antibody derived from a camelid species, or six immunoglobulin complementarity determining regions (CDRs) expressed in a heterologous framework, *e.g.*, a different germline or species, or in a different scaffold, *e.g.*, a fibronectin scaffold. A "binding molecule" as described herein can include one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve or more "antigen binding domains."

[0095] The terms "antibody" and "immunoglobulin" can be used interchangeably herein. An antibody (or a fragment, variant, or derivative thereof as disclosed herein) includes at least the

variable domain of a heavy chain (for camelid species) or at least the variable domains of a heavy chain and a light chain. Basic immunoglobulin structures in vertebrate systems are relatively well understood. *See, e.g.,* Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988). Unless otherwise stated, the term “antibody” encompasses anything ranging from a small antigen-binding fragment of an antibody to a full sized antibody, *e.g.,* an IgG antibody that includes two complete heavy chains and two complete light chains, an IgA antibody that includes four complete heavy chains and four complete light chains and optionally includes a J chain and/or a secretory component, or an IgM antibody that includes ten or twelve complete heavy chains and ten or twelve complete light chains and optionally includes a J chain.

[0096] As will be discussed in more detail below, the term “immunoglobulin” comprises various broad classes of polypeptides that can be distinguished biochemically. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon, (γ , μ , α , δ , ϵ) with some subclasses among them (*e.g.,* $\gamma 1$ - $\gamma 4$ or $\alpha 1$ - $\alpha 2$). It is the nature of this chain that determines the "class" of the antibody as IgG, IgM, IgA, IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) *e.g.,* IgG₁, IgG₂, IgG₃, IgG₄, IgA₁, IgA₂, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernible to the skilled artisan in view of the instant disclosure and, accordingly, are within the scope of this disclosure.

[0097] Light chains are classified as either kappa or lambda (κ , λ). Each heavy chain class can be associated with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain. The basic structure of certain antibodies, *e.g.,* IgG antibodies, includes two heavy chain subunits and two light chain subunits covalently connected via disulfide bonds to form a “Y” structure, also referred to herein as an “H2L2” structure.

[0098] Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be

appreciated that the variable domains of both the variable light (VL) and variable heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminal portion is a variable region and at the C-terminal portion is a constant region; the CH3 (or CH4 in the case of IgM) and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

[0100] As indicated above, a binding domain, *e.g.*, an antibody variable region allows the binding molecule to selectively recognize and specifically bind a receptor, or an epitope on an antigen. That is, the VL domain and VH domain, or subset of the complementarity determining regions (CDRs), of a binding molecule, *e.g.*, an antibody combine to form the variable region that defines a three dimensional antigen binding site. More specifically, the antigen binding site is defined by three CDRs on each of the VH and VL chains. Certain antibodies form larger structures. For example, IgA can form a molecule that includes two H2L2 units, a J chain, and a secretory component, all covalently connected via disulfide bonds, and IgM can form a pentameric or hexameric molecule that includes five or six H2L2 units and optionally a J chain covalently connected via disulfide bonds.

[0101] The six “complementarity determining regions” or “CDRs” present in an antibody antigen-binding domain are short, non-contiguous sequences of amino acids that are specifically positioned to form the binding domain as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the amino acids in the binding domain, referred to as “framework” or “FW” regions, show less inter-molecular variability. The framework regions largely adopt a β -sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β -sheet structure. Thus, framework regions act to form a scaffold that provides for positioning the CDRs in correct orientation by inter-chain, non-covalent interactions. The binding domain formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to its cognate epitope. The amino acids that make up the CDRs and the framework

regions, respectively, can be readily identified for any given heavy or light chain variable region by one of ordinary skill in the art, since they have been defined in various different ways (see, "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services, (1983); and Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987), which are incorporated herein by reference in their entireties).

[0102] In the case where there are two or more definitions of a term which is used and/or accepted within the art, the definition of the term as used herein is intended to include all such meanings unless explicitly stated to the contrary. A specific example is the use of the term "complementarity determining region" ("CDR") to describe the non-contiguous antigen combining sites found within the variable region of both heavy and light chain polypeptides. These particular regions have been described, for example, by Kabat et al., U.S. Dept. of Health and Human Services, "Sequences of Proteins of Immunological Interest" (1983) and by Chothia et al., J. Mol. Biol. 196:901-917 (1987), which are incorporated herein by reference. The Kabat and Chothia definitions include overlapping or subsets of amino acids when compared against each other. Nevertheless, application of either definition (or other definitions known to those of ordinary skill in the art) to refer to a CDR of an antibody or variant thereof is intended to be within the scope of the term as defined and used herein, unless otherwise indicated. The appropriate amino acids which encompass the CDRs as defined by each of the above cited references are set forth below in Table 1 as a comparison. The exact amino acid numbers which encompass a particular CDR will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which amino acids comprise a particular CDR given the variable region amino acid sequence of the antibody.

Table 1 CDR Definitions¹

	Kabat	Chothia
VH CDR1	31-35	26-32
VH CDR2	50-65	52-58
VH CDR3	95-102	95-102
VL CDR1	24-34	26-32
VL CDR2	50-56	50-52

VL CDR3	89-97	91-96
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¹Numbering of all CDR definitions in Table 1 is according to the numbering conventions set forth by Kabat *et al.* (see below).

[0103] Kabat *et al.* also defined a numbering system for antibody heavy and light chains, *e.g.*, antibody variable domain sequences that is applicable to any antibody. One of ordinary skill in the art can unambiguously assign this system of "Kabat numbering" to any variable domain sequence, without reliance on any experimental data beyond the sequence itself. As used herein, "Kabat numbering" refers to the numbering system set forth by Kabat *et al.*, U.S. Dept. of Health and Human Services, "Sequence of Proteins of Immunological Interest" (1983). Unless use of the Kabat numbering system is explicitly noted, however, consecutive numbering is used for all amino acid sequences in this disclosure.

[0104] Binding molecules, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof include, but are not limited to, polyclonal, monoclonal, human, humanized, or chimeric antibodies, single chain antibodies, epitope-binding fragments, *e.g.*, Fab, Fab' and F(ab')₂, Fd, Fvs, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv), fragments comprising either a VL or VH domain, fragments produced by a Fab expression library. ScFv molecules are known in the art and are described, *e.g.*, in US patent 5,892,019. Immunoglobulin or antibody molecules encompassed by this disclosure can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

[0105] By "specifically binds," it is generally meant that a binding molecule, *e.g.*, an antibody or fragment, variant, or derivative thereof binds to an epitope via its antigen binding domain, and that the binding entails some complementarity between the antigen binding domain and the epitope. According to this definition, a binding molecule is said to "specifically bind" to an epitope when it binds to that epitope via its antigen binding domain more readily than it would bind to a random, unrelated epitope. The term "specificity" is used herein to qualify the relative affinity by which a certain binding molecule binds to a certain epitope. For example, binding molecule "A" can be deemed to have a higher specificity for a given epitope than binding molecule "B," or binding molecule "A" can be said to bind to epitope "C" with a higher specificity than it has for related epitope "D."

- [0106] A binding molecule, *e.g.*, an antibody or fragment, variant, or derivative thereof disclosed herein can be said to bind a target antigen with an off rate ($k(\text{off})$) of less than or equal to, *e.g.*, $5 \times 10^{-2} \text{ sec}^{-1}$, 10^{-2} sec^{-1} , $5 \times 10^{-3} \text{ sec}^{-1}$, 10^{-3} sec^{-1} , $5 \times 10^{-4} \text{ sec}^{-1}$, 10^{-4} sec^{-1} , $5 \times 10^{-5} \text{ sec}^{-1}$, or 10^{-5} sec^{-1} , $5 \times 10^{-6} \text{ sec}^{-1}$, 10^{-6} sec^{-1} , $5 \times 10^{-7} \text{ sec}^{-1}$ or 10^{-7} sec^{-1} .
- [0107] A binding molecule, *e.g.*, an antibody or antigen-binding fragment, variant, or derivative disclosed herein can be said to bind a target antigen with an on rate ($k(\text{on})$) of greater than or equal to, *e.g.*, $10^3 \text{ M}^{-1} \text{ sec}^{-1}$, $5 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$, $10^4 \text{ M}^{-1} \text{ sec}^{-1}$, $5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, $10^5 \text{ M}^{-1} \text{ sec}^{-1}$, $5 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$, $10^6 \text{ M}^{-1} \text{ sec}^{-1}$, or $5 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ or $10^7 \text{ M}^{-1} \text{ sec}^{-1}$.
- [0108] A binding molecule, *e.g.*, an antibody or fragment, variant, or derivative thereof is said to competitively inhibit binding of a reference antibody or antigen binding fragment to a given epitope if it preferentially binds to that epitope to the extent that it blocks, to some degree, binding of the reference antibody or antigen binding fragment to the epitope. Competitive inhibition can be determined by any method known in the art, for example, competition ELISA assays. A binding molecule can be said to competitively inhibit binding of the reference antibody or antigen binding fragment to a given epitope by at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.
- [0109] As used herein, the term "affinity" refers to a measure of the strength of the binding of an individual epitope with one or more binding domains, *e.g.*, of an immunoglobulin molecule. *See, e.g.*, Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) at pages 27-28. As used herein, the term "avidity" refers to the overall stability of the complex between a population of binding domains and an antigen. *See, e.g.*, Harlow at pages 29-34. Avidity is related to both the affinity of individual binding domains in the population with specific epitopes, and also the valencies of the immunoglobulins and the antigen. For example, the interaction between a bivalent monoclonal antibody and an antigen with a highly repeating epitope structure, such as a polymer, would be one of high avidity. An interaction between a bivalent monoclonal antibody with a receptor present at a high density on a cell surface would also be of high avidity.
- [0110] Binding molecules or antigen-binding fragments, variants or derivatives thereof as disclosed herein can also be described or specified in terms of their cross-reactivity. As used herein, the term "cross-reactivity" refers to the ability of a binding molecule, *e.g.*, an antibody or fragment, variant,

or derivative thereof, specific for one antigen, to react with a second antigen; a measure of relatedness between two different antigenic substances. Thus, a binding molecule is cross reactive if it binds to an epitope other than the one that induced its formation. The cross reactive epitope generally contains many of the same complementary structural features as the inducing epitope, and in some cases, can actually fit better than the original.

[0111] A binding molecule, *e.g.*, an antibody or fragment, variant, or derivative thereof can also be described or specified in terms of their binding affinity to an antigen. For example, a binding molecule can bind to an antigen with a dissociation constant or K_D no greater than, *e.g.*, 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

[0112] Antibody fragments including single-chain antibodies or other binding domains can exist alone or in combination with one or more of the following: hinge region, CH1, CH2, CH3, or CH4 domains, J chain, or secretory component. Also included are antigen-binding fragments that can include any combination of variable region(s) with one or more of a hinge region, CH1, CH2, CH3, or CH4 domains, a J chain, or a secretory component. Binding molecules, *e.g.*, antibodies, or antigen-binding fragments thereof can be from any animal origin including birds and mammals. The antibodies can be human, murine, donkey, rabbit, goat, guinea pig, camel, llama, horse, or chicken antibodies. In another embodiment, the variable region can be chondrichthoid in origin (*e.g.*, from sharks). As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and can in some instances express endogenous immunoglobulins and some not, as described *infra* and, for example in, U.S. Pat. No. 5,939,598 by Kucherlapati *et al.*

[0113] As used herein, the term "heavy chain subunit" includes amino acid sequences derived from an immunoglobulin heavy chain, a binding molecule, *e.g.*, an antibody comprising a heavy chain subunit includes at least one of: a VH domain, a CH1 domain, a hinge (*e.g.*, upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, a CH4 domain, or a variant or fragment thereof. For example, a binding molecule, *e.g.*, an antibody or fragment, variant, or derivative thereof can include, in addition to a VH domain, a CH1 domain; CH1 domain, a hinge,

and a CH2 domain; a CH1 domain and a CH3 domain; a CH1 domain, a hinge, and a CH3 domain; or a CH1 domain, a hinge domain, a CH2 domain, and a CH3 domain. In certain aspects a binding molecule, *e.g.*, an antibody or fragment, variant, or derivative thereof can include, in addition to a VH domain, a CH3 domain and a CH4 domain; or a CH3 domain, a CH4 domain, and a J chain. Further, a binding molecule for use in the disclosure can lack certain constant region portions, *e.g.*, all or part of a CH2 domain. It will be understood by one of ordinary skill in the art that these domains (*e.g.*, the heavy chain subunit) can be modified such that they vary in amino acid sequence from the original immunoglobulin molecule.

[0114] The heavy chain subunits of a binding molecule, *e.g.*, an antibody or fragment thereof, can include domains derived from different immunoglobulin molecules. For example, a heavy chain subunit of a polypeptide can include a CH1 domain derived from an IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, a heavy chain subunit can include a hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, a heavy chain subunit can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule.

[0115] As used herein, the term "light chain subunit" includes amino acid sequences derived from an immunoglobulin light chain. The light chain subunit includes at least one of a VL or CL (*e.g.*, Cκ or Cλ) domain.

[0116] Binding molecules, *e.g.*, antibodies or antigen-binding fragments, variants, or derivatives thereof can be described or specified in terms of the epitope(s) or portion(s) of an antigen that they recognize or specifically bind. The portion of a target antigen that specifically interacts with the antigen binding domain of an antibody is an "epitope," or an "antigenic determinant." A target antigen can comprise a single epitope or at least two epitopes, and can include any number of epitopes, depending on the size, conformation, and type of antigen.

[0117] As previously indicated, the subunit structures and three dimensional configuration of the constant regions of the various immunoglobulin classes are well known. As used herein, the *term* "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain and the term "CH1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of a typical immunoglobulin heavy chain molecule.

- [0118] As used herein the term “CH2 domain” includes the portion of a heavy chain molecule that extends, *e.g.*, from about amino acid 244 to amino acid 360 of an IgG antibody using conventional numbering schemes (amino acids 244 to 360, Kabat numbering system; and amino acids 231-340, EU numbering system; see Kabat EA *et al. op. cit.* The CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 amino acids. Certain immunoglobulin classes, *e.g.*, IgM, further include a CH4 region.
- [0119] As used herein, the term “hinge region” includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 amino acids and is flexible, thus allowing the two N-terminal antigen binding regions to move independently.
- [0120] As used herein the term “disulfide bond” includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In certain IgG molecules, the CH1 and CL regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system). In certain aspects provided herein, a human IgG4 Fc domain can be mutated in the hinge region to insure disulfide bond formation between two hinge regions, specifically, a serine to proline mutation at position 228 (according to EU numbering). Human IgG4 Fc domains comprising the S228P mutation are referred to herein as “IgG4P Fc domains.”
- [0121] As used herein, an “Fc-TM region” is a human IgG Fc region that comprises amino acid substitutions of L234F, L235E and P331S as numbered by the EU index as set forth in Kabat and exhibits reduced or ablated effector (ADCC and/or CDC) function, reduced or ablated binding to Fc receptors, and/or reduced or ablated toxicities. *See, e.g.*, U.S. Patent Application Publication No. 2011/0059078, which is incorporated herein by reference in its entirety.
- [0122] As used herein, the term “chimeric antibody” refers to an antibody in which the immunoreactive region or site is obtained or derived from a first species and the constant region (which can be intact, partial or modified) is obtained from a second species. In some embodiments the target binding region or site will be from a non-human source (*e.g.* mouse or primate) and the constant region is human.

- [0123] The terms “multispecific antibody, or “bispecific antibody” refer to an antibody that has binding domains for two or more different epitopes within a single antibody molecule. Other binding molecules in addition to the canonical antibody structure can be constructed with two binding specificities. Epitope binding by bispecific or multispecific antibodies can be simultaneous or sequential. Triomas and hybrid hybridomas are two examples of cell lines that can secrete bispecific antibodies. Bispecific antibodies can also be constructed by recombinant means. (Ströhlein and Heiss, *Future Oncol.* 6:1387-94 (2010); Mabry and Snively, *IDrugs.* 13:543-9 (2010)). A bispecific antibody can also be a diabody.
- [0124] As used herein, the term “engineered antibody” refers to an antibody in which the variable domain in either the heavy and light chain or both is altered by at least partial replacement of one or more amino acids in either the CDR or framework regions. In certain aspects entire CDRs from an antibody of known specificity can be grafted into the framework regions of a heterologous antibody. Although alternate CDRs can be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, CDRs can also be derived from an antibody of different class, *e.g.*, from an antibody from a different species. An engineered antibody in which one or more “donor” CDRs from a non-human antibody of known specificity are grafted into a human heavy or light chain framework region is referred to herein as a “humanized antibody.” In certain aspects not all of the CDRs are replaced with the complete CDRs from the donor variable region and yet the antigen binding capacity of the donor can still be transferred to the recipient variable domains. Given the explanations set forth in, *e.g.*, U. S. Pat. Nos. 5,585,089, 5,693,761, 5,693,762, and 6,180,370, it will be well within the competence of those skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional engineered or humanized antibody.
- [0125] As used herein the term “engineered” includes manipulation of nucleic acid or polypeptide molecules by synthetic means (*e.g.* by recombinant techniques, *in vitro* peptide synthesis, by enzymatic or chemical coupling of peptides or some combination of these techniques).
- [0126] As used herein, the terms “linked,” “fused” or “fusion” or other grammatical equivalents can be used interchangeably. These terms refer to the joining together of two more elements or components, by whatever means including chemical conjugation or recombinant means. An “in-frame fusion” refers to the joining of two or more polynucleotide open reading frames (ORFs) to

form a continuous longer ORF, in a manner that maintains the translational reading frame of the original ORFs. Thus, a recombinant fusion protein is a single protein containing two or more segments that correspond to polypeptides encoded by the original ORFs (which segments are not normally so joined in nature.) Although the reading frame is thus made continuous throughout the fused segments, the segments can be physically or spatially separated by, for example, in-frame linker sequence. For example, polynucleotides encoding the CDRs of an immunoglobulin variable region can be fused, in-frame, but be separated by a polynucleotide encoding at least one immunoglobulin framework region or additional CDR regions, as long as the "fused" CDRs are co-translated as part of a continuous polypeptide.

[0127] In the context of polypeptides, a "linear sequence" or a "sequence" is an order of amino acids in a polypeptide in an amino to carboxyl terminal direction in which amino acids that neighbor each other in the sequence are contiguous in the primary structure of the polypeptide. A portion of a polypeptide that is "amino-terminal" or "N-terminal" to another portion of a polypeptide is that portion that comes earlier in the sequential polypeptide chain. Similarly a portion of a polypeptide that is "carboxy-terminal" or "C-terminal" to another portion of a polypeptide is that portion that comes later in the sequential polypeptide chain. For example in a typical antibody, the variable domain is "N-terminal" to the constant region, and the constant region is "C-terminal" to the variable domain.

[0128] The term "expression" as used herein refers to a process by which a gene produces a biochemical, for example, a polypeptide. The process includes any manifestation of the functional presence of the gene within the cell including, without limitation, gene knockdown as well as both transient expression and stable expression. It includes without limitation transcription of the gene into messenger RNA (mRNA), and the translation of such mRNA into polypeptide(s). If the final desired product is a biochemical, expression includes the creation of that biochemical and any precursors. Expression of a gene produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, proteolytic cleavage, and the like.

- [0129] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to therapeutic measures that cure, slow down, lessen symptoms of, and/or halt or slow the progression of an existing diagnosed pathologic condition or disorder. Terms such as "prevent," "prevention," "avoid," "deterrence" and the like refer to prophylactic or preventative measures that prevent the development of an undiagnosed targeted pathologic condition or disorder. Thus, "those in need of treatment" can include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented.
- [0130] OX40, or "OX40 receptor" is a protein (also variously termed CD134, tumor necrosis factor receptor superfamily member 4, and ACT-35) expressed on the surface of activated T cells, *e.g.*, CD4⁺ and CD8⁺ T cells, as well as on Foxp3⁺CD4⁺ regulatory T cells (Tregs). Naive CD4⁺ and CD8⁺ T cells do not express OX40 (Croft, M., (2010) *Ann Rev Immunol* 28:57-78).
- [0131] "OX40 ligand" ("OX40L") (also variously termed tumor necrosis factor ligand superfamily member 4, gp34, TAX transcriptionally-activated glycoprotein-1, and CD252) is found largely on antigen presenting cells (APCs), and can be induced on activated B cells, dendritic cells (DCs), Langerhans cells, plasmacytoid DCs, and macrophages (*Id.*). Other cells, including activated T cells, NK cells, mast cells, endothelial cells, and smooth muscle cells can express OX40L in response to inflammatory cytokines (*Id.*). OX40L specifically binds to the OX40 receptor. The human protein is described in PCT Publication No. WO 95/21915. The mouse OX40L is described in U.S. Pat. No. 5,457,035. OX40L is expressed on the surface of cells and includes an intracellular, a transmembrane and an extracellular receptor-binding domain. A functionally active soluble form of OX40L can be produced by deleting the intracellular and transmembrane domains as described, *e.g.*, in U.S. Pat. Nos. 5,457,035 and 6,312,700, and WO 95/21915, the disclosures of which are incorporated herein for all purposes. A functionally active form of OX40L is a form that retains the capacity to bind specifically to OX40, that is, that possesses an OX40 "receptor binding domain." Methods of determining the ability of an OX40L molecule or derivative to bind specifically to OX40 are discussed below. Methods of making and using OX40L and its derivatives (such as derivatives that include an OX40 binding domain) are described in WO 95/21915, which also describes proteins comprising the soluble form of OX40L linked to other peptides, such as human immunoglobulin ("Ig") Fc regions, that can be produced to facilitate purification of OX40 ligand from cultured cells, or to enhance the stability of the molecule after *in*

vivo administration to a mammal (see also, U.S. Pat. No. 5,457,035 and PCT Publication No. WP 2006/121810, both of which are incorporated by reference herein in their entireties).

[0132] By "subject" or "individual" or "animal" or "patient" or "mammal," is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, and zoo, sports, or pet animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, swine, cows, bears, and so on.

[0133] As used herein, phrases such as "a subject that would benefit from therapy" and "an animal in need of treatment" includes subjects, such as mammalian subjects, that would benefit from administration of a humanized anti-OX40 antibody. Such antibodies, can be used, *e.g.*, for a diagnostic procedures and/or for treatment or prevention of a disease, *e.g.*, cancer.

HUMANIZED ANTI-OX40 ANTIBODIES AND ANTIGEN-BINDING FRAGMENTS THEREOF

[0134] The present disclosure relates to antibodies, *e.g.*, humanized antibodies, which specifically bind to OX40. In certain aspects, an antibody or fragment thereof as provided herein can be isolated. In certain aspects, an antibody or fragment thereof as provided herein can be substantially pure. In certain aspects an antibody or fragment thereof as provided herein can be non-naturally occurring.

[0135] In certain aspects, this disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising the VH and VL of OX40mAb5, OX40mAb8, OX40mAb10, OX40mAb11, OX40mAb12, OX40mAb13, OX40mAb14, OX40mAb15, OX40mAb16, OX40mAb17, OX40mAb18, OX40mAb19, OX40mAb20, OX40mAb21, OX40mAb22, OX40mAb23, OX40mAb24, OX40mAb25, OX40mAb25a, OX40mAb26, OX40mAb27, OX40mAb28, OX40mAb29, OX40mAb30, OX40mAb31, OX40mAb32, or OX40mAb37. In certain aspects, this disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising the heavy chain and light chain of OX40mAb5, OX40mAb8, OX40mAb10, OX40mAb11, OX40mAb12, OX40mAb13, OX40mAb14, OX40mAb15, OX40mAb16, OX40mAb17, OX40mAb18, OX40mAb19, OX40mAb20, OX40mAb21, OX40mAb22, OX40mAb23, OX40mAb24, OX40mAb25, OX40mAb25a, OX40mAb26, OX40mAb27, OX40mAb28, OX40mAb29, OX40mAb30, OX40mAb31, or OX40mAb32.

[0136] In certain aspects this disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody VH and an antibody VL, wherein the VL comprises an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference amino acid sequence SEQ ID NO: 29 or SEQ ID NO: 32.

[0137] In certain aspects this disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody VH and an antibody VL, where the VL comprises SEQ ID NO: 29 or SEQ ID NO: 32.

[0138] The disclosure further provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody VH and an antibody VL, wherein the VH comprises VH-CDR1, VH-CDR2, and VH-CDR3 amino acid sequences identical to, or identical except for eight, seven, six, five, four, three, two, or one single amino acid substitutions, deletions, or insertions in one or more of the VH-CDRS to: the VHCDR1 amino acid sequence SEQ ID NO: 8, the VHCDR2 amino acid sequence SEQ ID NO: 14, SEQ ID NO: 15, or SEQ ID NO: 16, and the VHCDR3 amino acid sequence SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27.

[0139] The disclosure further provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody VH and an antibody VL, wherein the VH comprises an amino acid sequence with the formula:

HFW1-HCDR1-HFW2-HCDR2-HFW3-HCDR3-HFW4,

wherein HFW1 is SEQ ID NO: 6 or SEQ ID NO: 7, HCDR1 is SEQ ID NO: 8, HFW2 is SEQ ID NO: 9, HCDR2 is SEQ ID NO: 14, SEQ ID NO: 15 or SEQ ID NO: 16, HFW3 is SEQ ID NO: 17, HCDR3 is SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27, and HFW4 is SEQ ID NO: 28. In certain aspects the amino acid sequence of HFW2 is SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13. In certain aspects the amino acid sequence of HFW3 is SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0140] Moreover, the disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody VH and an antibody VL, wherein the VH comprises an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference amino acid sequence SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51,

SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, or SEQ ID NO: 67.

[0141] In one aspect, the disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody VH and an antibody VL, where the VL comprises the amino acid sequence SEQ ID NO: 29 and the VH comprises the amino acid sequence SEQ ID NO: 59.

[0142] A humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can include, in addition to a VH and a VL, a heavy chain constant region or fragment thereof. In certain aspects the heavy chain constant region is a human heavy chain constant region, *e.g.*, a human IgG constant region, *e.g.*, a human IgG1 constant region or a human IgG4 constant region. As described elsewhere herein, in certain aspects a heavy chain constant region or fragment thereof, *e.g.*, a human IgG constant region or fragment thereof, can include one or more amino acid substitutions relative to a wild-type IgG constant region, where the modified IgG has one or more desirable properties relative to a wild-type IgG constant region. For example, the human IgG constant region can be an IgG4P constant region, or an IgG1-TM constant region, as described elsewhere herein.

[0143] A humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can include, in addition to a VH and a VL, and optionally a heavy chain constant region or fragment thereof, a light chain constant region or fragment thereof. In certain aspects the light chain constant region is a kappa or lambda light chain constant region, *e.g.*, a human kappa constant region or a human lambda constant region. In a specific aspect, the light chain constant region is a human kappa constant region.

[0144] In certain aspects the disclosure provides a humanized anti-OX40 antibody or an antigen-binding fragment thereof comprising an antibody heavy chain or fragment thereof and an antibody light chain or fragment thereof, where the heavy chain comprises the amino acid sequence SEQ ID NO: 71, and the light chain comprises the amino acid sequence SEQ ID NO: 30.

[0145] A humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can be, *e.g.*, monoclonal, polyclonal, recombinant, multispecific, or any combination thereof. A humanized anti-OX40 antibody or antigen-binding fragment can be, *e.g.*, an Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, an sc(Fv)₂

fragment, an Fd fragment, a disulfide linked Fv fragment, a V-NAR domain, an IgNar, an intrabody, an IgGΔCH2, a minibody, a F(ab')₃ fragment, a tetrabody, a triabody, a diabody, a single-domain antibody, a DVD-Ig, a Fcab fragment, an mAb2 fragment, an (scFv)₂ fragment, or a scFv-Fc fragment.

[0146] In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can specifically bind to human, cynomolgus monkey, or rhesus monkey OX40. In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can specifically bind to the surface of primary human, cyno, or rhesus CD4 T cells or Jurkat cells. In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can specifically bind to OX40 as expressed on activated CD4⁺ or CD8⁺ T cells from human, cynomolgus monkey, rhesus monkey, or any combination thereof. In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can specifically bind to OX40 as expressed on primary activated CD4⁺ T cells from human, cynomolgus monkey, rhesus monkey, or any combination thereof. In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein does not bind to murine or rat OX40. In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein does not cross react with related TNFRSF proteins, *e.g.*, the TNFRSF proteins listed in Table 3-1 in Example 3 below.

[0147] A humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can contain one or more conservative amino acid changes, *e.g.*, up to ten conservative changes (*e.g.*, two substituted amino acids, three substituted amino acids, four substituted amino acids, or five substituted amino acids, etc.), provided that the changes can be made in the polypeptide without changing a biochemical function of the humanized anti-OX40 antibody or antigen-binding fragment thereof, *e.g.*, specifically binding to OX40, thereby triggering signaling. For example, one or more conservative changes can be made in a receptor binding domain of an antibody as provided herein without blocking its ability to bind to OX40.

[0148] Additionally, part of a polypeptide domain can be deleted without impairing or eliminating all of its functions. Similarly, insertions or additions can be made in the polypeptide chain, for example, adding epitope tags, without impairing or eliminating its functions, as described below. Other modifications that can be made without materially impairing one or more functions of a

polypeptide include, for example, *in vivo* or *in vitro* chemical and biochemical modifications that incorporate unusual amino acids. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, labeling, *e.g.*, with radionuclides, and various enzymatic modifications, as will be readily appreciated by those of ordinary skill in the art. A variety of methods for labeling polypeptides, and labels useful for such purposes, are well known in the art, and include radioactive isotopes such as ^{32}P , fluorophores, chemiluminescent agents, enzymes, and antiligands.

[0149] A humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can further include a heterologous agent, *e.g.*, a stabilizing agent, an immune response modifier, or a detectable agent. In certain aspects the heterologous agent comprises one or more additional polypeptide sequences fused to the polypeptide subunit via a peptide bond, such as a signal sequence (*e.g.*, a secretory signal sequence), a linker sequence, an amino acid tag or label, or a peptide or polypeptide sequence that facilitates purification. In certain aspects, the heterologous polypeptide can be fused to the N-terminus or the C-terminus of either a heavy chain or light chain antibody subunit, or fragment thereof, as long as the functional characteristics of the domains are maintained.

[0150] In certain aspects, the heterologous agent can be chemically conjugated to a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein. Exemplary heterologous agents that can be chemically conjugated to the polypeptide subunit include, without limitation, linkers, drugs, toxins, imaging agents, radioactive compounds, organic and inorganic polymers, and any other compositions which can provide a desired activity that is not provided by the polypeptide subunit itself. Specific agents include, without limitation, polyethylene glycol (PEG), a cytotoxic agent, a radionuclide, an imaging agent, biotin.

[0151] In certain aspects, the disclosure provides certain anti-OX40 antibodies to be used as controls or research tools. For example, the disclosure provides a humanized anti-OX40 antibody or antigen-binding fragment thereof as described above, in which the VH and VL are fused to a murine IgG1 heavy chain and a murine kappa light chain, respectively. This “reverse chimera” is useful for *in vivo* characterization in rhesus monkeys. In another example, the VH region on a humanized anti-OX40 antibody as provided herein can be attached to various heavy chain constant

regions with altered effector functions, *e.g.*, human IgG4P or human IgG1TM, described elsewhere herein.

[0152] A humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can specifically bind to OX40 as expressed on primary activated T cells, *e.g.*, primary activated CD4⁺ T cells, primary activated CD8⁺ T cells and/or regulatory T cells, from human, cynomolgus monkey, rhesus monkey, or any combination thereof. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein, *e.g.*, OX40mAb24, can bind to human OX40 expressed on primary activated human CD4⁺ T cells with a binding affinity of about 0.01 pM to about 1 nM, *e.g.*, about 1 pM to about 500 pM, *e.g.*, about 100 pM to about 400 pM, *e.g.*, about 250 pM to about 370 pM, all as measured by flow cytometry. For example, a humanized anti-OX40 antibody or an antigen-binding fragment thereof can bind to human OX40 expressed on primary activated human CD4⁺ T cells with a binding affinity of about 0.1 pM, about 0.5 pM, about 1 pM, about 10 pM, about 50 pM, about 100 pM, about 150 pM, about 200 pM, about 250 pM, about 275 pM, about 300 pM, about 325 pM, about 350 pM, about 370 pM, about 400 pM, about 425 pM, about 450 pM, about 475 pM, about 500 pM, about 550 pM, about 600 pM, about 650 pM, about 700 pM, about 750 pM, or about 1 nM, all as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to human OX40 expressed on primary activated human CD4⁺ T cells with a binding affinity of about 312 pM. Binding affinity can be measured by a number of different methods and/or instruments, and the relative binding affinities can vary depending on the method or instrument, as is well understood by persons of ordinary skill in the art.

[0153] A humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can occupy and cross-link some or all of the OX40 molecules on the surface of a cell, *e.g.*, a primary activated human CD4⁺ T cell. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to human OX40 expressed on primary activated human CD4⁺ T cells, and can achieve 20% receptor occupancy on primary activated human CD4⁺ T cells (EC₂₀) at a concentration of about 0.01 pM to about 1 nM, *e.g.*, about 0.1 pM to about 500 pM, *e.g.*, about 1 pM to about 200 pM, *e.g.*, about 10 pM to about 100 pM, *e.g.*, about 50 pM to about 100 pM, *e.g.*, about 63 pM to about 93 pM, *e.g.*, about 1 pM, about 10 pM, about 20 pM, about 30 pM, about 40 pM, about 50 pM, about 60 pM, about 70 pM, about

78 pM, about 80 pM, about 90 pM, about 100 pM, or about 150 pM, as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can achieve 20% receptor occupancy on primary activated human CD4⁺ T cells (EC₂₀) at a concentration of about 78 pM., as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to human OX40 expressed on primary activated human CD4⁺ T cells, and can achieve 50% receptor occupancy on primary activated human CD4⁺ T cells (EC₅₀) at a concentration of about 0.01 pM to about 1 nM, *e.g.*, about 1 pM to about 500 pM, *e.g.*, about 100 pM to about 400 pM, *e.g.*, about 250 pM to about 370 pM, *e.g.*, about 100 pM, about 150 pM, about 200 pM, about 250 pM, about 275 pM, about 300 pM, about 325 pM, about 350 pM, about 370 pM, about 400 pM, about 425 pM, about 450 pM, about 475 pM, or about 500 pM, all as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can achieve 50% receptor occupancy on primary activated human CD4⁺ T cells (EC₅₀) at a concentration of about 312 pM., as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof can bind to human OX40 expressed on primary activated human CD4⁺ T cells, and can achieve 90% receptor occupancy on primary activated human CD4⁺ T cells (EC₉₀) at a concentration of about 100 pM to about 100 nM, *e.g.*, about 500 pM to about 10 nM, *e.g.*, about 1 nM to about 500 nM, *e.g.*, about 2 nM to about 4 nM, *e.g.*, about 1 nM, about 2 nM, about 3 nM, about 4 nM, or about 5 nM, as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can achieve 90% receptor occupancy on primary activated human CD4⁺ T cells (EC₉₀) at a concentration of about 2290 pM to about 3330 pM, *e.g.*, about 2810 pM., as measured by flow cytometry.

[0154] In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to human OX40 expressed on OX40-overexpressing Jurkat cells with a binding affinity of about 250 pM to about 600 pM, *e.g.*, about 424 pM as measured by flow cytometry.

[0155] In certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof can bind to human OX40 expressed on OX40-overexpressing Jurkat cells, and can achieve EC₂₀ at a concentration of about 60 to about 150 pM, EC₅₀ at a concentration of about 250 to about 600

pM, and EC₉₀ at a concentration about 2260 to about 4390 pM as measured by flow cytometry. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to human OX40 expressed on OX40-overexpressing Jurkat cells and can achieve EC₂₀ at a concentration of about 106 pM, EC₅₀ at a concentration of about 424 pM, and EC₉₀ at a concentration of about 3820 pM, as measured by flow cytometry.

[0156] In another example, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to cynomolgus monkey OX40 expressed on primary activated cynomolgus monkey CD4⁺ T cells with a binding affinity of about 340 pM to about 820 pM, *e.g.*, about 580 pM, as measured by flow cytometry. In another example, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can bind to rhesus monkey OX40 expressed on primary activated rhesus monkey CD4⁺ T cells with a binding affinity of about 130 pM to about 600 pM, *e.g.*, about 370 pM, as measured by flow cytometry.

[0157] In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can induce dose-dependent proliferation of activated CD4⁺ T cells in a plate-based assay. For example, in an *in vitro* assay a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein, *e.g.*, OX40mAb24, a 20% maximal proliferation response (EC₂₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 14 pM to about 28 pM, *e.g.*, about 21 pM, a 50% maximal proliferation response (EC₅₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 0.3 pM to about 130 pM, *e.g.*, about 28 pM, and a 90% maximal proliferation response (EC₉₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 50 pM to about 90 pM, *e.g.*, about 72 pM, all as measured by flow cytometry.

[0158] In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can induce dose-dependent cytokine release from activated CD4⁺ T cells, *e.g.*, human primary activated CD4⁺ T cells. In certain aspects, the released cytokine is IFN γ , TNF α , IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1 β , or any combination thereof. In certain aspects, the cytokine is IFN γ , TNF α , IL-5, IL-10, IL-13 or any combination thereof. Similarly, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can achieve CD4⁺ T cell proliferation and cytokine release in primary activated cynomolgus monkey CD4⁺ T cells and in primary activated rhesus monkey CD4⁺ T cells.

- [0159] In additional aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can activate the NFκB pathway in OX40 expressing T cells in the presence of FcγR-expressing cells. For example, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can activate the NFκB pathway in OX40-expressing Jurkat NFκB-luciferase reporter cells that produce luciferase in response to stimulation of the NFκB signaling pathway. Alternatively, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can activate the NFκB pathway in cells expressing human OX40, cynomolgus monkey OX40 or rhesus monkey OX40.
- [0160] In yet another aspect a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can facilitate cancer treatment, *e.g.*, by slowing tumor growth, stalling tumor growth, or reducing the size of existing tumors, when administered as an effective dose to a subject in need of cancer treatment. In certain aspects the facilitation of cancer treatment can be achieved in the presence of T cells. In certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein, when administered as an effective dose to a subject in need of treatment, can reduce tumor growth by at least 10%, at least 20%, at least 30%, at least 40%, and least 50%, at least 60%, or at least 70% compared to administration of an isotype-matched control antibody.
- [0161] In yet further aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can induce proliferation of activated, OX40-expressing T cells through binding to OX40, and at the same time trigger complement-dependent or antibody-dependent cellular cytotoxicity against the OX40-expressing T cells, *e.g.*, activated CD4⁺ T cells, activated CD8⁺ T cells and/or regulatory T cells. Moreover in certain aspects, a humanized anti-OX40 antibody or antigen-binding fragment thereof as provided herein can induce proliferation of OX40-expressing T cells, *e.g.*, activated, OX40-expressing CD4⁺, CD8⁺ T cells and/or regulatory T cells through binding to OX40, and at the same time bind to C1q or trigger NK cell-mediated antibody-dependent cellular cytotoxicity of OX40-expressing T cells, *e.g.*, activated CD4⁺ T cells, activated CD8⁺ T cells and/or regulatory T cells.
- [0162] In certain aspects the disclosure provides an anti-OX40 antibody or fragment thereof comprising a humanized VH attached to a murine heavy chain constant region and a humanized VL attached to a murine light chain constant region, wherein the heavy chain comprises SEQ ID

NO: 81 and the light chain comprises SEQ ID NO: 83. In certain aspects the heavy chain constant region can be, *e.g.*, a murine IgG1 constant region. In certain aspects the light chain constant region can be, *e.g.*, a murine kappa constant region.

[0163] In certain aspects the disclosure provides a rat-anti-mouse OX40 antibody or antigen-binding fragment thereof comprising a rat VH and a rat VL, where the VH comprises the amino acid sequence SEQ ID NO: 85 and the VL comprises the amino acid sequence SEQ ID NO: 88. In certain aspects, this antibody or fragment thereof further comprises a light chain constant region or fragment thereof fused to the C-terminus of the VL. The light chain constant region can be, *e.g.*, a murine kappa constant region. In certain aspects, this antibody or fragment thereof further comprises a heavy chain constant region or fragment thereof fused to the C-terminus of the VH. The heavy chain constant region can be, *e.g.*, a murine IgG2a constant region. In certain aspects this rat-anti-mouse OX40 antibody comprises the heavy chain amino acid sequence SEQ ID NO: 86 and the light chain amino acid sequence SEQ ID NO: 89. In certain aspects, a rat-anti-mouse OX40 antibody or fragment thereof as provided herein can specifically bind to mouse OX40. In certain aspects, administration of an effective dose of a rat-anti-mouse OX40 antibody or fragment thereof as provided herein to mouse can inhibit mouse cancer cell line growth in the mouse.

[0164] In certain aspects a rat-anti-mouse OX40 antibody fragment as provided herein can be, *e.g.*, an Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, or an sc(Fv)₂ fragment, or any combination thereof.

POLYNUCLEOTIDES ENCODING HUMANIZED ANTI-OX40 ANTIBODIES, FRAGMENTS, OR SUBUNITS

[0165] This disclosure provides polynucleotides comprising nucleic acid sequences that encode a humanized anti-OX40 antibody or an antigen-binding fragment thereof. For example, the disclosure provides a polynucleotide, or two or more polynucleotides, comprising a nucleic acid sequence that encodes a humanized anti-OX40 antibody or a subunit of a humanized anti-OX40 antibody, or encodes an antigen-binding fragment of such an antibody. The polynucleotides of the disclosure can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, *e.g.*, modified genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand.

- [0166] In certain aspects, a polynucleotide can be isolated. In certain aspects, a polynucleotide can be substantially pure. In certain aspects a polynucleotide can be non-naturally occurring. In certain aspects a polynucleotide can be cDNA or derived from cDNA. In certain aspects a polynucleotide can be recombinantly produced. In certain aspects a polynucleotide can comprise the coding sequence for the mature polypeptide fused in the same reading frame to a polynucleotide which aids, for example, in expression and secretion of a polypeptide from a host cell (*e.g.*, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell). The polypeptide having a leader sequence is a preprotein and can have the leader sequence cleaved by the host cell to form the mature form of the polypeptide.
- [0167] In certain aspects the disclosure provides a polynucleotide comprising a nucleic acid that encodes a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein, or a polypeptide subunit of a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein.
- [0168] Also provided are polynucleotides comprising nucleic acid sequences comprising one or a small number of deletions, additions and/or substitutions. Such changes can be contiguous or can be distributed at different positions in the nucleic acid. A substantially identical nucleic acid sequence can, for example, have 1, or 2, or 3, or 4, or even more nucleotide deletions, additions and/or substitutions. In certain aspects, the one or more deletions, additions and/or substitutions do not alter the reading frame encoded by the polynucleotide sequence, such that a modified ("mutant") but substantially identical polypeptide is produced upon expression of the nucleic acid.
- [0169] In certain aspects this disclosure provides an isolated polynucleotide comprising a nucleic acid encoding an antibody VL, wherein the VL comprises an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference amino acid sequence SEQ ID NO: 29 or SEQ ID NO: 32. In certain aspects, the polynucleotide encodes an antibody light chain and comprises a nucleotide sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference nucleic acid sequence SEQ ID NO: 31.
- [0170] In certain aspects this disclosure provides an isolated polynucleotide comprising a nucleic acid encoding an antibody VL, wherein the VL comprises SEQ ID NO: 29 or SEQ ID NO: 32.
- [0171] The disclosure further provides an isolated polynucleotide comprising a nucleic acid encoding an antibody VH, wherein the VH comprises VH-CDR1, VH-CDR2, and VH-CDR3

amino acid sequences identical to, or identical except for eight, seven, six, five, four, three, two, or one single amino acid substitutions, deletions, or insertions in one or more of the VH-CDRS to: the VHCDR1 amino acid sequence SEQ ID NO: 8, the VHCDR2 amino acid sequence SEQ ID NO: 14, SEQ ID NO: 15, or SEQ ID NO: 16, and the VHCDR3 amino acid sequence SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27.

[0172] The disclosure further provides an isolated polynucleotide comprising a nucleic acid encoding an antibody VH, wherein the VH comprises an amino acid sequence with the formula:

HFW1-HCDR1-HFW2-HCDR2-HFW3-HCDR3-HFW4,

wherein HFW1 is SEQ ID NO: 6 or SEQ ID NO: 7, HCDR1 is SEQ ID NO: 8, HFW2 is SEQ ID NO: 9, HCDR2 is SEQ ID NO: 14, SEQ ID NO: 15 or SEQ ID NO: 16, HFW3 is SEQ ID NO: 17, HCDR3 is SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27, and HFW4 is SEQ ID NO: 28. In certain aspects the amino acid sequence of HFW2 is SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13. In certain aspects the amino acid sequence of HFW3 is SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0173] Moreover, the disclosure provides an isolated polynucleotide comprising a nucleic acid encoding an antibody VH, wherein the VH comprises an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference amino acid sequence SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, or SEQ ID NO: 67. In certain aspects, the polynucleotide comprises a nucleotide sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference nucleic acid sequence SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, SEQ ID NO: 46, SEQ ID NO: 48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 60, SEQ ID NO: 62, SEQ ID NO: 64, SEQ ID NO: 66, or SEQ ID NO: 68.

[0174] In certain aspects the disclosure provides a polynucleotide comprising the nucleic acid of SEQ ID NO: 60, the nucleic acid of SEQ ID NO: 31, the nucleic acid of SEQ ID NO: 72, or any combination thereof.

[0175] Further provided is a vector comprising a polynucleotide as described above. Suitable vectors are described elsewhere herein, and are known to those of ordinary skill in the art.

[0176] In certain aspects, the disclosure provides a composition, *e.g.*, a pharmaceutical composition, comprising a polynucleotide or vector as described above, optionally further comprising one or more carriers, diluents, excipients, or other additives.

[0177] In certain aspects, the disclosure provides a polynucleotide composition comprising: a polynucleotide that comprises a nucleic acid encoding a VH, and polynucleotide that comprises a nucleic acid encoding a VL. According to this aspect, the VL and VH together can comprise a VL amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference amino acid sequence SEQ ID NO: 29 or SEQ ID NO: 32, and a VH comprising: (a) VH-CDR1, VH-CDR2, and VH-CDR3 amino acid sequences identical to, or identical except for eight, seven, six, five, four, three, two, or one single amino acid substitutions, deletions, or insertions in one or more of the VH-CDRS to: the VHCDR1 amino acid sequence SEQ ID NO: 8, the VHCDR2 amino acid sequence SEQ ID NO: 14, SEQ ID NO: 15, or SEQ ID NO: 16, and the VHCDR3 amino acid sequence SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27; (b) an amino acid sequence with the formula:

HFW1-HCDR1-HFW2-HCDR2-HFW3-HCDR3-HFW4,

wherein HFW1 is SEQ ID NO: 6 or SEQ ID NO: 7, HCDR1 is SEQ ID NO: 8, HFW2 is SEQ ID NO: 9, HCDR2 is SEQ ID NO: 14, SEQ ID NO: 15 or SEQ ID NO: 16, HFW3 is SEQ ID NO: 17, HCDR3 is SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27, and HFW4 is SEQ ID NO: 28; or (c) an amino acid sequence at least 70%, 75%, 80%, 85%, 90%, 95%, or 100% identical to the reference amino acid sequence SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, or SEQ ID NO: 67. For VH (b), the amino acid sequence of HFW2 can be SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13; and/or the amino acid sequence of HFW3 can be SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, or SEQ ID NO: 24.

[0178] In a polynucleotide composition as described above, the polynucleotide comprising a nucleic acid encoding a VH and the polynucleotide comprising a nucleic acid encoding a VL can

reside in a single vector, or can be on separate, non-identical vectors. Accordingly the disclosure provides one or more vectors comprising the polynucleotide composition described above.

[0179] In some cases, a polynucleotide composition encoding a VH and VL as described above can encode a humanized antibody or antigen-binding fragment thereof that can specifically bind to OX40, *e.g.*, human OX40, cynomolgus monkey OX40, and/or rhesus monkey OX40.

[0180] In certain aspects the disclosure provides an isolated polynucleotide comprising a nucleic acid that encodes an anti-OX40 antibody or fragment thereof comprising a humanized VH attached to a murine heavy chain constant region and a humanized VL attached to a murine light chain constant region, wherein the heavy chain comprises SEQ ID NO: 81 and the light chain comprises SEQ ID NO: 83. In certain aspects the heavy chain constant region can be, *e.g.*, a murine IgG1 constant region. In certain aspects the light chain constant region can be, *e.g.*, a murine kappa constant region.

[0181] In certain aspects the disclosure provides an isolated polynucleotide comprising a nucleic acid that encodes a rat-anti-mouse OX40 antibody or antigen-binding fragment thereof comprising a rat VH and a rat VL, where the VH comprises the amino acid sequence SEQ ID NO: 85 and the VL comprises the amino acid sequence SEQ ID NO: 88. In certain aspects, this antibody or fragment thereof further comprises a light chain constant region or fragment thereof fused to the C-terminus of the VL. The light chain constant region can be, *e.g.*, a murine kappa constant region. In certain aspects, this antibody or fragment thereof further comprises a heavy chain constant region or fragment thereof fused to the C-terminus of the VH. The heavy chain constant region can be, *e.g.*, a murine IgG2a constant region. In certain aspects this rat-anti-mouse OX40 antibody comprises the heavy chain amino acid sequence SEQ ID NO: 86 and the light chain amino acid sequence SEQ ID NO: 89. In certain aspects, a rat-anti-mouse OX40 antibody or fragment thereof as provided herein can specifically bind to mouse OX40. In certain aspects, administration of an effective dose of a rat-anti-mouse OX40 antibody or fragment thereof as provided herein to mouse can inhibit mouse cancer cell line growth in the mouse.

[0182] This disclosure further provides a host cell comprising a polynucleotide, polynucleotide composition, or vector as provided above, where the host cell can, in some instances, express an antibody or antigen-binding fragment thereof that specifically binds to OX40, *e.g.*, human OX40, cynomolgus monkey OX40, or rhesus monkey OX40. Such a host cell can be utilized in a method

of making an antibody or antigen-binding fragment thereof as provided herein, which method includes (a) culturing the host cell and (b) isolating the antibody or antigen-binding fragment thereof expressed from the host cell.

[0183] Polynucleotide variants are also provided. Polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some aspects polynucleotide variants contain alterations that produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In some aspects, polynucleotide variants are produced by silent substitutions due to the degeneracy of the genetic code. Polynucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to those by a bacterial host such as *E. coli*). Vectors and cells comprising the polynucleotides described herein are also provided.

[0184] In some aspects, a DNA sequence encoding a humanized anti-OX40 antibody or an antigen-binding fragment thereof can be constructed by chemical synthesis using an oligonucleotide synthesizer. Such oligonucleotides can be designed based on the amino acid sequence of the desired polypeptide and selecting those codons that are favored in the host cell in which the recombinant polypeptide of interest will be produced. Standard methods can be applied to synthesize an isolated polynucleotide sequence encoding an isolated polypeptide of interest. For example, a complete amino acid sequence can be used to construct a back-translated gene. Further, a DNA oligomer containing a nucleotide sequence coding for the particular isolated polypeptide can be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and then ligated. The individual oligonucleotides can contain 5' or 3' overhangs for complementary assembly.

[0185] Once assembled (by synthesis, site-directed mutagenesis or another method), the polynucleotide sequences encoding a particular isolated polypeptide of interest can be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the protein in a desired host. Proper assembly can be confirmed, *e.g.*, by nucleotide sequencing, restriction mapping, and/or expression of a biologically active polypeptide in a suitable host. In order to obtain high expression levels of a transfected gene in a host, the gene can be operatively linked to or associated with transcriptional and translational expression control sequences that are functional in the chosen expression host.

[0186] In certain aspects, recombinant expression vectors are used to amplify and express DNA encoding a humanized anti-OX40 antibody or an antigen-binding fragment thereof. Recombinant expression vectors are replicable DNA constructs which have synthetic or cDNA-derived DNA fragments encoding a polypeptide chain of a humanized anti-OX40 antibody or an antigen-binding fragment thereof, operatively linked to suitable transcriptional or translational regulatory elements derived from mammalian, microbial, viral or insect genes. In one example, a transcriptional unit can comprise an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, transcriptional promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences, as described in detail below. Such regulatory elements can include an operator sequence to control transcription. The ability to replicate in a host, conferred, *e.g.*, by an origin of replication, and a selection gene to facilitate recognition of transformants can additionally be incorporated. DNA regions are operatively linked when they are functionally related to each other. For example, DNA for a signal peptide (secretory leader) is operatively linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operatively linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operatively linked to a coding sequence if it is positioned so as to permit translation. Structural elements intended for use in yeast expression systems include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where a recombinant protein is expressed without a leader or transport sequence, the protein can include an N-terminal methionine. This methionine can optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

[0187] The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations can be employed. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pCR 1, pBR322, pMB9 and their derivatives, wider host range plasmids, such as M13 and filamentous single-stranded DNA phages.

[0188] Suitable host cells for expression of a humanized anti-OX40 antibody or an antigen-binding fragment thereof include prokaryotes, yeast, insect or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or bacilli. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed. Additional information regarding methods of protein production, including antibody production, can be found, *e.g.*, in U.S. Patent Publication No. 2008/0187954, U.S. Patent Nos. 6,413,746 and 6,660,501, and International Patent Publication No. WO 04009823, each of which is hereby incorporated by reference herein in its entirety.

[0189] Various mammalian or insect cell culture systems can also be employed to express a humanized anti-OX40 antibody or an antigen-binding fragment thereof. Expression of recombinant proteins in mammalian cells can be performed because such proteins are generally correctly folded, appropriately modified and completely functional. Examples of suitable mammalian host cell lines include HEK-293 and HEK-293T, the COS-7 lines of monkey kidney cells, described by Gluzman (Cell 23:175, 1981), and other cell lines including, for example, L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. Mammalian expression vectors can comprise nontranscribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, BioTechnology 6:47 (1988).

[0190] A humanized anti-OX40 antibody or an antigen-binding fragment thereof produced by a transformed host, can be purified according to any suitable method. Such standard methods include chromatography (*e.g.*, ion exchange, affinity and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for protein purification. Affinity tags such as hexahistidine, maltose binding domain, influenza coat sequence and glutathione-S-transferase can be attached to the protein to allow easy purification by passage over an appropriate affinity column. Isolated proteins can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance and x-ray crystallography.

[0191] For example, supernatants from systems that secrete recombinant protein into culture media can be first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a suitable purification matrix. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, *e.g.*, silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a humanized anti-OX40 antibody or an antigen-binding fragment thereof. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a homogeneous recombinant protein.

[0192] A humanized anti-OX40 antibody or an antigen-binding fragment thereof produced in bacterial culture, can be isolated, for example, by initial extraction from cell pellets, followed by one or more concentration, salting-out, aqueous ion exchange or size exclusion chromatography steps. High performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of a recombinant protein can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

[0193] Methods known in the art for purifying antibodies and other proteins also include, for example, those described in U.S. Patent Publication Nos. 2008/0312425, 2008/0177048, and 2009/0187005, each of which is hereby incorporated by reference herein in its entirety.

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION METHODS

[0194] Methods of preparing and administering a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein, to a subject in need thereof, *e.g.*, to enhance an immune response in a cancer patient, *e.g.*, to inhibit or reduce tumor growth, are well known to or can be readily determined by those skilled in the art. The route of administration of a humanized anti-OX40 antibody or an antigen-binding fragment thereof can be, for example, oral, parenteral,

by inhalation or topical. The term parenteral as used herein includes, *e.g.*, intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal, or vaginal administration. While all these forms of administration are clearly contemplated as suitable forms, another example of a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition can comprise, without limitation, a buffer (*e.g.* acetate, phosphate or citrate buffer), a surfactant (*e.g.* polysorbate), a stabilizer agent (*e.g.* human albumin), etc. In other methods compatible with the teachings herein, a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

[0195] Certain pharmaceutical compositions provided herein can be orally administered in an acceptable dosage form including, *e.g.*, capsules, tablets, aqueous suspensions or solutions. Certain pharmaceutical compositions also can be administered by nasal aerosol or inhalation. Such compositions can be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, and/or other conventional solubilizing or dispersing agents.

[0196] The amount of a humanized anti-OX40 antibody or an antigen-binding fragment thereof that can be combined with carrier materials to produce a single dosage form will vary depending upon the subject treated and the particular mode of administration. The composition can be administered as a single dose, multiple doses or over an established period of time in an infusion. Dosage regimens also can be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response).

[0197] By "therapeutically effective dose or amount" or "effective amount" is intended an amount of a humanized anti-OX40 antibody or an antigen-binding fragment thereof, that when administered brings about a positive therapeutic response with respect to treatment of a patient with a disease or condition to be treated.

KITS

[0198] This disclosure further provides kits that comprise a humanized anti-OX40 antibody or an antigen-binding fragment thereof as described herein and that can be used to perform the methods

described herein. In certain embodiments, a kit comprises at least one purified humanized anti-OX40 antibody or an antigen-binding fragment thereof, in one or more containers. One skilled in the art will readily recognize that the disclosed humanized anti-OX40 antibody can be readily incorporated into one of the established kit formats that are well known in the art.

IMMUNOASSAYS

[0199] A humanized anti-OX40 antibody or an antigen-binding fragment thereof can be assayed for specific and/or selective binding by any method known in the art. The immunoassays that can be used include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), fluorescent focus assay (FFA), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, to name but a few. Such assays are routine and well known in the art (see, *e.g.*, Ausubel *et al.*, eds, (1994) Current Protocols in Molecular Biology (John Wiley & Sons, Inc., NY) Vol. 1, which is incorporated by reference herein in its entirety).

[0200] Methods and reagents suitable for determination of binding characteristics of a humanized anti-OX40 antibody as provided herein are known in the art and/or are commercially available. Equipment and software designed for such kinetic analyses are commercially available (*e.g.*, BIAcore®, BIAevaluation® software, GE Healthcare; KINEXA® Software, Sapidyne Instruments).

METHODS OF IMMUNE ENHANCEMENT AND TREATMENT

[0201] The enhancement of an antigen-specific immune response in a subject (*e.g.*, a mammalian subject, such as a human subject) by engaging OX40 on activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells, during or after antigen activation can be accomplished using a wide variety of methods. The method of choice will primarily depend upon the type of antigen against which it is desired to enhance the immune response, and various methods available are discussed below. Whatever method is selected, a humanized anti-OX40 antibody or an antigen-

binding fragment thereof can be administered to a subject, *e.g.*, a human patient such that it is presented to T cells of the subject during or shortly after priming of the T cells by antigen.

[0202] In certain aspects, the disclosure provides a method to promote survival or proliferation of activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells, comprising contacting the activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells, with a humanized anti-OX40 antibody or an antigen-binding fragment thereof, under conditions where the humanized anti-OX40 antibody can specifically bind to OX40 on the surface of the T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells. In certain aspects the contacting is *in vitro*. In certain aspects the contacting is *in vivo*, *e.g.* via administration of an effective dose of the humanized anti-OX40 antibody to a subject in need of treatment. In certain aspects the contacting can occur at the same time as T cell activation, *e.g.*, antigen activation, in certain aspects the contacting can occur after T cell activation.

[0203] In further aspects, the disclosure provides a method of inducing cytokine release from activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells, comprising contacting the activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells, with a humanized anti-OX40 antibody or an antigen-binding fragment thereof as provided herein, wherein the humanized anti-OX40 antibody can specifically bind to OX40 on the surface of the activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells. In certain aspects the contacting is *in vitro*. In certain aspects the contacting is *in vivo*, *e.g.* via administration of an effective dose of the humanized anti-OX40 antibody to a subject in need of treatment. In certain aspects the contacting can occur at the same time as T cell activation, *e.g.*, antigen activation, in certain aspects the contacting can occur after T cell activation. In certain aspects the cytokine can be IFN γ , TNF α , IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1 β , or any combination thereof. In certain aspects the cytokine is IFN γ , TNF α , IL-5, IL-10, IL-13, or any combination thereof.

[0204] In certain aspects, the activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells are human T cells, cynomolgus monkey T cells, rhesus monkey T cells, or a combination thereof.

[0205] The disclosure further provides a method of promoting T cell activation, comprising contacting T cells with a humanized anti-OX40 antibody as provided herein, wherein the

humanized anti-OX40 antibody can specifically bind to OX40 on the surface of the T cells. In certain aspects the contacting occurs in the presence of antigen, *e.g.*, a tumor antigen. In certain aspects the method further comprises interaction of an Fc domain of the humanized anti-OX40 antibody with a cell expressing FcγR, *e.g.*, a B cell, monocyte, macrophage, myeloid or plasmacytoid dendritic cell, follicular dendritic cell, Langerhans cell, endothelial cell, NK cell, activated T cell, neutrophil, eosinophil, platelet, mast cell, a CD45⁺ cell from a primary human tumor or tumor-draining or non-draining lymph node, a CD45⁺ cell from other secondary or tertiary lymphoid structures, or a combination thereof. In certain aspects, the T cell activation can be measured through stimulation of the NFκB signal transduction pathway. In certain aspects the contacting is *in vitro*. In certain aspects the contacting is *in vivo*, *e.g.* via administration of an effective dose of the humanized anti-OX40 antibody to a subject in need of treatment.

[0206] The disclosure further provides a method of treating cancer in a subject, comprising administering to a subject in need of treatment an effective amount of a humanized anti-OX40 antibody or an antigen-binding fragment thereof, or a composition or formulation comprising the humanized anti-OX40 antibody. In certain aspects, the cancer is a solid tumor. According to this method, administration of humanized anti-OX40 antibody or composition can inhibit tumor growth; can promote tumor reduction, or both. In certain aspects, the tumor growth inhibition is achieved in the presence of T cells.

[0207] The terms "cancer", "tumor", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancers include but are not limited to, carcinoma including adenocarcinomas, lymphomas, blastomas, melanomas, sarcomas, and leukemias. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer (including hormonally mediated breast cancer, see, *e.g.*, Innes *et al.* (2006) Br. J. Cancer 94:1057-1065), colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, various types of head and neck cancer

including, but not limited to, squamous cell cancers, and cancers of mucinous origins, such as, mucinous ovarian cancer, cholangiocarcinoma (liver) and renal papillary carcinoma.

[0208] This disclosure further provides a method of preventing or treating a cancer in a subject in need thereof, comprising administering to the subject an effective amount of a humanized anti-OX40 antibody or an antigen-binding fragment thereof, a composition or formulation comprising the humanized anti-OX40 antibody, or a polynucleotide, a vector, or a host cell as described herein.

[0209] Effective doses of compositions for treatment of cancer vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but non-human mammals including transgenic mammals can also be treated. Treatment dosages can be titrated using routine methods known to those of skill in the art to optimize safety and efficacy.

[0210] The compositions of the disclosure can be administered by any suitable method, *e.g.*, parenterally, intraventricularly, orally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term “parenteral” as used herein includes subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques.

[0211] The disclosure further provides a method of enhancing an immune response in a subject comprising administering to a subject in need thereof a therapeutically effective amount of a humanized anti-OX40 antibody or an antigen-binding fragment thereof, or a composition or formulation comprising the humanized anti-OX40 antibody.

[0212] The subject to be treated can be any animal, *e.g.*, mammal, in need of treatment, in certain aspects, subject is a human subject.

[0213] In its simplest form, a preparation to be administered to a subject is a humanized anti-OX40 antibody or an antigen-binding fragment thereof, administered in conventional dosage form, which can be combined with a pharmaceutical excipient, carrier or diluent as described elsewhere herein.

[0214] A humanized anti-OX40 antibody or an antigen-binding fragment thereof can be administered by any suitable method as described elsewhere herein, *e.g.*, via IV infusion. In

certain aspects, a humanized anti-OX40 antibody or an antigen-binding fragment thereof can be introduced into a tumor, or in the vicinity of a tumor cell.

[0215] All types of tumors are potentially amenable to treatment by this approach including, without limitation, carcinoma of the breast, lung, pancreas, ovary, kidney, colon and bladder, as well as melanomas, sarcomas and lymphomas.

[0216] Engagement of the OX40 receptor on activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells during, or shortly after, priming by an antigen results in an increased response of the activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells to the antigen. In the context of the present disclosure, the term "engagement" refers to binding to and stimulation of at least one activity mediated by the OX40 receptor. For example, engagement of the OX40 receptor on antigen specific activated T cells, *e.g.*, activated CD4⁺ T cells and/or activated CD8⁺ T cells, results in increased T cell proliferation as compared to the response to antigen alone, and increased cytokine production. The elevated response to the antigen can be maintained for a period of time substantially longer than in the absence of OX40 receptor engagement. Thus, stimulation via the OX40 receptor enhances the antigen specific immune response by boosting T cell recognition of antigens, *e.g.*, tumor antigens.

[0217] OX40 agonists can enhance antigen specific immune responses in a subject, such as a human subject, when administered to the subject during or shortly after priming of T cells by an antigen. OX40 agonists include OX40 ligand ("OX40L"), such as soluble OX40L fusion proteins and anti-OX40 antibodies or fragments thereof. A specific example is a humanized antibody that specifically binds to OX40, thereby triggering signaling. A collection of humanized anti-OX40 monoclonal antibodies are provided by this disclosure. Also described are nucleic acids including polynucleotide sequences that encode such antibodies. This disclosure also provides methods for enhancing an antigen specific immune response in a subject using humanized anti-OX40 monoclonal antibodies.

OX40 EPITOPES

[0218] The portion of a target molecule, *e.g.*, an OX40 polypeptide, which specifically interacts with the antigen binding domain of an antibody is an "epitope," or an "antigenic determinant." A target molecule, *e.g.*, a polypeptide, can be a single epitope, but typically includes at least two

epitopes, and can include any number of epitopes, depending on the size, conformation, and type of target molecule.

[0219] The minimum size of an epitope that can be bound by an antibody on a target polypeptide is thought to be about four to five amino acids. Peptide or polypeptide epitopes can contain at least seven, at least nine, at least ten, or at least about 15 or more amino acids. Since an antibody can recognize a polypeptide antigen in its tertiary form, the amino acids comprising an epitope need not be contiguous. An epitope of OX40, *e.g.*, human OX40 as provided herein can include at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or between about 10 to about 30 contiguous or non-contiguous amino acids of OX40, *e.g.*, human OX40. In certain aspects, an epitope of OX40, *e.g.*, human OX40 as provided herein consists of a peptide of 100 or fewer amino acids, 75 or fewer amino acids, 50 or fewer amino acids, 40 or fewer amino acids, 35 or fewer amino acids, 30 or fewer amino acids, 25 or fewer amino acids, 20 or fewer amino acids, or 15 or fewer amino acids, and can include at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or between about 10 to about 30 contiguous or non-contiguous amino acids of OX40, *e.g.*, human OX40. On the other hand, an epitope of OX40, *e.g.*, human OX40 as provided herein can include no more than 4, no more than 5, no more than 6, no more than 7, no more than 8, no more than 9, no more than 10, no more than 15, no more than 20, no more than 25, or can consist of between about 10 to about 30 contiguous or non-contiguous amino acids of OX40, *e.g.*, human OX40.

[0220] In certain aspects, an anti-OX40 antibody or fragment thereof as provided herein binds to an epitope of OX40, *e.g.*, human OX40, rhesus monkey OX40, or cynomolgus monkey OX40 that falls within the third cysteine rich domain (CRD3) of OX40, *e.g.*, within amino acids 108 to 146 of human OX40 (SEQ ID NO: 91), or a peptide at least 17%, at least 75%, at least 80%, at least 85%, and least 90%, at least 95%, or at least 100% identical to amino acids 108 to 146 of SEQ ID NO: 91. By “falls within” the CRD3 of OX40, means that the means that the epitope can include 4 or more, 5 or more, 6 or more 7 or more 8 or more 9 or more, 10 or more, 15 or more, or 30 or more contiguous or non-contiguous amino acids amino acids of the region of OX40 consisting of the CRD3 region, *e.g.*, amino acids 108 to 146 of SEQ ID NO: 91, or a peptide at least 70%, at least 75%, at least 80%, at least 85%, and least 90%, at least 95%, or at least 100% identical to amino acids 108 to 146 of SEQ ID NO: 91.

[0221] In certain aspects the OX40 CRD3 peptide that binds the antibody provided herein retains a leucine at the position corresponding to amino acid 116 of SEQ ID NO: 91, and an alanine at the position corresponding to amino acid 126 of SEQ ID NO: 91. For example, certain anti-OX40 antibodies or fragments thereof as provided herein bind to human OX40 but do not bind to mouse or rat OX40. The CRD3 region of mouse OX40 stretches from about amino acid 104 to about amino acid 144 of SEQ ID NO: 92. Amino acid Q113 of mouse OX40, SEQ ID NO: 92, corresponds to amino acid L116 of human OX40, SEQ ID NO: 91, and amino acid V124 of mouse OX40, SEQ ID NO: 92, corresponds to amino acid A126 of human OX40, SEQ ID NO: 91. As shown in Example 10, an OX40 antibody as provided herein, *e.g.*, OX40mAb24, can bind to a variant of mouse OX40 comprising SEQ ID NO: 92 except for a Q113L mutation and a V124A.

[0222] In certain aspects an isolated peptide is provided, the peptide consisting of or comprising an epitope that specifically binds an OX40 antibody as provided herein, *e.g.*, OX40mAb24. In certain aspects, the peptide consists of 100 or fewer amino acids, 75 or fewer amino acids, 50 or fewer amino acids, 40 or fewer amino acids, 35 or fewer amino acids, 30 or fewer amino acids, 25 or fewer amino acids, 20 or fewer amino acids, or 15 or fewer amino acids, and includes at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, or between about 10 to about 30 contiguous or non-contiguous amino acids of the CRD3 of OX40, *e.g.*, an OX40 region at least 70%, at least 75%, at least 80%, at least 85%, and least 90%, at least 95%, or at least 100% identical to amino acids 108 to 146 of SEQ ID NO: 91. In certain aspects, the peptide retains a leucine at the position corresponding to amino acid 116 of SEQ ID NO: 91, and an alanine at the position corresponding to amino acid 126 of SEQ ID NO: 91.

[0223] Such an isolated peptide can be used, *e.g.*, for screening libraries for binding molecules that specifically bind to OX40, or as an immunogen to raise anti-OX40 antibodies in a subject animal.

[0224] This disclosure employs, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, ed. (1989) *Molecular Cloning A Laboratory Manual* (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook *et al.*, ed. (1992) *Molecular Cloning: A Laboratory Manual*, (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) *DNA*

Cloning, Volumes I and II; Gait, ed. (1984) Oligonucleotide Synthesis; Mullis *et al.* U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) Nucleic Acid Hybridization; Hames and Higgins, eds. (1984) Transcription And Translation; Freshney (1987) Culture Of Animal Cells (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) A Practical Guide To Molecular Cloning; the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) Gene Transfer Vectors For Mammalian Cells, (Cold Spring Harbor Laboratory); Wu *et al.*, eds., Methods In Enzymology, Vols. 154 and 155; Mayer and Walker, eds. (1987) Immunochemical Methods In Cell And Molecular Biology (Academic Press, London); Weir and Blackwell, eds., (1986) Handbook Of Experimental Immunology, Volumes I-IV; Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); and in Ausubel *et al.* (1989) Current Protocols in Molecular Biology (John Wiley and Sons, Baltimore, Md.).

[0225] General principles of antibody engineering are set forth in Borrebaeck, ed. (1995) Antibody Engineering (2nd ed.; Oxford Univ. Press). General principles of protein engineering are set forth in Rickwood *et al.*, eds. (1995) Protein Engineering, A Practical Approach (IRL Press at Oxford Univ. Press, Oxford, Eng.). General principles of antibodies and antibody-hapten binding are set forth in: Nisonoff (1984) Molecular Immunology (2nd ed.; Sinauer Associates, Sunderland, Mass.); and Steward (1984) Antibodies, Their Structure and Function (Chapman and Hall, New York, N.Y.). Additionally, standard methods in immunology known in the art and not specifically described are generally followed as in Current Protocols in Immunology, John Wiley & Sons, New York; Stites *et al.*, eds. (1994) Basic and Clinical Immunology (8th ed; Appleton & Lange, Norwalk, Conn.) and Mishell and Shiigi (eds) (1980) Selected Methods in Cellular Immunology (W.H. Freeman and Co., NY).

[0226] Standard reference works setting forth general principles of immunology include Current Protocols in Immunology, John Wiley & Sons, New York; Klein (1982) J., Immunology: The Science of Self-Nonself Discrimination (John Wiley & Sons, NY); Kennett *et al.*, eds. (1980) Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses (Plenum Press, NY); Campbell (1984) "Monoclonal Antibody Technology" in Laboratory Techniques in Biochemistry and Molecular Biology, ed. Burden *et al.*, (Elsevier, Amsterdam); Goldsby *et al.*, eds. (2000) Kuby Immunology (4th ed.; H. Freeman & Co.); Roitt *et al.* (2001) Immunology (6th

ed.; London: Mosby); Abbas *et al.* (2005) Cellular and Molecular Immunology (5th ed.; Elsevier Health Sciences Division); Kontermann and Dubel (2001) Antibody Engineering (Springer Verlag); Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press); Lewin (2003) Genes VIII (Prentice Hall 2003); Harlow and Lane (1988) Antibodies: A Laboratory Manual (Cold Spring Harbor Press); Dieffenbach and Dveksler (2003) PCR Primer (Cold Spring Harbor Press).

[0227] All of the references cited above, as well as all references cited herein, are incorporated herein by reference in their entireties.

[0228] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

[0229] Abbreviations and definitions of terms are listed in Table 2.

[0230] Table 2. List of Abbreviations and Definitions of Terms

Abbreviation or Term	Definition
1A7	Isotype control mouse IgG1 kappa antibody
9B12	Mouse anti-human OX40 IgG1κ monoclonal antibody
A375	human melanoma cell line
Aa	amino acid
ADCC	Antibody-dependent cellular cytotoxicity
ADPE	Antibody Development and Protein Engineering
ANOVA	Analysis of variance
Asp	aspartic acid
BSA	Bovine serum albumin
BLASTP	protein sequence homology Basic Local Alignment Search Tool
C	Final tumor volumes from the control group
CD	cluster of differentiation
CDC	Complement-dependent cytotoxicity
CD4 ⁺ , OX40 ⁺	CD4 positive, OX40 positive
CDR	complementarity-determining regions
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence Interval
Clq	Complement component Clq
CR	Complete response
CRD	Cysteine rich domain
Cyno	cynomolgus

Abbreviation or Term	Definition
DM-L	density media- lymphocyte
E:T	effector-to-target (ratio)
EC	effective concentration
EC _f	effective concentration resulting in f % of maximal effect
EC ₂₀	effective concentration resulting in 20% of maximal effect
EC ₅₀	half maximal effective concentration
EC ₉₀	effective concentration resulting in 90% of maximal effect
F	fraction of maximal
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
Fc	Fragment, crystallizable
Fcγ1g-/-	Genetically engineered mouse strain; lacks expression of activating Fc gamma receptors (Fc gamma I, III, and IV)
Fcγ2b-/-	Genetically engineered mouse strain; lacks expression of inhibitory Fc gamma IIb receptor
FCS	Flow cytometry standard
Fcγ	fragment, crystallizable gamma
FMO	Fluorescence minus-one
FP	Fusion protein
G	Force of gravity
H	hour
H ⁺ L	heavy plus light chains
HEK293	Human embryonic kidney cell line
Hr	hour
HSC	Hematopoietic stem cell
Hu	Human
ICOS	Inducible T-cell co-stimulator
IgG	Immunoglobulin
IgG1	Immunoglobulin G1
IL-2	interleukin 2
IP	intraperitoneal
IU	International Units
K _d	equilibrium binding dissociation constant
KI	knock-in
KLH	Keyhole limpet hemocyanin
KO	knock-out
K _p	equilibrium dissociation constant
Leu	Leucine
M	Mouse
mAb	monoclonal antibody
mL	milliliter
OX40mAb24	Humanized anti-human OX40 IgG1κ monoclonal antibody

Abbreviation or Term	Definition
MFI	mean fluorescence intensity
mOX40L FP	Mouse OX40 ligand mouse IgG1 fusion protein
mOX40L FP (Y182A)	Mouse OX40 ligand mouse IgG1 fusion protein engineered to have reduced binding to OX40
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIP228	Mouse IgG1 kappa monoclonal antibody against 4-hydroxy-3-iodo-5-nitrophenylacetic acid
NK	natural killer
NOD/SCID	non-obese diabetic/severe combined immunodeficient
NSG	Mice with genetic background <i>Nod.Cg-Prkd^{cscid}112rg^{tm/Wy}/SzJ</i>
OX40L	OX40 ligand
OX40L FP IgG4P Y180A	human OX40 ligand IgG4P fusion protein engineered to have reduced binding to OX40
OX40mAb24	Humanized anti-human OX40 IgG1 κ monoclonal antibody
OX86	Rat anti-mouse OX40 IgG1κ monoclonal antibody
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA-L	Phytohemagglutinin-Leucoagglutinin
PI	Propidium iodide
PK/PD	Pharmacokinetic/pharmacodynamics
RBC	red blood cell
RBCL	Red blood cell lysis
RBD	receptor binding domain
Rh	recombinant human
RLU	Relative light units
RPMI	Roswell Park Memorial Institute 1640 medium
ROA	Route of administration
SC	subcutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
Ser	serine
T	Final tumor volumes from the test group
Tcm	Central Memory T cell
TCR	T cell receptor
Teff	Effector T cell
Tem	Effector memory T cell
TGI	tumor growth inhibition
TIL	tumor infiltrating leukocyte
TM	transmembrane domain
TNFR	tumor necrosis factor receptor
TNFRSF	tumor necrosis factor receptor superfamily
TRAF2	tumor necrosis factor receptor-associated factor 2

Abbreviation or Term	Definition
Treg	regulatory T cells
μL	microliter
μg	Microgram
V	volume

EXAMPLE 1: Humanization of Anti-human OX40 Murine MAb 9B12

[0231] Murine mAb 9B12 was humanized by grafting its CDRs onto selected human germline frameworks. The sequences of the heavy chain variable region (VH) and the light chain variable region (VL) of murine mAb 9B12 were compared with the human antibody germline sequences available in the public NCBI databases. Human acceptor frameworks (FR) were identified based on the highest sequence homology. When selecting an optimal acceptor framework several criteria were considered such as matching residues that could impact binding (residues in the Vernier zone, canonical class residues, and VH/VL interface residues), and the potential for immunogenicity (low germline frequency). An optimal hybrid human acceptor FR sequence was designed for VH and VL independently by selecting the most homologous human immunoglobulin germline segment for each individual framework region. Three different germline acceptor sequences were combined to form the humanized VH framework backbone, while two different germline acceptor sequences were selected for VL. The fully human germline acceptor template chosen for the VH chain (9B12VH-hu) was a combination of IG VH4-34*09 (FR 1), VH4-39 (FR 2), VH6-1(FR 3) and JH4 (FR 4). VL (9B12VL-hu1) was a combination of O18 (FR 1), O18 (FR 2), L23 (FR 3) and JK1 (FR 4). The framework homology between the murine sequence and human template acceptor sequence was approximately 72% for VH and 77% for VL.

[0232] Murine framework residues which could influence or maintain the functional conformation of the parent CDR and did not match the human germline sequence were identified and selectively reintroduced into the human acceptor FR to best preserve the 9B12 binding affinity and functionality. In this case, mouse FR residues 27D, 39K, 47Y, 48M, 71R, 78Y and 91F in the VH chain and 44V and 68R in the VL chain were mutated back in the human template. CDR residues as defined by Kabat were fused into the designed acceptor frameworks for both VH and VL for generating the humanized antibody. Two humanized VH genes (9B12VH-hu and 9B12VH-hu39K71R) and two VL genes (9B12VL-hu1 and 9B12VL-hu2) were synthesized by GeneART

(Thermo Fisher Scientific, Waltham, MA), then cloned into the in-house pOE IgG1 expression vector.

[0233] A panel of humanized variants has been generated and characterized in T cell-based binding and proliferation assays. The humanized variants, containing several framework amino acids reverted to mouse FR residues in the heavy chain variable region, bound to OX40 on activated CD4⁺ T cells with comparable affinities and similar potencies (T cell proliferation) to 9B12. The variable light chain paired with all humanized VH variants encodes for fully humanized frameworks. To reduce the risk of immunogenicity, the number of mouse residues in the VH human FR was further reduced to 3 or 4 by replacing non-influential mouse residues with the corresponding human residues. To remove potential sequence liabilities, the NG deamidation site in VH-CDR2, the RYD integrin binding site and the DG isomerization site in VH-CDR3 were proactively removed either independently or in combination. To avoid ADCC mediated by human IgG1 Fc effector function, IgG4P and IgG1TM Fc variants were made for the humanized lead mAbs. The resultant IgG4P and IgG1TM variants exhibited the same binding activity to OX40 as the IgG1 variants, but significantly reduced ADCC activity which is most similar to the murine mAb 9B12. In summary, the humanized variants exhibited cell-binding affinities and *in vitro* potencies comparable to the parent mouse mAb 9B12. The amino acid differences among the humanized VH variant, all of which are paired with the humanized VL, are summarized in FIG. 1.

[0234] Reverse chimera was engineered for *in vivo* characterization in rhesus monkeys. Briefly, the VH and VL of humanized mAb24 were grafted onto the constant heavy and constant light chains of murine 9B12. *In vitro* characterization demonstrated the Fab portions of the reverse chimera bound to human OX40 comparable to mAb24.

EXAMPLE 2: Binding Affinity and Receptor Occupancy of OX40mAb24 to Native OX40 Expressed on the Surface of Activated Human, Non-human Primate, Rat and Mouse T cells

2.1 MATERIALS

[0235] Materials used in this example are listed in Table 2-1.

Table 2-1 Materials

Item	Source
AlexaFluor® A488 goat anti-human IgG (H+L)	Life Technologies, Carlsbad, CA
AlexaFluor® A488 goat anti-mouse IgG (H+L)	Life Technologies, Carlsbad, CA
AlexaFluor® A488 goat anti-rat IgG (H+L)	Life Technologies, Carlsbad, CA

Item	Source
Antibiotic/antimycotic solution, 100X	Life Technologies, Carlsbad, CA
Anti-rat CD134 (OX40 clone) antibody	Biolegend, San Jose, CA
Anti-rat CD3 antibody	BD Biosciences, San Jose, CA
Anti-mouse CD134 (OX86 clone) antibody	MedImmune, Gaithersburg, MD
Anti-rat IgG1, κ isotype control mAb clone RTK2071	Biolegend, San Jose, CA
Balb/C mouse	Harlan, Indianapolis, IN
Beta mercaptoethanol (BME)	Life Technologies, Carlsbad, CA
Concanavalin A	Sigma, St. Louis, MO
Ethylenediaminetetraacetic acid (EDTA)	Life Technologies, Carlsbad, CA
Heat inactivated newborn calf serum (FBS)	Life Technologies, Carlsbad, CA
Hamster anti-mouse CD3 antibody	BD Biosciences, San Jose, CA
Hamster anti-mouse CD28 antibody	BD Biosciences, San Jose, CA
IL-2, recombinant human	Preprotech, Rocky Hill, NJ
Lymphocyte separation medium (LSM)	MP Biomedicals, Santa Ana, CA
Magselect rat CD4 T cell isolation kit	R&D Systems, Minneapolis, MN
Miltenyi MACS buffer	Miltenyi San, Diego, CA
Mouse CD4 T cell isolation kit	Miltenyi San, Diego, CA
Mouse IgG1, κ isotype control mAb clone MOPC-21	Biolegend, San Jose, CA
Non-human primate CD4 T cell isolation kit	Miltenyi San, Diego, CA
Non-TC treated round-bottom 96 well plates	VWR, Radnor, PA
Percoll	Sigma, St. Louis, MO
Phytohemagglutinin-Leucoagglutinin (PHA-L)	Roche Applied Science, Indianapolis, IN
Phosphate buffered saline, pH 7.2 (PBS)	Life Technologies, Carlsbad, CA
RosetteSep CD4 T cell enrichment kit	Stem Cell Technologies, Vancouver, BC
RPMI-1640	Life Technologies, Carlsbad, CA
Sprague Dawley rat	Harlan, Indianapolis, IN
Whole blood, sodium heparin anti-coagulated	MedImmune Blood Donor Program, Gaithersburg, MD

[0236] In this example, cell-based equilibrium binding assays were performed to measure the apparent affinity of OX40mAb24 binding to OX40 expressed on the cell surface of human, non-human primate, rat and mouse T cells. Additionally, the equilibrium binding assays were utilized to determine the concentrations of OX40mAb24 that achieve 20%, 50%, or 90% human OX40 receptor occupancy on activated human CD4⁺ T cells.

[0237] OX40mAb24 concentrations required to achieve 20%, 50% or 90% receptor occupancy were also determined for binding of 9B12, the murine anti-human OX40 monoclonal antibody

from which OX40mAb24 was derived, to human and non-human primate OX40 expressed on the surface of CD4⁺ T cells for a comparison of OX40mAb24 and 9B12 binding.

2.2 Assays

2.2.1 Binding of OX40mAb24 to Primary Human CD4⁺ T cells and OX40-expressing Jurkat T cells

[0238] The apparent equilibrium binding constant (K_d) for OX40mAb24 binding to human OX40 and the concentrations required to bind 20%, 50%, or 90% of cell surface human OX40 receptor at equilibrium were calculated from binding curves of OX40mAb24 to OX40-expressing activated primary human CD4⁺ T cells or human OX40-overexpressing Jurkat T cells. Similar experiments were performed at the same time to assess binding of 9B12 to these cells for comparison with OX40mAb24 values.

[0239] Primary human CD4⁺ T cells were first isolated from sodium heparin anti-coagulated whole blood obtained from healthy donors through the MedImmune Blood Donor Program using a RosetteSep CD4⁺ T cell enrichment kit (Stem Cell Technologies, Vancouver, BC) and a modified manufacturer's protocol.

[0240] Primary human CD4⁺ T cells were cultured for 48h with 2 μ g/mL PHA-L and 20 IU/mL rhIL-2 to activate T cells and up-regulate OX40. Activated T cells, which were >95% viable, were subsequently used in OX40mAb24 binding experiments. All donors represent unique individuals; that is, repeat binding experiments were not performed with CD4⁺ T cells from the same donor.

[0241] Human OX40-overexpressing Jurkat NF κ B-luciferase clone 64 cells were cultured in complete RPMI +10% FBS prior to binding experiments, without the need for activation.

[0242] OX40mAb24 (10 μ g/mL) or 9B12 (10 μ g/mL) was diluted over a 17 point 2-fold dilution series. OX40mAb24 was added to 100,000 cells (activated primary CD4⁺ T cells or human OX40-overexpressing Jurkat NF κ B-luciferase clone 64 cells) per well and incubated for one hour at 4°C. For background binding subtraction, cells were incubated in the presence of secondary antibody alone. The control R347 human IgG1 monoclonal antibody and mouse IgG1 isotype clone MOPC-21 were used in experiments with OX40 over-expressing Jurkat T cells to demonstrate the specificity for OX40 binding of OX40mAb24 or 9B12, respectively. Following incubation, cells were washed three times with 200 μ L of cold (4°C) FACS buffer and incubated with 100 μ L of FACS buffer (PBS + 2% heat-inactivated newborn calf serum) containing 10 μ g/mL AlexaFluor®

647 labeled goat anti-human IgG secondary antibody (for binding to OX40mAb24) or 10 µg/mL AlexaFluor® 488 labeled goat anti-mouse IgG secondary antibody (for binding to 9B12) and 5 µg/mL propidium iodide (PI). Following secondary antibody incubation, cells were washed and suspended in 100 µL FACS buffer for flow cytometry analysis on a BD LSRII flow cytometer as described below.

2.2.2 OX40mAb24 binding to Mouse CD4⁺ T cells

[0243] OX40mAb24 was investigated for binding to mouse OX40 expressed on activated primary CD4⁺ T cells. Similar experiments were performed at the same time to assess binding of 9B12 to these cells in comparison with OX40mAb24. Mouse CD4⁺ T cells were isolated from harvested normal Balb/C mouse spleens according to the following protocol:

[0244] Spleens were mashed against a 70 µM nylon filter to release splenocytes and the filter rinsed with 1 mL complete medium (RPMI-1640 plus 10% FBS, 1% antibiotic/antimycotic solution and 55 µM beta mercaptoethanol [BME]). Splenocytes were pelleted and the supernatant discarded. The pellet was treated with 5 mL of IX red blood cell (RBC) lysis buffer and incubated to lyse RBCs. Osmolarity was restored by addition of complete medium at the end of incubation time.

[0245] Cells were pelleted, washed in Miltenyi MACS buffer (PBS pH 7.2 + 0.5% bovine serum albumin (BSA) + 2 mM ethylenediaminetetraacetic acid [EDTA]) and the supernatant was discarded. The pellet was suspended in cold MACS buffer and counted with a ViCell counter to determine cell number and viability.

[0246] Mouse CD4⁺ T cell isolation was performed with a Miltenyi process kit (San Diego, CA) according to manufacturer's instructions, and isolated cells were suspended in complete medium

[0247] Mouse CD4⁺ T cells (150,000 per well in 100 µL complete medium) were cultured overnight in 96-well plates coated with 2 µg/mL each hamster anti-mouse CD3 and hamster anti-mouse CD28 antibodies to activate T cells and induce OX40 expression. Activated CD4⁺ T cells were removed from the incubation plate and 100,000 cells were transferred to each well of a non-tissue culture-treated 96-well round-bottom plate for binding assays and washed once with FACS buffer. Binding was performed with 10 µg/mL of OX40mAb24, 9B12 and rat anti-mouse OX40, clone OX86 (positive control) antibodies each serially diluted 3-fold in FACS buffer for a 10-point data curve. For negative controls, 10 µg/mL R347 human IgG1, MOPC-21 mouse IgG1 or RTK2071 rat IgG1 were diluted 6-fold for a 3 point data curve. FACS buffer (50 µL) containing

OX40mAb24, or antibodies, was added to CD4⁺ T cells in duplicate and incubated. Following primary incubation, cells were washed with 200 μ L of 4°C FACS buffer and incubated with 50 μ L of FACS buffer containing 10 μ g/mL AlexaFluor® 488 labeled goat anti-human secondary, 10 μ g/mL AlexaFluor® 488 labeled goat anti-mouse secondary or AlexaFluor® 488 labeled goat anti-rat secondary antibody and 5 μ g/mL PI. Following secondary antibody incubation, cells were washed with 4°C FACS buffer (200 μ L per wash) and suspended in 100 μ L FACS buffer for flow cytometry analysis on a BD LSRII flow cytometer as described in Section 2.3.

2.2.3 OX40mAb24 Binding to Rat CD4⁺ T cells

[0248] OX40mAb24 was investigated for binding to rat OX40 expressed on activated primary CD4⁺ T cells. Similar experiments were performed at the same time with 9B12 to allow comparison with OX40mAb24. Rat CD4⁺ cells were isolated from freshly harvested normal Sprague-Dawley rat spleens according to the protocol described above for the isolation of mouse splenocytes, except that rat CD4⁺ T cell isolation was performed with an R&D Systems Magellect kit (Minneapolis, MN) according to manufacturer's instructions.

[0249] Rat CD4⁺ T cells (1×10^6 per mL complete medium) were cultured overnight in a T75 cell culture flask with 1 μ g/mL concanavalin A (Con A) and 500 IU/mL IL-2, to activate T cells and induce OX40 expression, and incubated overnight. Activated CD4⁺ T cells were removed from the flask and 100,000 cells were transferred to each well of a non-tissue culture treated 96-well round-bottom plate for binding assays and washed with FACS buffer. Binding was performed with 10 μ g/mL OX40mAb24, 9B12 or mouse anti-rat CD134, clone OX40 (positive control) antibody serially diluted 3-fold for a 10 point data curve in FACS buffer. For negative controls, 10 μ g/mL R347 human IgG1 or mouse IgG1 clone MOPC-21 were serially diluted 6-fold for a 3 point data curve. 100 μ L of OX40mAb24 control protein, 9B12 or clone OX40 antibody was added to CD4⁺ T cells in duplicate and incubated. Following primary incubation, cells were washed with 200 μ L of 4°C FACS buffer per wash and incubated with 100 μ L of FACS buffer containing 10 μ g/mL AlexaFluor® 488 labeled goat and human, or AlexaFluor® 488 labeled goat anti-mouse secondary antibody and 5 μ g/mL PI. Following secondary antibody incubation, cells were processed for flow cytometry as described in Section 2.3.

2.2.4 OX40mAb24 binding to Cynomolgus Monkey CD4⁺ T cells

- [0250] OX40mAb24 was investigated for binding to cynomolgus monkey (cyno) OX40 expressed on activated primary CD4⁺ T cells. Similar experiments were performed at the same time with 9B12 to allow comparison with OX40mAb24 values. Cyno CD4⁺ T cells were isolated from sodium heparin anti-coagulated whole blood obtained from healthy cyno donors (N=2) from World Wide Primates (Miami, FL) according to the following protocol:
- [0251] Whole blood was layered onto 30 mL of 60% Percoll in a 50 mL conical centrifuge tube. Blood was centrifuged and peripheral blood mononuclear cells (PBMC) were collected at the interface and washed with cold (4°C) Miltenyi MACS buffer at 1200 RPM for 10 minutes. Supernatant was discarded and the pellet was treated with 5 mL of 1X RBC lysis buffer and incubated. Complete medium (RPMI with 10% FBS and 1% antibiotics/antimycotics) was added to the pellet to stop the lysis process at the end of incubation time.
- [0252] Cells were pelleted and washed with 20 mL of cold (4°C) Miltenyi MACS buffer. The supernatant was discarded and the cell pellet was suspended in cold (4°C) MACS buffer and counted with a ViCell counter to determine cell number and viability.
- [0253] Cyno CD4⁺ T cell isolation was performed with a Miltenyi non-human primate kit according to manufacturer's instructions, then CD4⁺ T cells counted on a ViCell counter and suspended at 1×10^6 per mL in complete medium as described above.
- [0254] Cyno CD4⁺ T cells (1×10^6 per mL in complete medium) were incubated for 48 hours in a T75 cell culture flask with 2 µg/mL PHA-L and 20 IU/mL IL-2, to activate T cells and induce OX40 expression. Activated CD4⁺ T cells were removed from the flask and 100,000 cells were transferred to each well of a non-tissue culture treated 96-well round-bottom plate for binding assays and washed with 200 µL FACS buffer. Binding was performed with 10 µg/mL OX40mAb24 or 9B12 serially diluted 4-fold in FACS buffer for an 11-point data curve, or both R347 human IgG1 or mouse IgG1 clone MOPC-21 (negative controls) serially diluted 6-fold for a 3 point data curve. OX40mAb24, 9B12 or control protein was added to CD4⁺ T cells and incubated. Following primary incubation, cells were washed with 200 µL of cold (4°C) FACS buffer per wash and incubated with 100 µL of FACS buffer containing 10 µg/mL AlexaFluor® 488 labeled goat anti-human secondary antibody or 10 µg/mL AlexaFluor® 488 labeled goat anti-

mouse secondary and 5 $\mu\text{g/mL}$ PI. Following secondary antibody incubation, cells were processed for flow cytometry as described in Section 2.3.

2.2.5 OX40mAb24 Binding to Rhesus Monkey CD4⁺ T cells

[0255] OX40mAb24 was investigated for binding to rhesus macaque OX40 expressed on activated primary CD4⁺ T cells. Similar experiments were performed at the same time with 9B12 to allow comparison with OX40mAb24 values. Rhesus CD4⁺ T cells were isolated from sodium heparin anti-coagulated whole blood obtained from healthy rhesus donors (N=2) from World Wide Primates (Miami, FL) according to the following protocol:

[0256] Heparinized rhesus blood (20 mL) was diluted 1:1 with PBS and layered onto 15 ml of 95% LSM in a 50 ml conical centrifuge tube. Blood was centrifuged, PBMC were collected at the interface and washed twice with cold Miltenyi MACS buffer at 400xg for 30 minutes. Supernatant was discarded and the pellet was treated with 5 mL of IX RBC lysis buffer and incubated. Complete medium (RPMI with 10% FBS and 1% antibiotics/antimycotics) was added to the pellet to stop the lysis process at the end of incubation time. Cells were pelleted and washed with 20 mL of cold Miltenyi MACS buffer. Supernatant was discarded and the pellet was suspended in cold MACS buffer and counted with a ViCell counter to determine cell number and viability. Rhesus CD4⁺ T cell isolation was performed with a Miltenyi non-human primate kit (San Diego, CA) according to manufacturer's instructions.

[0257] Rhesus CD4⁺ T cells (1×10^6 per mL in complete medium) were cultured for 48 hours in a T75 cell culture flask with 5 $\mu\text{g/mL}$ Con-A and 1000 IU/mL IL-2 to activate T cells and induce OX40 expression. Binding to 100,000 activated rhesus CD4⁺ T cells was performed with 10 $\mu\text{g/mL}$ OX40mAb24 and 9B12 serially diluted 3-fold in FACS buffer for a 10 point (experiment 1) or 12 point data curve (experiment 2) and 10 $\mu\text{g/mL}$ human and mouse isotype controls diluted 6-fold for 2 data points (experiment 1) or 4 data points (experiment 2). AlexaFluor® 488-labeled goat anti-human secondary antibody and AlexaFluor® 488 labeled goat anti-mouse secondary binding were as described above for cyno CD4⁺ T cells, and flow cytometry as described in Section 2.3.

2.3 Flow Cytometry

[0258] Flow cytometry in the assays described in Section 2.1 was performed using an LSRII flow cytometer (Becton-Dickinson, San Jose, CA). Flow Jo cytometry analysis software (TreeStar, Ashland, OR) was used to determine OX40mAb24, 9B12 and control protein binding to cells. Wells containing OX40-expressing cells (unstained, no PI or secondary antibody), cells bound to AlexaFluor® 488- or AlexaFluor® 647-labeled secondary antibody reagent only or cells permeabilized with 0.1% saponin and treated with 10 µg/mL PI were prepared for single-stain compensation controls. After fluorescence compensation, live (PI negative) cells were gated and the mean fluorescence intensity (MFI) of secondary antibody was determined.

2.4 Calculations

2.4.1 Determination of Apparent Equilibrium Dissociation Constant (K_d)

[0259] GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com, was used to plot MFI of OX40mAb24 binding versus protein concentration (M) to create binding curves from which apparent K_d was determined. To determine the apparent K_d for OX40mAb24 and 9B12 binding to human, mouse, rat, cyno, or rhesus monkey OX40, a non-linear regression (curve fit) equation for one site (specific) binding was employed.

2.4.2 Determination of 20%, 50%, and 90% Receptor Occupancy Values

[0260] The amount of a monoclonal antibody (mAb) bound to its receptor can be estimated from the following binding relationship:



[0261] The binding dissociation constant (K_d) of the respective antibody is represented by:

Equation 2:

$$K_d = \frac{[A][B]}{[AB]}$$

Where [A] and [B] represent the concentrations of free receptor and antibody, respectively.

Finally, the fractional occupancy, fraction (F) of all receptor molecules that are bound to the antibody, can be calculated by:

Equation 3:

$$F = \frac{[AB]}{[A] + [AB]}$$

Formation of equation 2 and substitution into equation 3 results in:

Equation 4:

$$F = \frac{[A][B]/Kd}{[A] + ([A][B]/Kd)}$$

Simplification of equation 4 results in:

Equation 5:

$$F = \frac{[B]}{[B] + Kd}$$

Therefore, at equilibrium condition, the fraction of all receptor molecules that are bound to the antibody can be calculated if the concentration of free mAb and the dissociation constant K_d of the respective antibody are known.

Derivation of this formula for the calculation of the concentration of antibody required for a fractional receptor occupancy expressed as F , leads to the following formula:

Equation 6:

$$[B] = F * Kd / (1 - F)$$

Where $[B]$ equals the concentration of mAb, in this case OX40mAb24. This formula (Equation 6) was used for the calculation of the concentrations of OX40mAb24 required for 20, 50, and 90% receptor occupancy ($F = 0.20, 0.50$ or 0.90) from cell binding experiments from which the K_d value was calculated using the non-linear regression (curve fit) equation for one site (specific) binding described above.

2.5 Statistical Methods

[0262] A 2-sided unpaired Student's t test with 95% confidence level and Welch's correction to account for data sets with different standard deviations was utilized in Graphpad Prism software to determine statistically significant differences between apparent K_d values or apparent receptor occupancy values determined for OX40mAb24 binding to OX40 on activated primary human CD4 T cells versus OX40-expressing Jurkat T cells. Descriptive statistics (*i.e.*, mean and standard error of the mean) are presented in the summary figures and tables.

2.6 RESULTS

2.6.1 Binding of OX40mAb24 to Primary Human CD4⁺ T cells

[0263] OX40mAb24 bound to activated human CD4⁺ T cells with a mean apparent K_d of 312 pM and 20%, 50% and 90% receptor occupancy values of 78.1, 312, and 2810 pM, respectively; $n = 6$ binding assays with six independent T cell donors (Figures 2A and 2B, and Table 2-2).

[0264] In comparison, 9B12 bound to activated human CD4⁺ T cells with a mean K_d of 669 pM, and 20%, 50% and 90% receptor occupancy values of 167, 669, and 6020 pM, respectively (Figures 2C and 2D and Table 2-2). The ratio between 9B12 and OX40mAb24 apparent K_d values was therefore 2.1 to 1, and reflects a similar binding affinity to human OX40 for the murine and humanized monoclonal antibodies

Table 2-2 Apparent Affinity (K_d) and Receptor Occupancy Values for Binding of OX40mAb24 or 9B12 to OX40-expressing Activated Primary Human CD4⁺ T cells

Binding Protein	N donors	K_d (StdErr), pM	EC_{20} (StdErr), pM	EC_{50} (StdErr), pM	EC_{90} (StdErr), pM
OX40mAb24	6	312 (57.9)	78.1 (14.5)	312 (57.9)	2810 (521)
9B12	6	669 (137)	167 (34.3)	669 (137)	6020 (1230)

EC_{20} = effective concentration resulting in 20% of maximal effect; EC_{50} = half maximal effective concentration; EC_{90} = effective concentration resulting in 90% of maximal effect; K_d = equilibrium binding dissociation constant; StdErr = standard error of the mean.

2.6.2 Binding of OX40mAb24 to a Jurkat T cell Line Engineered to Over-express Human OX40

[0265] OX40mAb24 bound to OX40-expressing Jurkat T cells with a mean K_d of 424 pM and 20%, 50% and 90% receptor occupancy values of 106, 424, and 3820 pM, respectively. Figure 3A-C and Table 2-3. There were no statistically significant differences in apparent binding affinity or receptor occupancy of OX40mAb24 to OX40 expressed by activated primary human CD4⁺ T cells and OX40 over-expressing Jurkat T cells ($p=0.59$).

[0266] In comparison, 9B12 bound these cells with a mean K_d of 726 pM and 20%, 50% and 90% receptor occupancy values of 182, 726, and 6540 pM, respectively (Figures 3D-F and Table 2-3). The ratio between the apparent K_d values for 9B12 and OX40mAb24 was therefore 1.7 to 1, similar to the ratio calculated for binding to OX40 on activated human CD4⁺ T cells

Table 2-3 Apparent Affinity (K_d) and Receptor Occupancy Values for Binding of OX40mAb24 or 9B12 to Human OX40-overexpressing Jurkat NFkB-luciferase Clone 64 cells

Binding Protein	N experiments	K_d (StdErr), pM	EC_{20} (StdErr), pM	EC_{50} (StdErr), pM	EC_{90} (StdErr), pM
OX40mAb24	3	424 (173)	106 (43.3)	424 (173)	3820 (1560)
9B12	3	726 (308)	182 (76.9)	726 (308)	6540 (2770)

EC_{20} = effective concentration resulting in 20% of maximal effect; EC_{50} = half maximal effective concentration; EC_{90} = effective concentration resulting in 90% of maximal effect; K_d = equilibrium binding dissociation constant; StdErr = standard error of the mean.

2.6.3 Binding of OX40mAb24 to Mouse or Rat CD4⁺ T cells

[0267] Neither OX40mAb24 nor 9B12 bound to activated mouse or rat CD4⁺ T cells (data not shown). Positive staining of activated mouse and rat CD4⁺ T cells was observed with commercial anti-mouse and anti-rat OX40 antibodies, clones OX86 and OX40, respectively. 9B12 did not bind activated mouse nor rat CD4⁺ T cells (data not shown).

2.6.4 Binding of OX40mAb24 to Cynomolgus and Rhesus Monkey CD4⁺ T cells

[0268] OX40mAb24 bound to activated cynomolgus cells with a mean K_d of 581 pM. The cyno K_d was 1.9-fold higher than the human K_d (Table 2-4).

[0269] 9B12 bound the activated cyno CD4⁺ T cells with a mean K_d of 1088 pM (Table 2-4), which resulted in a ratio between 9B12 and OX40mAb24 apparent K_d values of 1.9 to 1.

[0270] OX40mAb24 bound to activated rhesus monkey CD4⁺ T cells with a mean K_d of 369 pM (Table 2-4). The rhesus K_d was 1.2-fold higher than the human K_d .

[0271] 9B12 bound the activated rhesus monkey CD4⁺ T cells with a mean K_d of 713 pM (Table 2-4), which results in a ratio between 9B12 and OX40mAb24 apparent K_d values not shown of 2.8 to 1

Table 2-4 Apparent Affinity (K_d) for Binding of OX40mAb24 or 9B12 to Cynomolgus and Rhesus Monkey OX40-expressing Activated Primary CD4⁺ T cells

Binding Protein	Cynomolgus		Rhesus	
	N experiments	K_d (StdErr), pM	N experiments	K_d (StdErr), pM
OX40mAb24	2	581 (238)	2	369 (236)
9B12	2	1088 (37)	2	713 (559)

K_d = equilibrium binding dissociation constant; StdErr = standard error of the mean.

EXAMPLE 3: Binding Specificity of OX40mAb24 for Human OX40

[0272] In this example, flow cytometry-based cell binding assays were performed to determine the specificity of OX40mAb24 for human OX40, relative to other human TNFRSF members with related amino acid sequences, which included: NGFR (TNFRSF16), LT β R (TNFRSF3), TNFR2 (TNFRSF1 β), GITR (TNFRSF18), CD137 (TNFRSF9), and HVEM (TNFRSF14). In addition, the binding specificity of OX40mAb24 for recombinant human TNFRSF members was tested in an ELISA format, which included those mentioned above as well as DR6 (TNFRSF21), osteoprotegerin (OPG; TNFRSF11B), RANK (TNFRSF11A), FAS (TNFRSF6), and CD40 (TNFRSF5).

3.1 Materials

[0273] Materials used in this study are listed in Table 3-1.

Table 3-1 Materials

Item	Source
AlexaFluor® 647 goat anti-human IgG (H+L)	Life Technologies, Carlsbad, CA
BioTek® plate washer	BioTek®, Wincoski, VT
CD137 (TNFRSF9) pCMV6-XL5 expression vector	Origene Technologies, Inc., Rockville, MD
GITR (TNFRSF18) pCMV6-XL5 expression vector	Origene Technologies, Inc., Rockville, MD
Goat anti-human IgG kappa light chain HRP conjugate	Sigma-Aldrich, St. Louis, MO
Goat-antimouse IgG (Fab specific) HRP conjugate	Sigma-Aldrich, St. Louis, MO
HVEN (TNFRSF14) pCMV6-XL4 expression vector	Origene Technologies, Inc., Rockville, MD
Lipofectamine 2000	Life Technologies, Carlsbad, CA
AF488 anti-mouse IgG1 isotype	Biolegend, San Diego, CA
AF488 anti-human GITR	Ebioscience, San Diego, CA
AF647 anti-human NGFR	BD, San Jose, CA
APC anti-human CD137	BD, San Jose, CA
APC anti-mouse IgG1 isotype	Biolegend, San Diego, CA
Clone Manager v9 software	Sci-Ed Software, Cary, NC
Fetal bovine serum (FBS), heat inactivated	Life Technologies, Carlsbad, CA
LT β R (TNFRSF3) pCMV6-XL4 expression vector	Origene Technologies, Inc., Rockville, MD
MaxiSorp 96 well flat bottom plate	VWR, Radnor, PA
Newborn calf serum, heat inactivated	Life Technologies, Carlsbad, CA
NGFR (TNFRSF16) pCMV6-XL5 expression vector	Origene Technologies, Inc., Rockville, MD
PE anti-human HVEM	Ebioscience, San Diego, CA
PE anti-human Lt β R	R&D systems, Minneapolis, MN
PE anti-human TNFRSF 1B	BD, San Jose, CA
PE anti-mouse IgG1 isotype	Biolegend, San Diego, CA
PE anti-rat isotype	Ebioscience, San Diego, CA
PBS, pH 7.2 without calcium and magnesium	Life Technologies, Carlsbad, CA
Recombinant human TNFRSF1 β (TNFR1I)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF3 (LT β R)	R&D systems, Minneapolis, MN

Item	Source
Recombinant human TNFRSF4 (OX40)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF5 (CD40)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF6 (FAS)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF9 (CD137)	In house protein; lot# AMPur19
Recombinant human TNFRSF11A (RANK)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF11B (OPG)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF14 (HVEM)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF16 (NGFR)	R&D systems, Minneapolis, MN
Recombinant human TNFRSF18 (GITR)	In house protein; lot# LBPur0025
Recombinant human TNFRSF21 (DR6)	R&D systems, Minneapolis, MN
TNFRSF1B pCMV6-XL5 expression vector	Origene Technologies, Inc., Rockville, MD
Tween-20	Sigma-Aldrich, St. Louis, MO

3.2 Methods

3.2.1 Search for Proteins with Close Sequence Homology to Human OX40

[0274] In order to identify human proteins with amino acid sequence identity to human OX40, a protein sequence homology Basic Local Alignment Search Tool (BLASTP) search was conducted using the protein sequence of OX40 (CCDS 11 / UniProt P43489). Nineteen TNFRSF family members / isoforms were identified. The full-length sequences of these proteins were verified using both the CCDS and UniProt databases (www.uniprot.org). Clone Manager version 9 software was used to perform an assembled alignment against the human OX40 reference using a blosum62 scoring matrix to determine the percentage of amino acid identity between human OX40 and the proteins identified in the BLASTP search (Table 3-2).

3.2.2 Binding specificity of OX40mAb24 for OX40 relative to other TNFRSF members expressed in HEK293 cells

[0275] cDNA constructs capable of directing the expression of individual TNFRSF members, when transfected into mammalian cells, were obtained from Origene Technologies, Rockville, MD. These cDNA constructs were amplified and purified by the Protein Sciences group at MedImmune in Gaithersburg, MD for use in transient transfections. For individual expression of each of the TNFRSF members, HEK293 cells were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) combined with 0.5 µg DNA of an expression vector encoding one of the TNFRSF members, according to the manufacturer's suggested protocol for Lipofectamine 2000. Forty-eight hours post transfection, cells were removed from tissue culture plates by trypsinization. Trypsin

was neutralized by the addition of serum-containing complete medium RPMI-1640 plus 10% FBS), followed by cell pelleting and washes in complete medium. Cells were then suspended in cold FACS buffer (PBS + 2% FBS) and plated into 96 well non-tissue culture-treated plates for binding studies with TNFRSF member-specific mAbs (Table 3-3) and OX40mAb24.

[0276] For binding of antibodies or OX40mAb24, HEK cells were pelleted, FACS buffer removed, and cells suspended in FACS buffer containing 2 $\mu\text{g/mL}$ propidium iodide (PI) and either a fluorochrome-labeled mAb, specific for the transfected TNFRSF member, at a concentration recommended by the manufacturer or with OX40mAb24 at a concentration of 1 $\mu\text{g/mL}$. For binding controls, cells were separately incubated with fluorochrome-labeled isotype control antibodies. Cells were incubated with antibodies for 1 hour at 4°C in the dark. Thereafter, cells incubated with fluorochrome-labeled monoclonal antibodies were washed in cold FACS buffer and then binding events collected and analyzed by flow cytometry using an LSRII flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software, as described in Section 3.2.3. Cells incubated with OX40mAb24 were washed in ice cold FACS buffer and then suspended 25 $\mu\text{g/mL}$ of Alexa Fluor® 647 goat antihuman IgG (H+L) secondary antibody and incubated for a further 30 minutes at 4°C in the dark. For a secondary antibody binding control cells were incubated in the absence of OX40mAb24, but in the presence of fluorochrome-labeled secondary antibody alone. Thereafter, cells were washed and suspended in cold FACS buffer for analysis on an LSRII flow cytometer.

3.2.3 Flow Cytometry Analysis

[0277] Flow cytometry standard (FCS) data was examined using FlowJo software (Ashland, OR). To analyze mAb binding, cells were first gated for viable (PI negative) cells, and then the mean fluorescence intensity (MFI) of events was plotted versus total number of events to generate binding histograms. The geometric MFI of all viable cells was determined for each sample so that the fold MFI over background (isotype control or secondary antibody alone) could be determined.

3.2.4 OX40mAb24 binding specificity ELISA

[0278] An eight point, two-fold dilution series of each recombinant human TNFRSF protein was prepared after dilution of stock proteins to 5 $\mu\text{g/mL}$ in PBS. Subsequently, 50 μL of each antigen dilution was transferred in duplicate to wells of a Nunc 96-well MaxiSorp flat bottom plate, and incubated overnight at 4°C to adsorb proteins to the plate. Afterwards, plates were washed three

times with PBS in a BioTek® plate washer to remove unbound protein. Anti OX40 mAb29, 9B12, and control antibodies were diluted in PBS to a final concentration of 10 µg/mL, and 50 µL of mAb were added to each well and incubated at room temperature for one hour to bind mAb to plate-bound protein. Thereafter, wells were washed with PBS/Tween-20 0.1% (volume/volume) using a BioTek® plate washer. HRP conjugated goat anti-human or goat anti-mouse secondary antibodies at 10 µg/mL was added to each well and incubated at room temperature for 1 hour. After three washes in PBS/Tween-20 0.1%, 50 µL of TMB substrate was added to each well and incubated at room temperature for 5 minutes to develop the colorimetric product. Reactions were stopped by adding 50 µL of 0.5 molar H₂SO₄ to wells, and plates read immediately at 450 nm using an Envision C plate reader for detection of the colorimetric product. Results were graphed in GraphPad Prism software for Windows, version 5.01, and binding curves generated using non-linear regression analysis for single site binding.

3.3 Results

[0279] The protein sequence homology BLASTP search on human OX40 identified 19 human TNFRSF proteins or isoforms that shared 15-27% amino acid sequence identity with the full-length OX40 sequence. The proteins and their percentages of sequence identity are listed in Table 3-2.

Table 3-2 Amino Acid Sequence Identity of Twelve TNFRSF Members with the Highest Homology to Human OX40.

TNFRSF member	Alternate names	% identity with OX40	UniProt protein sequence ID
TNFRSF11A	RANK, CD265	27	Q9Y6Q6 iso 2 (delta 7,8,9)
TNFRSF6B	DcR3	25	O95407
TNFRSF18	GITR, AITR	25	Q9Y5U5 iso 2
TNFRSF10C	DCR1, TRAIL-R3	24	O14798
TNFRSF5	CD40	23	P25942 iso 2
TNFRSF18	GITR, AITR	23	Q9Y5U5 iso 3
TNFRSF18	GITR, AITR	23	Q9Y5U5
TNFRSF9	CD137, 4-1BB	22	Q07011
TNFRSF5	CD40	21	P25942
TNFRSF14	TR2, HVEM-A	21	Q92956
TNFRSF16	NGF receptor	21	P08138
TNFRSF3	LTβR, TNFRIII	20	P36941

TNFRSF member	Alternate names	% identity with OX40	UniProt protein sequence ID
TNFRSF6	Fas	20	P25445 iso 6 Tmdl (A)
TNFRSF6	Fas	20	P25445
TNFRSF3	LT β R, TNFRIII	19	P36941 iso 2
TNFRSF6	Fas	19	P25445 iso 7 FasExo8Del
TNFRSF11B	Osteoprotegerin	18	O0030
TNFRSF1B	TNFR1b, TNFR2, CD120b	18	P20333
TNFRSF11A	RANK, CD265	16	Q9Y6Q6

CCDS = consensus coding sequence; ID = identifier; NA = not applicable; TNFRSF = tumor necrosis factor receptor superfamily; UniProt - Universal Protein Resource

[0280] Binding of OX40mAb24 to transiently transfected HEK293 cells that expressed human NGFR, LT β R, TNFR2, GITR, CD137 or HVEM was assessed by flow cytometry as described in Section 3.1.3 above. Cell surface expression of human NGFR, LT β R, TNFR2, GITR, CD137 and HVEM was confirmed using commercially available antibodies specific for each human TNFRSF protein; fold increase in MFI compared to isotype control antibodies for each TNFRSF protein is shown in Table 3-3. Binding of OX40mAb24 to HEK293 cells that expressed human NGFR, LT β R, TNFR2, GITR, CD137 or HVEM was not substantially above that seen for binding of the secondary antibody alone to those same cells (Table 3-3, Figure 4A). In contrast, binding of OX40mAb24 to a Jurkat cell line that constitutively overexpresses OX40 was 48-fold greater, by mean MFI, than the binding of fluorochrome labeled secondary antibody alone (Table 3-3 and Figure 4B).

Table 3-3: Fold Binding of Fluorochrome-labeled TNFRSF-specific Monoclonal Antibodies and OX40mAb24 to TNFRSF-overexpressing HEK293 Cells or OX40mAb24 to OX40-overexpressing Jurkat Cells.

TNFRSF member expressed	Cell Line Transferred with TNFRSF Member	Receptor-Specific Commercial mAb binding (ratio to isotype control MFI)	OX40mAb24 binding (ratio to secondary antibody alone MFI)
OX40	Jurkat	ND	48
TNFRSF16 (NGFR)	HEK293	17	2.2
TNFRSF3 (LT β R)	HEK293	146	1.0
TNFRSF1B	HEK293	5.0	1.0
TNFRSF18 (GITR)	HEK293	37	1.1
TNFRSF9 (CD137)	HEK293	24	1.2
TNFRSF14 (HVEM)	HEK293	69	1.1

mAb = monoclonal antibody; MFI = mean fluorescence intensity; ND = not determined; TNFRSF = tumor necrosis factor receptor superfamily

[0281] In an ELISA format, binding of an antibody containing the Fab arms of OX40mAb24 but with IgG1 Fc domain containing three amino acid modifications (mAb29) was specific for OX40 (Figure 5A), showing no specific binding above background to recombinant human NGFR (TNFRSF16), LT β R (TNFRSF3), TNFR2 (TNFRSF1B), GITR (TNFRSF18), CD137 (TNFRSF9), HVEM (TNFRSF14), DR6 (TNFRSF21), osteoprotegerin (OPG; TNFRSF11B), RANK (TNFRSF11A), FAS (TNFRSF6), and CD40 (TNFRSF5). 9B12, the mouse anti-human OX40 IgG1 monoclonal antibody that was “humanized” to create OX40mAb24, demonstrated similar lack of binding to these TNFRSF proteins (Figure 5B).

3.4: Conclusions

[0282] Binding of OX40mAb24 and 9B12 to human OX40 is specific, and do not cross-react with highly related TNFRSF proteins.

EXAMPLE 4: Ability of OX40mAb24 to Co-stimulate Primary Human CD4⁺ T Cells through OX40 *In Vitro*

[0283] In this example, the ability of OX40mAb24 to enhance activation of T cells, in combination with activation through the CD3/T cell receptor (TCR) complex, was assessed using a plate-based human CD4⁺ T cell proliferation and cytokine release assay. Soluble OX40mAb24 activity was also examined, as were the activity of soluble and plate-bound OX40mAb24 in the absence of CD3/TCR signaling.

4.1 MATERIALS

[0284] Materials used in this study are listed in Table 4-1.

Table 4-1 Materials

Item	Source
AlexaFluor® 647 goat anti-human IgG (H+L)	Life Technologies, Carlsbad, CA
Anti-human CD4 EFluor450®	eBioscience, San Diego, CA
Bovine serum albumin (BSA)	Sigma, Saint Louis, MO
CFSE cell labeling kit	Life Technologies, Carlsbad, CA
Complete RPMI medium: RPMI-1640 + 10% FBS	Materials from Life Technologies, Carlsbad, CA
Deep well plates, polypropylene, 2 mL	VWR, Radnor, PA
EasySep CD4 ⁺ T cell enrichment kit	Stem Cell Technologies, Vancouver, BC Canada
FlowJo software	TreeStar, Ashland, OR
Goat anti-human IgG, Fc γ -specific	Jackson ImmunoResearch, West Grove, PA
Goat anti-mouse IgG, Fc γ -specific	Jackson ImmunoResearch, West Grove, PA

Item	Source
Heat inactivated newborn calf serum	Life Technologies, Carlsbad, CA
IL-2, recombinant human	Preprotech, Rocky Hill, NJ
Leuko Pak	AllCells, Alameda, CA
LSM	MP Biomedicals, Santa Ana, CA
LSR II flow cytometer	BD Biosciences, San Jose, CA
Mouse anti-human CD3 antibody clone OKT3	Biolegend, San Diego, CA
Newborn calf serum, heat inactivated (FBS)	Life Technologies, Carlsbad, CA
Non-tissue culture treated round-bottom 96 well plates	VWR, Radnor, PA
PHA-L	Roche Applied Science, Indianapolis, IN
Phosphate Buffered Saline (PBS) pH7.2 without Calcium and Magnesium	Life Technologies, Carlsbad, CA
Prism software, v 5.01	Graphpad Software, San Diego, CA
Propidium iodide (1 mg/mL solution)	Sigma, Saint Louis, MO
RosetteSep CD4 ⁺ T cell enrichment kit	StemCell Technologies, Vancouver, BC Canada
RPMI-1640	Life Technologies, Carlsbad, CA
Th1/Th2 multi-cytokine detection array	Mesoscale Discovery (MSD), Rockville, MD
Vi-Cell counter	Beckman Coulter, Indianapolis, IN
Whole blood, sodium heparin anti-coagulated	MedImmune Blood Donor Program, Gaithersburg, MD

4.2 Assays

4.2.1 Plate-immobilized bioactivity of OX40mAb24

[0285] The bioactivity of OX40mAb24 was determined by measurement of human CD4⁺ T cell proliferation and cytokine production in a plate-based drug capture assay (Figure 6).

[0286] Enriched human CD4⁺ T cells were isolated from healthy donor whole blood using a RosetteSep CD4⁺ T cell enrichment kit, according to the manufacturer's protocol. Assays were performed with cells from four independent donors.

[0287] CD4⁺ T cells were suspended in complete RPMI culture medium and the cell concentration adjusted to 1.0×10^6 per mL. Final concentrations of 2 μ g/mL phytohemagglutinin-leucoagglutinin (PHA-L) and 20 IU/mL recombinant human IL-2 were added, and cells were cultured at 37°C and 5% CO₂ in a humidified tissue culture incubator for 2 days to activate T cells and up-regulate OX40.

[0288] Non-tissue culture treated round-bottom 96 well assay plates were coated with 100 μ L of 2 μ g/mL goat anti-mouse Fc γ -specific IgG and 2 μ g/mL goat anti-human Fc γ -specific IgG in PBS. Goat anti-human IgG capture antibodies were not added to wells intended for assay of soluble OX40mAb24 activity. Plates were incubated overnight at 4°C, washed with 200 μ L of PBS, and

blocked for 90 minutes at 37°C with 1% BSA in PBS (1% BSA/PBS). The plates were washed with PBS and 2 ng/mL of mouse anti-human CD3 clone OKT3 reconstituted in 1% BSA/PBS was added to the plates for 90 minutes at 37°C. The plates were washed with PBS to remove unbound OKT3, OX40mAb24, R347 human IgG1 control mAb, 9B12, and mouse IgG1 control mAb clone MOPC-21 were each reconstituted in 1% BSA/PBS starting at 0.918 µg/mL (3.0 nM) and serially diluted over a 3-fold dilution series and then added to assay plates and incubated for 90 minutes at 37°C.

[0289] Activated primary human CD4⁺ T cells were collected, washed in complete RPMI medium, and the concentration adjusted to 1.0×10^6 viable cells/mL. Cells were labelled with carboxyfluorescein succinimidyl ester (CFSE), according to the manufacturer's instructions, with the exception of using 1.25 µM CFSE as opposed to the recommended 5 µM with an incubation of 10 minutes at 37°C. After labeling, cells were suspended in complete RPMI medium and the concentration was adjusted to 0.5×10^6 per mL. The plates were washed with PBS and 200 µL of CD4⁺ T cells (100,000/well) were added to each well. For wells containing soluble OX40mAb24, OX40mAb24 was diluted in complete RPMI medium to the highest final concentrations used for plate-bound OX40mAb24. Cells in the plate were pelleted by centrifugation at 380xg and incubated at 37°C for 3 days. After 72 hours incubation time, 40 µL of cell culture supernatant was removed for cytokine release measurement. CD4⁺ T cells were pelleted, and washed once with PBS containing 2% FBS (FACS buffer). Cells were suspended in binding mix containing anti-human CD4 eFluor450[®] labeled antibody for identification of CD4⁺ T cells, and propidium iodide (PI) for live/non-viable cell discrimination, and incubated for 30 minutes. Following incubation, cells were washed in FACS buffer, re-suspended in FACS buffer and analyzed by flow cytometry using an LSRII flow cytometer and FlowJo software for analysis of Flow Cytometry Standard (FCS) formatted data.

[0290] To analyze T cell proliferation, live (PI negative) events were gated using FlowJo software, and the percentage of CD4-gated cells showing dilution of CFSE determined as a measure of the percentage of cells undergoing proliferation.

[0291] To analyze cytokine release, cell culture supernatants obtained after 72 hours of culture were measured for cytokine content using a 10-plex human Th1/Th2 cytokine analysis kit from MesoScale Discovery (Gaithersburg, MD) according to the manufacturer's protocol. This kit

employs an electrochemical detection method to quantitatively measure the following human cytokines: IFN γ , IL-2, IL-4, IL-5, IL-8, IL-10, IL-12 p70, IL-13, and IL-1 β .

[0292] GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com was used to plot the log of mAb concentration versus either proliferation or cytokine release values. The effective concentrations resulting in 20%, 50% and 90% maximal effect (EC₂₀, EC₅₀, and EC₉₀) values for OX40mAb24 bioactivity were calculated from sigmoidal dose-response bioactivity curves using the ECAnything function.

4.3 RESULTS

[0293] Proliferation data from each of the four donors and cytokine release data from one donor are shown in Figure 7A-C and Figure 8A-E. EC₂₀, EC₅₀, and EC₉₀ potency values for human primary CD4⁺ T cell proliferation are shown in Table 4-2; potency values for human primary CD4⁺ T cell cytokine release assays in Table 4-3 through Table 4-7; Mean proliferation and cytokine release values for OX40mAb24 and 9B12 are shown in Table 4-8 and Table 4-9, respectively.

[0294] OX40mAb24 co-stimulated proliferation of primary human CD4⁺ T cells (n=4) in a concentration-dependent manner with EC₂₀, EC₅₀, and EC₉₀ values of 21, 28, and 72 pM, respectively. 9B12 co-stimulated proliferation of CD4⁺ T cells with EC₂₀, EC₅₀, and EC₉₀ values of 106, 218, and 622 pM. Therefore, the ratio of 9B12 to OX40mAb24 concentrations required to induce a 50% of maximum proliferative response was 8 to 1 (Table 4-2).

[0295] OX40mAb24 and 9B12 co-stimulated primary human CD4⁺ T cells to release cytokines (n=4). Mean EC₂₀, EC₅₀, and EC₉₀ values were less potent than values for proliferation, and are summarized in Table 4-8 and Table 4-9. Non-linear regression analysis could not be conducted for IL-2, IL-4, IL-8, IL-12 p70, and IL-1 β assay results for both mAbs, due to poorly formed or non-existent sigmoidal dose-response curves.

Table 4-2 Mean EC₂₀, EC₅₀ and EC₉₀ Values for OX40mAb24 and 9B12 in a Primary Human CD4⁺ T cell Proliferation Assay

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	OX40mAb24	32 (22, 46)	38 (20, 72)	71 (17, 29)	Not Tested	None
661	OX40mAb24	33 (28, 39)	51 (33, 77)	128 (96, 172)	Not Tested	None

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
645	OX40mAb24	7.5 (2.7, 21)	8.3 (2.8, 25)	35 (7.1, 173)	None	Not Tested
651	OX40mAb24	9.6 (6.3, 15)	16 (9.1, 26)	54 (30, 99)	None	Not Tested
Mean (Standard Error of the Mean), pM	OX40mAb24	21 (6.9)	28 (9.8)	72 (20)		
367	9B12	99.8 (80.0, 125)	156 (90.2, 269)	337 (214, 530)	Not Tested	Not Tested
661	9B12	128 (113, 144)	237 (164, 342)	535 (464, 617)	Not Tested	Not Tested
645	9B12	77.5 (30.2, 199)	169 (67.0, 425)	686 (152, 3080)	Not Tested	Not Tested
651	9B12	118 (80.8, 173)	309 (201, 475)	929 (511, 1690)	Not Tested	Not Tested
Mean (Standard Error of the Mean), pM	9B12	106 (11.1)	218 (35.2)	622 (125)		

CI = confidence interval; EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; TCR = T cell receptor.

Table 4-3: Mean EC₂₀, EC₅₀ and EC₉₀ Values for IFN γ Induced by OX40mAb24 or 9B12 in a Primary Human CD4⁺ T Cell Bioactivity Assay

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	OX40mAb24	38.6 (21.3, 70.0)	57.2 (31.2, 105)	106 (34.6, 328)	Not Tested	None
661	OX40mAb24	ND	ND	ND	Not Tested	None
645	OX40mAb24	32.1 (20.8, 49.7)	58.2 (42.0, 80.8)	150 (77.6, 289)	None	Not Tested
651	OX40mAb24	47.5 (24.5, 91.8)	77.4 (51.4, 116)	168 (86.7, 324)	None	Not Tested
Mean (Standard Error of the Mean), pM	OX40mAb24	39.4 (4.46)	64.3 (6.57)	141 (18.4)		
367	9B12	ND	ND	ND	Not Tested	Not Tested
661	9B12	ND	ND	ND	Not Tested	Not Tested
645	9B12	706 (518, 963)	2380 (1420, 3970)	16300 (5480, 48300)	Not Tested	Not Tested
651	9B12	344 (274, 430)	758 (639, 900)	2660 (780, 3990)	Not Tested	Not Tested
Mean (Standard Error of the Mean), pM	9B12	525 (148)	1570 (662)	9480 (5569)		

CI = confidence interval; EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; TCR = T cell receptor.

Table 4-4: Mean EC₂₀, EC₅₀ and EC₉₀ Values for TNF α Induced by OX40mAb24 or 9B12 in a Primary Human CD4⁺ T cell Bioactivity Assay

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	OX40mAb24	ND	ND	ND	Not Tested	None
661	OX40mAb24	ND	ND	ND	Not Tested	None
645	OX40mAb24	37.6 (22.5, 62.7)	54.1 (29.1, 101)	96.2 (26.7, 347)	None	Not Tested
651	OX40mAb24	ND	ND	ND	None	Not Tested
Mean (Standard Error of the Mean), pM	OX40mAb24	ND	ND	ND		
367	9B12	ND	ND	ND	Not Tested	Not Tested
661	9B12	ND	ND	ND	Not Tested	Not Tested
645	9B12	306 (258, 503)	670 (527, 853)	1800 (1100, 2920)	Not Tested	Not Tested
651	9B12	388 (300, 502)	764 (640, 919)	2260 (1500, 3410)	Not Tested	Not Tested
Mean (Standard Error of the Mean), pM	9B12	347 (33.5)	717 (38.4)	2030 (188)		

CI = confidence interval; EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; ND = not determined due to poor curve fit (EC₂₀, EC₅₀, EC₉₀) or lack of sufficient in values to determine mean and standard error of the mean; TCR = T cell receptor.

Table 4-5: Mean EC₂₀, EC₅₀ and EC₉₀ Values for IL10 Induced by OX40mAb24 or 9B12 in a Primary Human CD4⁺ T Cell Bioactivity Assay

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	OX40mAb24	41.6 (17.8, 97.0)	59.5 (25.4, 139)	105 (25.0, 440)	Not Tested	None
661	OX40mAb24	63.5 (29.6, 136)	89.5 (64.2, 125)	154 (93.3, 255)	Not Tested	None
645	OX40mAb24	ND	ND	ND	None	Not Tested
651	OX40mAb24	53.0 (31.0, 90.6)	86.4 (65.0, 115)	188 (110, 321)	None	Not Tested
Mean (Standard Error of the Mean), pM	OX40mAb24	52.7 (6.32)	78.5 (9.53)	149 (24.1)		

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	9B12	130 (83.3, 204)	198 (139, 284)	385 (224, 662)	Not Tested	Not Tested
661	9B12	ND	ND	ND	Not Tested	Not Tested
645	9B12	528 (363, 767)	1220 (858, 1740)	4630 (2050, 10400)	Not Tested	Not Tested
651	9B12	405 (300, 547)	796 (649, 976)	2320 (1440, 3730)	Not Tested	Not Tested
Mean (Standard Error of the Mean), pM	9B12	354 (118)	738 (296)	2445 (1227)		

CI = confidence interval; EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; ND = not determined due to poor curve fit (EC₂₀, EC₅₀, EC₉₀) or lack of sufficient values to determine mean and standard error of the mean; TCR = T cell receptor.

Table 4-6: Mean EC20, EC50 and EC90 Values for IL13 Induced by OX40mAb24 or 9B12 in a Primary Human CD4⁺ T Cell Bioactivity Assay

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	OX40mAb24	ND	ND	ND	Not Tested	None
661	OX40mAb24	ND	ND	ND	Not Tested	None
645	OX40mAb24	44.7 (26.5, 75.5)	73.8 (52.2, 104)	163 (93.3, 286)	None	Not Tested
651	OX40mAb24	36.2 (24.9, 52.7)	65.0 (49.6, 85.3)	164 (99.2, 273)	None	Not Tested
Mean (Standard Error of the Mean), pM	OX40mAb24	40.5 (4.25)	69.4 (4.40)	164 (0.50)		
367	9B12	ND	ND	ND	Not Tested	Not Tested
661	9B12	154 (841, 283)	238 (166, 341)	472 (278, 800)	Not Tested	Not Tested
645	9B12	1100 (413, 2910)	5450 (1060, 2800)	6930 (4310, 111000)	Not Tested	Not Tested
651	9B12	495 (324, 756)	1770 (896, 3510)	13400 (2980, 60400)	Not Tested	Not Tested
Mean (Standard Error of the Mean), pM	9B12	583 (277)	2486 (1550)	6930 (3730)		

CI = confidence interval; EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; ND = not determined due to poor curve fit (EC₂₀, EC₅₀, EC₉₀) or lack of sufficient values to determine mean and standard error of the mean; TCR = T cell receptor.

Table 4-7: Mean EC₂₀, EC₅₀ and EC₉₀ Values for IL5 Induced by OX40mAb24 or 9B12 in a Primary Human CD4⁺ T cell Bioactivity Assay

Donor Number	Monoclonal Antibody	EC ₂₀ (95% CI), pM	EC ₅₀ (95% CI), pM	EC ₉₀ (95% CI), pM	Soluble activity	Activity in absence of TCR stimulation
367	OX40mAb24	33.2 (21.2, 52.1)	46.7 (24.7, 88.0)	79.8 (12.3, 519)	Not Tested	None
661	OX40mAb24	43.4 (27.6, 68.4)	69.0 (49.7, 95.7)	144 (86.8, 238)	Not Tested	None
645	OX40mAb24	ND	ND	ND	None	Not Tested
651	OX40mAb24	56.2 (35.5, 88.9)	95.9 (76.1, 121)	224 (128, 391)	None	Not Tested
Mean (Standard Error of the Mean), pM	OX40mAb24	44.3 (6.65)	70.5 (14.2)	149 (41.7)		
367	9B12	ND	ND	ND	Not Tested	Not Tested
661	9B12	ND	ND	ND	Not Tested	Not Tested
645	9B12	ND	ND	ND	Not Tested	Not Tested
651	9B12	423 (285, 627)	1090 (742, 1600)	4870 (1900, 12500)	Not Tested	Not Tested
Mean (Standard Error of the Mean), pM	9B12	ND	ND	ND		

CI = confidence interval; EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; ND = not determined due to poor curve fit (EC₂₀, EC₅₀, EC₉₀) or lack of sufficient in values to determine mean and standard error of the mean; TCR = T cell receptor.

Table 4-8: Summary of Mean EC₂₀, EC₅₀ and EC₉₀ Values for Proliferation and Cytokine Release for OX40mAb24

Bioactivity Readout	EC ₂₀ (StdErr), pM	EC ₅₀ (StdErr), pM	EC ₉₀ (StdErr), pM
CD4 T cell proliferation	21 (6.9)	28 (98)	72 (20)
IFN γ release	39.4 (4.46)	64.3 (6.57)	141 (18.4)
TNF α release	ND	ND	ND
IL10	52.7 (6.32)	78.5 (9.53)	149 (24.1)
IL13	40.5 (4.25)	69.4 (4.40)	164 (0.50)
IL5	44.3 (6.65)	70.5 (14.2)	149 (41.7)

EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; ND = not determined due to insufficient n value to calculate a mean and standard error of the mean; StdErr = standard error of the mean.

Table 4-9: Summary of Mean EC₂₀, EC₅₀ and EC₉₀ Values for Proliferation and Cytokine Release for 9B12

Bioactivity Readout	EC ₂₀ (StdErr), pM	EC ₅₀ (StdErr), pM	EC ₉₀ (StdErr), pM
CD4 T cell proliferation	106 (11.1)	218 (35.2)	622 (125)
IFN γ release	525 (148)	1570 (662)	9480 (5569)
TNF α release	347 (33.5)	717 (38.4)	2030 (188)

Bioactivity Readout	EC ₂₀ (StdErr), pM	EC ₅₀ (StdErr), pM	EC ₉₀ (StdErr), pM
IL10	354 (118)	738 (296)	2445 (1227)
IL13	583 (277)	2486 (1547)	6934 (3732)
IL5	ND	ND	ND

EC₂₀ = effective concentration resulting in 20% of maximal effect; EC₅₀ = half maximal effective concentration; EC₉₀ = effective concentration resulting in 90% of maximal effect; ND = not determined due to insufficient n value to calculate a mean and standard error of the mean; StdErr = standard error of the mean.

[0296] The activity of non-plate bound, soluble OX40mAb24 was determined. Soluble OX40mAb24 did not induce either primary human CD4⁺ T cell proliferation (Figure 7A-C) or cytokine release (Figure 8A-E) above levels observed for either anti-CD3 antibody alone or in the presence of R347 human IgG1 control mAb. Plate bound anti-CD3 antibody alone produced a minimal-to-moderate level of proliferation and cytokine release. The lack of activity demonstrated here by soluble OX40mAb24 is in agreement with the absence of activity observed for soluble OX40mAb24 without cell-based cross-linking in a 2-cell bioactivity assay that measured OX40 mediated NFκB signaling (see Example 5 below).

[0297] Likewise, OX40mAb24, either immobilized on the plate surface or added as a soluble unbound protein, in the absence of a sub-mitogenic anti-CD3 antibody signal induced little-to-no CD4⁺ T cell proliferation (Figure 7A-C) or cytokine release (Figure 8A-E). These results demonstrated that, in this study, OX40mAb24 does not have activity in primary human CD4⁺ T cells in the absence of simultaneous CD3/TCR ligation.

4.4 Conclusions

[0298] OX40mAb24 induced proliferation and cytokine release of primary human CD4⁺ T cells in a concentration-dependent manner similar to that of the antibody from which it was humanized 9B12 (Figure 8A-E and Figure 9A-E). OX40mAb24 demonstrated activity as a plate-bound, but not as a soluble, protein. Furthermore, OX40mAb24 activity occurred concurrent with CD3/TCR signaling.

EXAMPLE 5: Determination of the *In Vitro* Activity of OX40mAb24 in 2-cell Based Bioactivity Assays Using Jurkat NFκB-luciferase Reporter T cells

[0299] In this example, the ability of OX40mAb24 and 9B12 to signal through human OX40 was assessed using a set of two-cell reporter bioactivity assays. Measurement of T cell activation through OX40 co-stimulation was accomplished by using an OX40-overexpressing Jurkat NFκB-

luciferase T cell reporter line that produces luciferase in response to stimulation of the NFκB signaling pathway (Figure 10). NFκB signaling has been reported to occur downstream of OX40 engagement, and can correlate with other measures of T cell activation, such as proliferation and cytokine release (Croft M, *et al.*, *Immunol Rev.* 229:173-91 (2009)). The amount of luciferase, and thus T cell activation, was measured by adding a luciferase substrate to cell lysates and measuring light emitted by the reaction product using a luminometer. Bioactivity of OX40mAb24 cross-linked using cells engineered to express different Fcγ receptor complements, as well as soluble, non-FcγR-crosslinked OX40mAb24 was measured.

5.1 Materials

[0300] Materials used in this study are listed in Table 5-1.

Table 5-1: Materials

Item	Source
CD45 ⁺ microbeads	Miltenyi, San Diego, CA
Collagenase III	Worthington Biochemical Corporation, Lakewood, NJ
DNAse I, from b ovine pancreas	Sigma, Saint Louis, MO
EDTA, 0.5M pH 8.0	Life Technologies, Carlsbad, CA
Envision luminescence reader	Perkin Elmer, Waltham, MA
Heat inactivated newborn calf serum (FBS)	Life Technologies, Carlsbad, CA
LS column	Miltenyi, San Diego, CA
MACS buffer: PBS + 0.5% BSA + 2 mM EDTA	Materials from Life Technologies, Carlsbad, CA
Non-TC treated round-bottom 96 well plates	VWR, Radnor, PA
Prism v5.01 software	GraphPad, San Diego, CA
Steady-Glo Luciferase Assay Solution	Promega, Madison, WI
ViCell counter	Beckman Coulter, Indianapolis, IN

5.2 Two-cell Bioactivity Assay

5.2.1 Isolation of Primary Human CD45⁺ Cells

[0301] Primary human CD45⁺ cells were isolated from human tumors. Tumor samples were removed from transport media and placed in sterile petri dish. Hank's Buffered Salt Solution was added and visible necrotic tissue or any normal tissue from tumor sample was dissected. Tissue was minced into small pieces (~1 mm) and placed in a 50 mL conical tube, and Collagenase III enzyme mix (250 IU/mL collagenase III, 3 mM CaCl₂, 315 μg/mL DNAase 1) was added, mixed and incubated. The digested sample was filtered through a 70 micron filter and washed with MACS buffer. Dissociated cells were pelleted and the cell number and viability were determined using a ViCell counter. Cells were suspended in MACS buffer with CD45 microbeads and

incubated on ice. Cells were washed and re-suspended in MACS buffer for positive selection of CD45⁺ cells using an LS column. Bead-bound CD45⁺ cells were eluted by removing the column from the magnet and adding MACS buffer to the column. Cells were pelleted and re-suspended in complete RPMI medium and used in bioactivity assays as described above.

5.2.2 Assay Protocol

[0302] OX40mAb24 and 9B12 were tested for bioactivity using a 2-cell bioactivity assay. Human OX40-overexpressing Jurkat NFκB-luciferase reporter clone 64 (OX40 Jurkat reporter) was used to measure OX40 agonism (NFκB activity). A second, FcγR-expressing cell line, was used to mediate OX40mAb24 cross-linking, which clusters and activates OX40 on the OX40 Jurkat reporter cells (Figure 10). The FcγR-expressing cell lines used for cross-linking included the Raji human B cell lymphoma line, CD32A-expressing HEK293, CD32B-expressing HEK293 or CD45⁺ immune cells isolated from primary human tumors.

[0303] To determine the soluble activity of OX40mAb24, bioactivity assays were also conducted using either parental HEK293 cells, which are FcγR null and therefore are unable to cross-link OX40mAb24, or in the absence of cross-linking cells altogether.

[0304] Prior to use, OX40 Jurkat reporter cells were cultured in complete RPMI medium in a tissue culture incubator at a density of 0.5-1.5 x 10⁶ per mL. Cells were passaged the day prior to the bioassay at a final density of 10⁶ cells per mL.

[0305] OX40 Jurkat reporter cells, FcγR-expressing cell lines, or HEK parental cells were collected, and pelleted. To isolate CD45⁺ cells from primary human tumors and normal adjacent tissues, tissues were dissociated and CD45⁺ cells isolated and re-suspended in complete RPMI medium for use in bioactivity assays, as described below.

[0306] OX40mAb24, 9B12 and various control antibodies were serially diluted 3-fold in complete RPMI medium.

[0307] OX40 Jurkat reporter cells plus FcγR-expressing cells, or HEK parental cells, were added to a 96 well plate at 100,000 cells per well. OX40mAb24, 9B12 or control antibodies were added to cells in a dilution series with a starting concentration of 10 μg/mL, and incubated at 37°C in a tissue culture incubator.

[0308] After 16-24 hours incubation time, 100 μL reconstituted Steady-Glo luciferase assay solution (Promega, Madison, WI) was added to each well and mixed to lyse cells and then

incubated to equilibrate luciferase signal. Steady-Glo/sample lysate (150 μ L) was transferred from each well to a 96 well, white walled assay plate for detection and luminescence read using a Perkin Elmer Envision luminescence reader.

[0309] GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA), was used to plot the concentration of OX40mAb24, 9B12, R347 human IgG1, mouse IgG1 clone MOPC-21 (x-axis is log 10 of protein concentration) versus luminescence RLU (y-axis). The EC₂₀, EC₅₀, and EC₉₀ values for bioactivity were determined using ECAanything (ECf) for f=20, f=50 and f=90 from sigmoidal dose-response (variable slope) bioactivity curves).

5.3 Results of 2-cell Bioactivity Assays

[0310] Results for the bioactivity of OX40mAb24 and control antibodies are shown in Figure 11A-D, Figure 12A-D and Table 5-2.

[0311] In the presence of an Fc γ R-expressing cell line (*e.g.*, CD32A-expressing HEK293, CD32B-expressing HEK293, Raji B cells, or CD45⁺ cells isolated from primary human tumors), OX40mAb24 demonstrated potent stimulation of OX40-overexpressing Jurkat NF κ B reporter cells, as measured by NF κ B pathway activation. In the absence of a second cell type or in the presence of HEK293 cells lacking exogenously expressed Fc γ Rs, OX40mAb24 exhibited minimal reporter activity (Figure 11A-D).

[0312] Potency values (EC₂₀, EC₅₀, and EC₉₀) for the two-cell bioassays are summarized in Table 5-2. The mean EC₂₀, EC₅₀, and EC₉₀ values across all assays were 228, 751, and 5630 pM, respectively.

[0313] Results for the bioactivity of 9B12 and control antibodies are shown in Figure 12A-D and the potency values are summarized in Table 5-3. The mean EC₂₀, EC₅₀, and EC₉₀ values for all assays were 519, 2530, and 41100 pM, respectively. Therefore, the ratio of 2-cell bioactivity (EC₅₀) for 9B12 relative to that of OX40mAb24 was calculated to be 3.4 to 1.

Table 5-2: Two-cell Bioactivity of OX40mAb24

Experiment Number	Fc γ R-expressing cell*	EC ₂₀ (95% CI) pM	EC ₅₀ (95% CI) pM	EC ₉₀ (95% CI) pM
1	Raji	298 (236, 378)	1140 (982, 1340)	9850 (6500, 14300)
2	CD32A-expressing HEK	104 (78.8, 138)	437 (370, 517)	4250 (2900, 6240)
3	CD32A-expressing HEK	100 (79.5, 126)	322 (281, 370)	2060 (1530, 2780)
4	Raji	350 (259, 467)	1040 (855, 1270)	5930 (3740, 9400)
5	CD32B-expressing HEK	90.6 (75.4, 109)	270 (242, 301)	1520 (1200, 1920)

Experiment Number	FcγR-expressing cell*	EC ₂₀ (95% CI) pM	EC ₅₀ (95% CI) pM	EC ₉₀ (95% CI) pM
6	Raji	180 (109, 296)	796 (577, 1100)	8410 (3820, 18500)
7	CD32B-expressing HEK	280 (198, 394)	899 (710, 1140)	5730 (3290, 9970)
8	CD32B-expressing HEK	237 (196, 286)	671 (592, 762)	3510 (2630, 4670)
9	Raji	676 (443, 1030)	1320 (970, 1800)	3820 (2000, 7320)
10	Raji	297 (127, 694)	1260 (666, 2390)	12500 (2570, 60500)
11	CD32B-expressing HEK	144 (113, 184)	700 (598, 818)	8540 (5770, 12600)
12	CD45 ⁺ cells, NSCLC	33.6 (24.7, 45.8)	81.6 (68.5, 97.3)	334 (236, 471)
13	CD45 ⁺ cells, RCC	92.2 (75.4, 113)	337 (296, 383)	2630 (1940, 3550)
14	CD45 ⁺ cells, NSCLC	247 (207, 295)	772 (688, 867)	4700 (3600, 6140)
15	CD45 ⁺ cells, RCC	351 (155, 798)	1410 (782, 2530)	12700 (2960, 54500)
16	CD32A-expressing HEK	166 (145, 191)	553 (507, 603)	3720 (3060, 4530)
	Mean (Standard Error of the Mean)	228 (38.9)	751 (101)	5630 (937)

NSCLC = non-small cell lung cancer; RCC = renal cell carcinoma

* As indicated, assays used Raji, CD32A-expressing HEK293, CD32B-expressing HEK293 cells, or CD45⁺ cells isolated from primary human tumor samples with OX40-expressing Jurkat NFκB-luciferase clone 64 reporter cells.

Table 5-3: Two-cell Bioactivity of 9B12

Experiment Number	FcγR-expressing cell*	EC ₂₀ pM	EC ₅₀ pM	EC ₉₀ pM
1	Raji	1110 (791, 1570)	9280 (4460, 19300)	267000 (57900, 1230000)
2	CD32A-expressing HEK	74.1 (53.0, 104)	338 (279, 410)	3760 (2440, 5790)
3	CD32A-expressing HEK	66.7 (43.3, 103)	225 (175, 289)	1550 (914, 2630)
4	Raji	1040 (834, 1300)	5040 (3880, 6550)	61200 (32200, 116000)
5	CD32B-expressing HEK	249 (206, 303)	726 (641, 823)	3950 (2980, 5240)
6	Raji	548 (408, 736)	2700 (2070, 3620)	33700 (17000, 66700)
7	CD32B-expressing HEK	585 (520, 658)	1770 (1620, 1930)	10200 (8280, 12500)
8	CD32B-expressing HEK	518 (449, 598)	1340 (1210, 1480)	6050 (4800, 7630)
9	Raji	872 (504, 1510)	3070 (1910, 4950)	22600 (7000, 73100)
10	Raji	571 (212, 1540)	3330 (1080, 10200)	54400 (3400, 872000)
11	CD32B-expressing HEK	650 (458, 923)	2360 (1790, 3100)	18200 (9200, 36000)
12	CD45 ⁺ cells, NSCLC	104 (76.3, 143)	223 (185, 269)	741 (501, 1100)
13	CD45 ⁺ cells, RCC	ND	ND	ND
14	CD45 ⁺ cells, NSCLC	ND	ND	ND
15	CD45 ⁺ cells, RCC	356 (132, 964)	2420 (938, 6250)	50500 (4140, 616000)
	Mean (Standard Error of the Mean)	519 (96.2)	2530 (687)	41100 (19700)

ND = not determined; NSCLC = non-small cell lung cancer; RCC = renal cell carcinoma

* As indicated, assays used Raji, CD32A-expressing HEK293, CD32B-expressing HEK293 cells, or CD45⁺ cells isolated from primary human tumor samples with OX40-expressing Jurkat NFκB-luciferase clone 64 reporter cells.

5-4: Conclusions

[0314] OX40mAb24 and 9B12 mediate the activation of human T cells as measured by the stimulation of the NFκB pathway in an OX40-overexpressing Jurkat NFκB-luciferase reporter cell line. In the absence of cells that express FcγRs capable of cross linking via the Fc domains of

OX40mAb24, minimal reporter cell-line activity was measured. However, in a 2-cell system comprising cells that express Fc γ R that are capable of cross-linking the mAb, and a Jurkat overexpressing NF κ B-luciferase reporter line for readout of T cell activation, OX40mAb24 and 9B12 mediated potent OX40 activation with mean EC₅₀ values of 751 pM and 2530 pM, respectively. Therefore, the bioactivity of OX40mAb24 in the 2-cell assay system was similar to that of 9B12.

EXAMPLE 6: Ability of OX40mAb24 to Trigger Effector Function

[0315] In this example OX40mAb24 was assessed with respect to its ability to trigger Fc effector function namely, the ability of OX40mAb24 to trigger human natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) against primary human CD4⁺ T cells expressing high levels of OX40, or to bind C1q, a prerequisite for complement-dependent cellular cytotoxicity (CDC) by the classical complement pathway. Versions of OX40mAb24 containing either a human IgG4P Fc domain (mAb28), or a triple mutation in the IgG1 Fc domain that reduces Fc γ RIIIa binding (mAb29) were utilized to assess the contribution of the OX40mAb24 IgG1 Fc domain to mediate ADCC activity. Also, the effector functions of OX40mAb24 were compared to that of 9B12, a mouse anti-human OX40 IgG1 monoclonal antibody that was humanized to create OX40mAb24. The anti-CD20 antibody rituximab binds to B cells expressing CD20 and was used as a positive control for ADCC experiments. Because primary activated human CD4⁺ T cells do not express CD20, a separate assay using the Toledo B cell line, which does express CD20, was conducted to validate the activity of NK cells used in the ADCC assay system.

6.1 MATERIALS

[0316] Materials used in this study are listed in Table 7-1.

Table 6-1: Materials

Item	Source
Complement protein C1q	Quidel, San Diego, CA
Complete RPMI medium: RPMI-1640+ 10% FBS	Materials from Life Technologies, Carlsbad, CA
DM-L medium	Stem Cell Technologies, Vancouver, BC Canada
GLC biosensor chip	Bio-Rad, Hercules, CA
FlowJo Software	FlowJo, Ashland, OR
IL-2, recombinant human	Preprotech, Rocky Hill, NJ
LSR II flow cytometer	BD Biosciences, San Jose, CA

Prism software, v 5.01	Graphpad Software, San Diego, CA
Propidium iodide (1 mg/mL solution)	Sigma, Saint Louis, MO
ProteOn Manager 2.1 software	Bio-Rad, Hercules, CA
ProteOn XPR36 instrument	Bio-Rad, Hercules, CA
RosetteSep CD4 T cell enrichment kit	Stem Cell Technologies, Vancouver, BC Canada
RosetteSep Human NK cell enrichment	Stem Cell Technologies, Vancouver, BC Canada
ViCell counter	Beckman Coulter, Indianapolis, IN
Vybrant DiO cell labeling solution	Life Technologies, Carlsbad, CA
Whole blood, sodium heparin anti-coagulated	MedImmune Blood Donor Program, MedImmune, Gaithersburg, MD

6.2 Assays

6.2.1 Antibody-dependent cellular cytotoxicity

[0317] The ADCC activity of OX40mAb24 relative to that of 9B12 and monoclonal antibodies containing the Fab arms of OX40mAb24 with either an IgG4P Fc domain or a triple mutant (TM) human IgG1 Fc domain, was tested using enriched primary human NK cells as effectors and OX40-expressing primary human CD4⁺ T cells as targets. As a positive control, the activity of each NK cell preparation was tested using rituximab directed killing of the Toledo B cell line.

[0318] For the isolation of primary human CD4⁺ T cells, heparin anti-coagulated, whole blood obtained from healthy donors through the MedImmune Blood Donor Program was processed according to the following protocol: Stem Cell Technologies RosetteSep CD4 T cell isolation kit antibody mix (1 mL, Stem Cell Technologies, Vancouver, BC Canada) was added per 20 mL of whole blood, mixed, and incubated for 20 minutes (mm) at room temperature (RT). Blood was diluted 1:1 with sterile room temperature FACS buffer (PBS, pH 7.2 plus 2% heat inactivated newborn calf serum) and layered onto DM-L medium followed by centrifugation for 20 min. After centrifugation, the buffy coat containing human CD4⁺ T cells was removed and the cells were washed once with RT FACS buffer and once with RT complete RPMI medium. Cells were counted on a ViCell counter to determine cell number and viability and CD4⁺ T cells were suspended in complete RPMI medium at a concentration of 1.0×10^6 per mL.

[0319] Primary human CD4⁺ T cells (1.0×10^6 per mL in complete RPMI medium) were cultured in a humidified tissue culture incubator at 37°C and 5% CO₂ for 48 hours with 2 µg/mL PHA-L and 20 IU/mL rhIL-2 to activate T cells and up-regulate OX40 and were subsequently used in OX40mAb24 ADCC assays. All donors in the figures referenced below represent unique

individuals; that is, CD4⁺ T cells were not isolated from the same donor for repeat ADCC experiments.

[0320] To discriminate target T cells, or cultured Toledo B cells, from purified human NK cells in cytotoxicity assays, the fluorescent dye, DiO, was incorporated into the cell membrane of target cells using the Vybrant DiO cell labeling solution (according to the manufacturer's protocol for suspension cell labeling). After activation and DiO labeling of primary human CD4⁺ T cells and Toledo B cells, effector NK cells were isolated from sodium heparin anticoagulated blood from the MedImmune Blood Donor Program using the same protocol as above for human CD4⁺ T cells, with the exception that 1 mL of RosetteSep NK cell isolation kit antibody mix was used in place of 1 mL of RosetteSep CD4⁺ T cell isolation kit antibody mix. Isolated primary human NK cells were washed two times with warm complete RPMI medium (RPMI-1640 plus 10% FBS), and then suspended in complete RPMI at a concentration of 2.67×10^6 per mL. Likewise, activated primary human CD4⁺ T cells were also washed two times in warm complete RPMI and suspended at a concentration of 2.67×10^5 per mL. Thereafter, 75 μ L of primary human NK cells (200,000) and 75 μ L of activated primary human CD4⁺ T cells (20,000) were added to wells of sterile non-tissue culture treated round bottom 96-well plates for an effector-to-target ratio of 10:1. In some experiments, complete RPMI medium (50 μ L) containing OX40mAb24 or control mAb was added to give a final concentration of 10 μ g/mL. In others, complete RPMI (50 μ L) containing a 3-fold dilution series of OX40mAb24 or control mAbs, resulting in a final concentration of 67 nM, or a 27-fold dilution series of 9B12 or R347 human IgG1 starting from 67 nM, was added to the plated cells. Rituximab (10 μ g/mL, control antibody) was used as a positive control in wells containing DiO labeled, CD20-expressing Toledo B cells, in place of OX40-expressing, activated primary human CD4⁺ T cells, because activated CD4⁺ T cells do not express CD20. Cells in the ADCC assay were gently pelleted by centrifugation at RT and subsequently cultured for 24 hours.

[0321] At the end of the incubation period, cells were pelleted by centrifugation and then suspended in FACS buffer containing 10 μ g/mL of propidium iodide (PT) for flow cytometry analysis on a BD LSRII flow cytometer. Non-viable (PT positive) cells among DiO-positive target CD4⁺ T cells or Toledo B cells were discriminated using FlowJo software after fluorescence compensation.

[0322] Graphical representation of the data for NK cell ADCC assays, including determination of mean values and standard error of the mean, was generated using GraphPad Prism version 5.01 for Windows.

6.2.2 OX40mAb24 Binding to C1q

[0323] A ProteOn XPR36 instrument was used to determine the binding of OX40mAb24 or 9B12 to human complement protein C1q purified from pooled human sera to OX40mAb24 or 9B12 by surface plasmon resonance. Standard amine coupling was used to immobilize OX40mAb24 or 9B12 to the surface of a GLC biosensor chip. Human C1q was suspended in PBS/0.005% Tween 20, pH 7.4 at five concentrations ranging from 26 nM to 1.6 nM. The samples were injected at 30 μ L/min for 200 seconds, and the dissociation phase was followed for 600 seconds. Sensorgram data was processed using ProteOn Manager 2.1 software (Bio-Rad) using 1:1 fitting.

6.3 Results

[0324] The ability of OX40mAb24, 9B12, and human IgG4P (mAb28) and IgG1-TM (mAb29) versions of OX40mAb24 to mediate ADCC was tested at saturating levels (10 μ g/mL [67 nM]) for OX40 binding (see Example 2), using both allogeneic and autologous mixtures of NK cells and OX40-expressing target cells (Figure 13A and Figure 14A). OX40mAb24 showed measurable ADCC activity against activated human CD4⁺ T cells. In contrast, 9B12, mAb28 and mAb29 showed no ADCC activity above that of the negative controls. A positive-control antibody against human CD20 (rituximab) demonstrated ADCC activity using the same NK cells, demonstrating that the NK cells were all capable of robust ADCC activity (Figure 13B and Figure 14B).

[0325] In dose-response experiments, OX40mAb24 demonstrated concentration-dependent ADCC activity against activated human CD4⁺ T cells (Figure 15A-D, Figure 16A-D, Figure 17A-B, and Table 6-2. In contrast, 9B12 did not show any detectable ADCC activity. In addition, the R347 human IgG1 control mAb did not show any detectable ADCC activity, which confirms that ADCC activity mediated by OX40mAb24 was dependent on engagement with OX40 on the target cells. A positive-control antibody against human CD20 also demonstrated ADCC against a CD20-expressing B cell lymphoma cell line (Figure 13B and Figure 14B); this supports the validity of the assay. Collectively, the results of these experiments confirmed that OX40mAb24 is capable of ADCC against target cells that have been cultured under conditions previously shown to stimulate expression of cell surface OX40.

Table 6-2 Summary of OX40mAb24-mediated NK cell ADCC Potency

Experiment Number	Donor Number	EC20 (pM)	EC50 (pM)	EC90 (pM)
3	363	134	2380	225000
3	504	309	1370	14600
4	464	37.0	3050	3330000
4	532	111	1310	66100
5	601	54.4	501	16900
5	602	61.4	527	16000
Mean (standard error of the mean), pM		118 (41.1)	1520 (415)	611000 (545000)

[0326] The potential for OX40mAb24 to bind to the human complement component C1q was assessed using a surface plasmon resonance assay. In this assay, binding to C1q was used as a surrogate for activity in a complement dependent cytotoxicity assay (Dall'Acqua *et al.*, J. Immunol 177:1129-1138 (2006)). 9B12 was used as a negative control antibody. OX40mAb24 demonstrated a concentration-dependent ability to bind to purified human C1q protein (Figure 18A). In contrast, 9B12 did not demonstrate any detectable binding to C1q protein (Figure 18B).

6.4 Conclusions

[0327] OX40mAb24 binds to C1q and triggers NK-mediated ADCC against activated CD4⁺ T cells.

EXAMPLE 7: *In Vitro* Comparability Studies with OX40mAb24 and Cynomolgus and Rhesus Monkey T Cells

[0328] In this example, the ability of OX40mAb24 to enhance activation of T cells was determined using two-cell reporter bioactivity assays with a Jurkat NFκB-luciferase T cell reporter line expressing cynomolgus (cyno)/rhesus monkey OX40. Fcγ receptor-mediated drug cross-linking was mediated by either a rhesus B cell line, or by rhesus Fcγ receptor-expressing cells in a post-red blood cell lysis whole blood sample. OX40 activation was measured as increased luciferase activity in response to stimulation of the NFκB signaling pathway downstream of primary human T cell activation. NFκB signaling is a well-studied downstream event in OX40 signaling, and can correlate with other measures of T cell activation such as proliferation and cytokine release (Croft M, *et al.*, Immunol Rev. 229:173-91 (2009)). In addition, the ability of OX40mAb24 to enhance T cell receptor-mediated activation (co-stimulation) of rhesus T cells was tested using a plate-

based bioactivity assay in which CD4⁺ T cell proliferation was assessed. An isotype control (NIP228 IgG1), was used to demonstrate specific OX40 engagement.

[0329] In parallel, the bioactivity of 9B12, the murine anti-OX40 monoclonal antibody from which OX40mAb24 was derived, was measured to provide for a comparison of OX40mAb24 and 9B12 activity on non-human primate OX40.

7.1 MATERIALS

[0330] Materials used in this study are listed in Table 7.1

Table 7-1 Materials

Item	Source
Ammonium Chloride	StemCell Technologies, Vancouver, BC
Anti-human CD3 antibody, clone SP34-2	BD Biosciences, San Jose, CA
Anti-human CD4-V450 antibody, clone L200	BD Biosciences, San Jose, CA
Anti-human CD28 antibody, clone CD28.2	BD Biosciences, San Jose, CA
CFSE cell labeling kit	Life Technologies, Carlsbad, CA
Concanavalin A	Sigma, Saint Louis, MO
Envision multilabel plate reader	Perkin Elmer, Waltham, MA
Goat anti-human Fc γ antibody	Jackson ImmunoResearch, West Grove, PA
Goat anti-mouse Fc γ antibody	Jackson ImmunoResearch, West Grove, PA
Heat inactivated fetal bovine serum	Life Technologies, Carlsbad, CA
Interleukin-2, recombinant human	Preprotech, Rocky Hill, NJ
LSR II flow cytometer	BD Biosciences, San Jose, CA
Lymphocyte separation medium (LSM)	MP Biomedicals, Santa Ana, CA
MACS buffer	Miltenyi, San Diego, CA
Nonhuman primate CD4 T cell isolation kit	Miltenyi, San Diego, CA
Propidium iodide (1 mg/mL solution)	Sigma, Saint Louis, MO
Rhesus monkey (Indian Origin) whole blood, sodium heparin anti-coagulated	Worldwide Primates, Miami, FL
RPMI-1640 medium	Life Technologies, Carlsbad, CA
SteadyGlo Luciferase Assay Solution	Promega, Madison, WI
ViCell counter	Beckman Coulter, Indianapolis, IN

7.2 Assays

7.2.1 Cyno/Rhesus 2-Cell Bioactivity Assays

[0331] OX40mAb24 was tested for bioactivity using a 2-cell reporter assay. As cyno and rhesus monkeys share identical OX40 amino acid sequences, a cyno/rhesus OX40-expressing Jurkat NF κ B-luciferase reporter cell line (clone B2) was used for readout of OX40 agonism (NF κ B activity). To mediate OX40mAb24 cross-linking and, consequently, OX40 clustering on Jurkat

reporter cells, either an Fc γ receptor-expressing rhesus B-cell line, LCL8664, or leukocytes from whole blood of a normal rhesus monkey, which contain Fc γ R expressing cells, were used. Background bioactivity was assessed both without the addition of OX40mAb24, and also in the absence of the Fc γ R-expressing cells. To demonstrate the role of OX40 engagement on reporter cells, an isotype control was used in place of OX40mAb24.

[0332] The OX40mAb24 2-cell bioactivity assay with LCL8664 was performed according to the following protocol:

[0333] The day prior to use, cyno/rhesus OX40-expressing Jurkat NF κ B-luciferase reporter clone B2 and LCL8664 were cultured in complete RPMI medium (RPMI with 10% fetal bovine serum [FBS] and 1% antibiotics/antimycotics) to achieve cell densities of approximately 5×10^6 cells/mL and 4×10^5 cells/mL, respectively. The next day, OX40 Jurkat reporter cells and LCL8664 were pelleted, suspended in complete medium and counted using a ViCell counter before cell concentrations for both cell lines were adjusted to 2.5×10^6 in complete medium.

[0334] Clone B2 and LCL8664 cells were each added to a 96 well round bottom non tissue-culture treated plate at 100,000 cells per well. OX40mAb24 was added to cells in complete RPMI medium, to a final concentration starting at 30 μ g/mL and diluted in 3-fold increments. Similarly, R347 IgG1 (isotype control) was used at a final concentration of 10 μ g/mL and diluted in 6-fold increments. 9B12 and MOPC-21 (isotype control) were diluted in the same manner. Plates were transferred to a 37°C incubator with a humidified 5% CO₂ atmosphere.

[0335] After 16 hours incubation time, 100 μ L reconstituted Steady-Glo luciferase assay solution was added to each well and mixed to lyse cells and then incubated to equilibrate luciferase signal. Steady-Glo/sample lysate (150 μ L) was transferred from each well to a 96 well, white walled assay plate for detection and luminescence read using a Perkin Elmer Envision luminescence reader.

[0336] The following protocol was used to acquire RBC-lysed rhesus cells from sodium heparin anti-coagulated whole blood obtained from a healthy Indian-origin rhesus macaque:

[0337] Fresh whole blood (5 mL) was added to a 50-mL conical tube and 45 mL of ammonium chloride solution was added. The cell mixture was incubated for 10 minutes on ice, then pelleted, and washed with complete RPMI medium after which the cells were ready for use in the 2-cell bioactivity assay.

[0338] The 2-cell bioactivity assay with rhesus RBC- lysed whole blood cells was performed as described with the LCL8664 rhesus B-cell line, but 10 µg/mL NIP228 IgG1 was used as the negative-control antibody.

7.2.2 Rhesus CD4 T cell Proliferation Assay

[0339] OX40mAb24 bioactivity was determined in a CD4⁺ T cell proliferation assay using activated primary rhesus CD4⁺ T cells and plate-captured drug according to the following protocol. Rhesus CD4⁺ T cells isolated from sodium heparin anti-coagulated whole blood obtained from healthy rhesus monkeys (N = 2) from World Wide Primates were used as the source of responding cells.

[0340] Fresh heparinized rhesus blood was diluted 1:1 with PBS at room temperature (RT). Then 20 mL of diluted whole blood was overlayed onto 15 mL of 95% lymphocyte separation medium (LSM) in a 50-mL conical centrifuge tube. Blood was centrifuged at 400 x g for 30 minutes at room temperature without the brake. Peripheral blood mononuclear cells were collected at the interface and washed twice with Miltenyi MACS buffer and pelleted. The cell pellet was treated with red cell lysis buffer for 5 minutes, and lysis buffer was deactivated with the addition of complete RPMI medium. The cells were washed and suspended in MACS buffer and counted with a ViCell counter to determine cell number and viability.

[0341] Rhesus CD4⁺ T cells were isolated with a Miltenyi nonhuman primate kit according to manufacturer's instructions. Then, CD4⁺ T cells were counted on a ViCell counter, suspended at 1×10^6 cells/ mL in complete RPMI medium with 5 µg/mL Concanavalin A and 1000 IU/mL of IL-2 and cultured at 37°C and 5% CO₂ in a humidified incubator for 2 days to activate T cells and induce OX40 expression. Non-tissue culture treated round-bottom 96 well assay plates were coated with 100 µL of 2 µg/mL goat anti-mouse Fcγ-specific IgG and 2 µg/mL goat anti-human Fcγ-specific IgG in PBS. Goat anti-human IgG capture antibodies were not added to wells intended for assay of soluble OX40mAb24 activity. Plates were incubated overnight at 4°C, washed with 200 µL of PBS, and blocked for 90 minutes at 37°C with 1% BSA in PBS (1% BSA/PBS). The plates were washed with PBS and 2 ng/mL of anti-CD3 (clone SP34-2) reconstituted in 1% BSA/PBS was added to the plates for 90 minutes at 37°C. The plates were washed with PBS to remove unbound OX40mAb24, R347 human IgG1 control mAb, 9B12, and

mouse IgG1 control mAb (clone MOPC-21) were each reconstituted in 1% BSA/PBS starting at 1.01 µg/mL (6.67 nM; experiment 1) or 1.5 µg/mL (10.0 nM; experiment 2) and serially diluted over a 3-fold dilution series and then added to assay plates and incubated for 90 minutes at 37°C.

[0342] Activated primary rhesus CD4⁺ T cells were collected, washed in complete RPMI medium, and the concentration adjusted to 1.0×10^6 viable cells/mL. Cells were labelled with carboxyfluorescein succinimidyl ester (CFSE), according to the manufacturer's instructions, with the exception of using 1.25 µM CFSE instead of the recommended 5 µM, with an incubation of 10 minutes at 37°C. After labeling, cells were suspended in complete RPMI and the concentration was adjusted to 1.0×10^6 per mL. The plates were washed with PBS and 200 µL of CD4⁺ T cells (200,000/well) was added to each well. Then, the plate was incubated at 37°C for 3 days.

[0343] After 72 hours incubation time, CD4⁺ T cells were pelleted, and washed once with PBS containing 2% FBS (FACS buffer). Cells were suspended in binding mix containing anti-CD4 V450[®] labeled antibody for identification of CD4⁺ T cells, and propidium iodide (PI) for live/non-viable cell discrimination, and incubated for 30 minutes. Following incubation, cells were washed in FACS buffer, re-suspended in FACS buffer and analyzed by flow cytometry using an LSRII flow cytometer and FlowJo software for analysis of Flow Cytometry Standard (FCS) formatted data.

[0344] The assay was repeated in two independent experiments using primary rhesus CD4⁺ T cells.

[0345] To assess cell proliferation, live (propidium iodide-negative) events were gated using FlowJo software and the percentage of CD4⁺-gated cells showing dilution of CFSE was determined. Specific activity of OX40mAb24 was calculated by subtracting the percentage of CD4⁺-gated cells showing dilution of CFSE in response to anti-CD3 alone from the percentage of CD4⁺-gated cells showing dilution of CSFE in response to anti-CD3 antibody plus OX40mAb24.

[0346] The concentrations of OX40mAb24 that achieved half-maximal (EC₅₀) responses in the Jurkat NFκB reporter assay and the primary rhesus CD4⁺ T cell assay were determined using non-linear regression analysis (log dose-response, 4-parameter fit curves) in GraphPad Prism, version 5.01 (San Diego, CA).

7.3 RESULTS

7.3.1 Cyno/Rhesus 2-Cell Bioactivity Assay

[0347] Results of the cyno/rhesus 2-cell bioactivity assays are shown in Table 7-2, Figure 19A-B, Figure 20A-B and Figure 21A-B. OX40mAb24 activated the OX40 signaling pathway, as measured by NFκB signaling, in cyno/rhesus OX40-expressing Jurkat T cells in the presence of the rhesus B-cell line, LCL8664, with a mean EC₅₀ of 1450 pM (N = 2 experiments). As shown in Table 7-2, the EC₅₀ value of OX40mAb24 activity in the cyno/rhesus 2-cell assay was 1.3 fold higher than in human 2-cell assays with the Raji B-cell line, while 9B12 had limited activity in cyno/rhesus 2-cell assays and an EC₅₀ value could not be determined.

[0348] In addition, as shown in Table 7-2, OX40mAb24 activated the OX40 signaling pathway in Jurkat T cells in the presence of rhesus RBC-lysed whole blood, which is expected to contain Fcγ receptor-expressing cells, with an EC₅₀ of 550 pM (N = 1 experiment). As Table 7-2 also shows, the EC₅₀ value of 9B12 in the same assay was 6052 pM, and was 4680 pM in a human 2-cell assay with the Raji B-cell line.

Table 7-2 Mean Half Maximal Effective Concentration for OX40mAb24 and 9B12 in 2-cell Bioactivity Assays

Test or Control Article	2-cell assay format		
	Rhesus LCL8664 B cells + cyno/rhesus OX40-expressing Jurkat NFκB-luciferase clone B2 bioactivity [mean EC ₅₀ (Std Err); n]	Rhesus RBC-lysed whole blood + cyno/rhesus OX40-expressing Jurkat NFκB-luciferase clone B2 bioactivity [mean EC ₅₀ (Std Err); n]	Human Raji B cells + human OX40 Jurkat NFκB-luciferase clone 64 bioactivity ^a [mean EC ₅₀ (Std Err); n]
OX40mAb24	1450 (204); n=2	550; n=1	1110 (92.5); n=5
R347 human IgG1	no activity; n=2	NT	no activity
NIP228 human IgG1	NT	no activity; n=1	NT
9B12	ND	6052; n=1	4680 (1220); n = 5

Std Err = standard error of the mean. n=number of experiments. NT=Not tested. ND=Not Determined.

^aData from Example 5.

7.3.2 Rhesus Primary C-cell Proliferation Assay

[0349] Results for the rhesus primary T cell proliferation assay are shown in Table 7-3, Table 7-4, and Figure 22A-D. OX40mAb24 induced proliferation of primary rhesus CD4⁺ T cells with a mean EC₅₀ of 436 pM (Table 7-3; N = 2 experiments). As shown in Table 7-5, the EC₅₀ value for 9B12 was 110 pM (Table 7-4; N = 2 experiments). In OX40mAb24 induced proliferation of activated primary human CD4⁺ T cells in a similar assay format with a mean EC₅₀ of 28 pM (Table 7-5; data from Example 4).

Table 7-3 Mean Half Maximal Effective Concentration for OX40mAb24 in Primary Rhesus CD4 T cell Proliferation Assays

Test or control antibody	No. of Experiments	Mean EC ₅₀ (Std Err)
OX40mAb24	2	436 (309)
NIP228 IgG1	2	No activity

Std Err = standard error of the mean.

Table 7-4 Mean Half Maximal Effective Concentration for 9B12 in Primary Rhesus CD4 T cell Proliferation Assays

Test or control antibody	No. of Experiments	Mean EC ₅₀ (Std Err)
9B12	2	110 (13)
MOPC-21 mouse IgG1	2	No activity

Std Err = standard error of the mean.

Table 7-5 Mean Half Maximal Effective Concentration for OX40mAb24 and 9B12 in Rhesus and Human CD4 T cell Proliferation Assays

Test or control antibody	Rhesus Mean (Std Err)	Human Mean (Std Err)
OX40mAb24	436 (309)	28 (98)
9B12	110 (13)	218 (35)

Std Err = standard error of the mean.

7.4 CONCLUSIONS

[0350] OX40mAb24 activated the OX40 signaling pathway in a cyno/rhesus OX40 expressing Jurkat T cell NFκB reporter cell line in the presence of rhesus B cells or Fcγ receptor-expressing cells contained within RBC-lysed rhesus whole blood. In addition, OX40mAb24 induced proliferation of primary rhesus CD4⁺ T cells.

EXAMPLE 8: Activity of OX40mAb24 in Mouse Models of Human Cancers

[0351] This example was designed to determine if OX40mAb24 is effective as a single-agent therapy for the treatment of cancers. This study was conducted in xenograft models of human cancers mixed with alloreactive human T cells in immunocompromised non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice.

8.1 Materials

[0352] Materials used in this study, and their source, are listed in Table 8-1.

Table 8-1 Materials

Item	Source
DMEM medium	Invitrogen, Carlsbad, CA
FBS	Invitrogen, Carlsbad, CA
Lymphocyte separation medium	VWR, West Chester, PA
PBS	Invitrogen, Carlsbad, CA
RPMI 1640	Invitrogen, Carlsbad, CA
RosetteSep CD4 ⁺ T cell enrichment kit	Stem Cell Technologies, Vancouver, BC, Canada
RosetteSep CD8 ⁺ T cell enrichment kit	Stem Cell Technologies, Vancouver, BC, Canada
RosetteSep DML medium	Stem Cell Technologies, Vancouver, BC, Canada
Mitomycin C	Sigma-Aldrich, St. Louis, MO

8.2 Experimental Protocols**8.2.1 Test Animals**

[0353] Female NOD/SCID mice aged 5 to 9 weeks, were obtained from Harlan Laboratories, Inc. The animals were humanely treated and housed according to Institutional Animal Care and Use Committee approved protocols in the Laboratory Animal Resources facility at MedImmune, an Association for Animal Accreditation of Laboratory Animal Care and United States Department of Agriculture-licensed facility. The animals were kept in sterile micro-isolator units, provided with sterile bedding and food, and acidified drinking water ad libitum. Environmental conditions were standardized (room temperature: 20°C +/- 1°C; relative humidity: 50% ± 10%; 12-hour light-dark cycle). The animals were monitored daily for adverse clinical signs and weekly for body weight.

8.2.2 Establishment of Xenografts

[0354] Human cancerous A375 cells originating from a human melanoma cell line were obtained from ATCC. The cells were grown in DMEM with 10% FCS at 37°C under 5% CO₂ in a

humidified incubator. They were then harvested, washed once with PBS, then resuspended in PBS.

[0355] Human cancerous A375 cells were harvested from cell cultures. They were resuspended in PBS. A375 cells were subsequently mixed with CD4⁺ and CD8⁺ T cell lines alloreactive to A375 tumor cell lines before implantation into animals.

[0356] To generate CD4⁺ and CD8⁺ T cell lines, human peripheral blood mononuclear cells (PBMCs) from healthy donors were enriched for CD4⁺ or CD8⁺ T cells by the addition of 1 mL RosetteSep T cell enrichment product per 20 mL of whole blood. This was followed by a 20-minute incubation and subsequent isolation by density gradient centrifugation using RosetteSep DM-L density medium. After centrifugation, the cells were washed 3 times with PBS supplemented with 2% fetal bovine serum (FBS) and resuspended in RPMI1640 medium supplemented with 10% FBS. Enriched CD4⁺ and CD8⁺ T cells were cultured separately for 7 to 10 days in medium supplemented with recombinant human interleukin 2 (rhIL-2) and each combined with mitomycin C-treated A375 cells. T cells were collected and separately cultured again for 7 to 10 days in medium supplemented with rhIL-2 and combined with mitomycin C-treated A375 cells. CD4⁺ and CD8⁺ T cells were collected and combined at a 2:1 ratio.

[0357] A375 cells and PMBCs enriched for CD4⁺ and CD8⁺ T cells were mixed at a ratio of 6 A375 cells to 1 T cell immediately before implantation.

[0358] Xenografts were established by subcutaneous (SC) injection of 3.5×10^6 cells (human T cells mixed with A375 cells at an effector-to-target (E:T) ratio of 1:6 and suspended in 200 μ L of PBS) into the right flanks of the animals.

8.2.3 Randomization, Group Designation, and Dose Levels

[0359] Six animals were randomly assigned to each experimental group prior to SC injection of cells. There were no animal substitutions. Group designations and dose levels for each experiment are listed in Table 8-2, Table 8-3 and Table 8-4. Test and control antibodies were diluted in PBS to appropriate concentrations and administered intraperitoneally (IP) in a total volume of 200 μ L. The first dose of test and control antibodies was administered on Day 3 or 4 after implantation of cancer/effector T cells. The animals received up to 3 additional doses of the test and control antibodies, as indicated in the figures and described in the corresponding figure description. The

formation of tumors was observed in each animal 1 or 2 times a week. The primary endpoints in this study was either a tumor volume of 2000 mm³ or gross tumor necrosis.

8.2.4 Tumor Measurements

[0360] Tumors were measured at intervals indicated in the figures and tables for each experiment by caliper and tumor volumes (V) were calculated using the following formula:

$$V \text{ (mm}^3\text{)} = (\text{length [mm]} \times \text{width [mm]} \times \text{width [mm]})/2.$$

For each group, the results are reported as the arithmetic mean. Antitumor effects were expressed as percent tumor growth inhibition (% TGI), which was calculated as follows:

$$\% \text{ TGI} = (1 - [\text{mean tumor V of treatment group}] \div [\text{mean tumor V of control group}]) \times 100$$

8.3 Statistical Methods

[0361] A comparison between OX40mAb24-treated or 9B12-treated, and isotype control-treated animals was made, and intergroup differences were analyzed for statistical significance by a Mann-Whitney rank sum test.

[0362] Significant p-values obtained from the Mann-Whitney rank sum test are presented in the summary tables and figures adjacent to the arithmetic mean and standard deviation of the mean.

8.4 Results

[0363] The activity of OX40mAb24 on growth of tumors in mouse models of human cancer was investigated in this study. Immunodeficient NOD/SCID female animals were implanted with human cancer cell lines mixed with alloreactive human CD4⁺ and CD8⁺ T cell lines. CD4⁺ and CD8⁺ T cells were derived from PBMCs isolated from healthy human donors. Animals received the first dose of the test and control antibodies there or four days after implantation of xenographs, and were administered additional doses of the test and control antibodies as indicated.

[0364] In three separate experiments, OX40mAb24 plus alloreactive human T cells significantly inhibited growth of A375 cells by up to 85% as compared to the isotype-control group (Table 8-2, Table 8-3, and Table 8-4; Figure 23A, Figure 24A and Figure 25). The control 9B12 plus alloreactive human T cells also significantly inhibited growth of A375 cells by up to 77% as compared to the isotype-control group (Tables 8-2 to 8-4, Figure 23B and Figure 24B).

Table 8-2 Treatment Groups and Percent TGI in A375 Xenograft Model on Day 18 (Experiment 2)

Group ^a	Test Antibody	Dose ^b (mg/kg)	% TGI ^c
1	None; no T cells	NA	NA
2	None	NA	NA
3	Isotype control	5	NA
4	OX40mAb24	5	79
5	OX40mAb24	2.5	75
6	OX40mAb24	1.0	85
7	9B12	5	53

IP = intraperitoneal; NA = not applicable; TGI = tumor growth inhibition; V = volume

^a Number of animals per group: 6.^b All animals received 200 µl of test antibody IP on Days 4, 7, 9, and 12^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of isotype control group)] x 100**Table 8-3 Treatment Groups and Percent TGI in A375 Xenograft Model on Day 25 (Experiment 1)**

Group ^a	Test Antibody	Dose ^b (mg/kg)	% TGI ^c
1	None; no T cells	NA	NA
2	None	NA	NA
3	Isotype control	5	NA
4	OX40mAb24	5	68
5	OX40mAb24	2.5	83
6	OX40mAb24	1.0	84
7	9B12	5	80

IP = intraperitoneal; NA = not applicable; TGI = tumor growth inhibition; V = volume

^a Number of animals per group: 6.^b All animals received 200 µl of test antibody IP on Days 4, 7, 9, and 12^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of isotype control group)] x 100**Table 8-4 Treatment Groups and Percent TGI in A375 Xenograft Model on Day 28 (Experiment 3)**

Group ^a	Test Antibody	Dose ^b (mg/kg)	% TGI ^c
1	None; no T cells	NA	NA
2	None	NA	NA
3	Isotype control	3.0	NA
4	OX40mAb24	3.0	75
5	OX40mAb24	1.0	73
6	OX40mAb24	0.3	68
7	OX40mAb24	0.1	84
8	OX40mAb24	0.03	73

IP = intraperitoneal; NA = not applicable; TGI = tumor growth inhibition; V = volume

^a n = 6.^b All animals received 200 µl of test antibody IP on Days 3, 6, 8, and 10^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of isotype control group)] x 100

8.5 Conclusions

[0365] OX40mAb24 demonstrated potent antitumor activity in mouse models of human cancer mixed with alloreactive human T cells. The antitumor activity of OX40mAb24 was similar to the antitumor activity of 9B12. These results provide evidence that OX40mAb24 can be effective as a single-agent therapy for the treatment of patients with cancer with a T cell infiltrate.

EXAMPLE 9: Rat/Mouse Anti-Mouse OX40 IgG2a Chimera Antibody Clone OX86 Inhibits the Growth of Mouse Cancer Cell Lines in Syngeneic Models

[0366] OX40mAb24 does not cross-react to mouse (m)OX40 (See Example 2); therefore, it is not possible to test its activity in immunocompetent mouse models. OX86 is a rat anti-mOX40 IgG1 antibody that specifically binds to and triggers signaling of mOX40 (al-Shamkhani *et al.*, *Eur. J. Immunol.* 26:1695-1699 (1996)), and has anti-tumor activity in immunocompetent mouse models of cancer (Weinberg AD, *et al.*, *J. Immunol.* 164:2160-2169 (2000)). To more fully study the effects of OX40 agonism using a mouse surrogate antibody with functional properties similar to OX40mAb24, a rat/mouse anti-mOX40 IgG2a chimera antibody (OX86 mIgG2a; rat anti-OX40 light and heavy chain variable regions with mouse IgG2a constant regions) was generated from OX86. This example evaluates the single-agent anti-tumor activity of OX86 mIgG2a in three mouse models of cancer.

9.1 Materials

[0367] Materials used in this study, and their source, are listed in Table 9-1.

Table 9-1 Materials

Item	Source
Phosphate-buffered saline, pH 7.2	Life Technologies, Carlsbad, CA
Fetal bovine serum, heat inactivated	Life Technologies, Carlsbad, CA
Roswell Park Memorial Institute 1640 medium	Life Technologies, Carlsbad, CA
0.25% Trypsin-EDTA (1x)	Life Technologies, Carlsbad, CA

EDTA = Ethylenediaminetetraacetic acid.

9.2 Experimental Protocols

9.2.1 Test Animals

[0368] BALB/c and C57BL/6 mice of 6-8 weeks of age were received at MedImmune from Harlan Laboratories, Inc. (Indianapolis, IN) and allowed to acclimatize for 3 days prior to study start.

Thereafter, mice were shaved at tumor implantation sites and implanted with microchip transponders for identification.

[0369] The animals were housed according to Institutional Animal Care and Use Committee approved protocols in the Laboratory Animal Resources facility at MedImmune, an Association for Animal Accreditation of Laboratory Animal Care and United States Department of Agriculture-licensed facility. The animals were kept in sterile micro-isolator units, provided with sterile bedding and food, and acidified drinking water ad libitum. Environmental conditions were standardized (room temperature: $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$; relative humidity: $50\% \pm 10\%$; 12-hour light-dark cycle). The animals were monitored daily for adverse clinical signs and bi-weekly for body weight. If hind limb paralysis, respiratory distress, 20% body weight loss, or tumor volume greater than 2000 mm^3 were noted, the animals were immediately sacrificed humanely by asphyxiation with CO_2 .

9.2.2 Establishment and Implantation of Syngeneic Tumors

[0370] CT26 and 4T1 were obtained from ATCC in Manassas, VA. MCA205 cells were obtained from Providence Cancer Center, Portland, OR. All cells were cultured in RPMI 1640 cell culture medium supplemented with 10% FBS and grown at 37°C at 5% CO_2 in a humidified tissue culture chamber, then harvested, washed once in FBS and then resuspended in PBS.

[0371] Allografts were established by subcutaneous (SC) injection of 5.0×10^5 CT26 cells, or 1.0×10^5 4T1 cells suspended in 0.1 mL of PBS into the right flank of 7- to 9-week-old BALB/c mice, while 2.5×10^5 MCA205 cells suspended in 0.1 mL of phosphate-buffered saline were injected into the right flank of 7- to 9-week-old C57BL/6 mice.

9.2.3 Randomization, Group Designation, and Dose Levels

[0372] BALB/c (total of 216) and C57BL/6 (total of 70) female mice were used in this study. BALB/c mice implanted with CT26 tumor cells were randomly assigned after tumors grew to a mean volume of 120 mm^3 per cohort, 9 days after implantation or 200 mm^3 per cohort, 13 days after implantation. BALB/c mice implanted with 4T1 tumor cells were randomly assigned after tumors grew to a mean volume of 120 mm^3 per cohort, 13 days after implantation. C57BL/6 mice were randomly assigned after tumors grew to a mean volume of 95 mm^3 per cohort, 11 days after implantation. Group designations number of animals, dose levels, and dose schedule are presented

in Table 9-2, Table 9-3, Table 9-4, and Table 9-5. All test antibodies and control antibodies were administered by intraperitoneal (IP) injection. There were no animal substitutions.

[0373] Animals from each group were sacrificed when tumor volumes reached approximately 2000 mm³ or when tumors became ulcerated or necrotic.

Table 9-2 Study Design: CT26 Syngeneic Model

Group	Number of animals (M/F)	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1	10 (F)	None	NA	NA	IP
2	10 (F)	Negative control (OX86 mIgG1 D265A)	9, 12	2.5	IP
3	10 (F)	OX86 mIgG2a	9, 12	2.5	IP
4	10 (F)	OX86 mIgG2a	9, 12	1.0	IP
5	10 (F)	OX86 mIgG2a	9, 12	0.25	IP
6	10 (F)	OX86 mIgG2a	9, 12	0.1	IP

F = female; IgG1 = immunoglobulin G1; IP = intraperitoneal; mAb = monoclonal antibody; OX86 mIgG2a = rat anti-OX40 light and heavy chain variable regions of clone OX86 with mouse IgG2a constant regions; OX86 mIgG1 D265A = mouse anti-mouse OX40 mIgG1 with a point mutation in the Fc domain that reduces its ability to bind Fcγ receptors; NA = not applicable because the animals were not treated; ROA = route of administration. ^a Dose volume: 0.2 mL.

Table 9-3 Study Design: CT26 Syngeneic Model

Group	Number of animals (M/F)	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1	12 (F)	None	NA	NA	IP
2	12 (F)	OX86 mIgG2a	13, 16	10	IP
3	12 (F)	OX86 mIgG2a	13, 16	3	IP
4	12 (F)	OX86 mIgG2a	13, 16	1	IP
5	12 (F)	OX86 mIgG2a	13, 16	0.3	IP
6	12 (F)	OX86 mIgG2a	13, 16	0.1	IP

F = female; IP = intraperitoneal; OX86 mIgG2a = rat anti-OX40 light and heavy chain variable regions of clone OX86 with mouse IgG2a constant regions; NA = not applicable because the animals were not treated; ROA = route of administration. ^a Dose volume: 0.2 mL.

Table 9-4 Study Design: MCA205 Syngeneic Model

Group	Number of animals (M/F)	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1	14 (F)	None	NA	NA	IP
2	14 (F)	Isotype control (mixture) ^b	11, 14	20	IP
3	14 (F)	OX86 mIgG2a	11, 14	10	IP
4	14 (F)	OX86 mIgG2a	11, 14	10	IP
5	14 (F)	OX86 mIgG2a	11, 14	5	IP

F = female; IP = intraperitoneal; OX86 mIgG2a = rat anti-OX40 light and heavy chain variable regions of clone OX86 with mouse IgG2a constant regions; NA = not applicable because the animals were not treated; ROA = route of administration. ^a

Dose volume: 0.2 mL. ^b Mixture contains isotype control antibodies with Fc domains of mIgG2a (10 mg/kg) and mIgG2b (10 mg/kg).

Table 9-5 Study Design: 4T1 Syngeneic Model

Group	Number of animals (M/F)	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1	12 (F)	None	NA	NA	IP
2	12 (F)	Isotype control (mixture) ^b	13, 16, 20, 23	70	IP
3	12 (F)	OX86 mIgG2a	13, 16, 20, 23	10	IP
4	12 (F)	OX86 mIgG2a	13, 16, 20, 23	5	IP
5	12 (F)	OX86 mIgG2a	13, 16, 20, 23	2.5	IP
6	12 (F)	OX86 mIgG2a	13, 16, 20, 23	1.0	IP
7	12 (F)	OX86 mIgG2a	13, 16, 20, 23	0.25	IP

F = female; IP = intraperitoneal; OX86 mIgG2a = rat anti-OX40 light and heavy chain variable regions of clone OX86 with mouse IgG2a constant regions; NA = not applicable because the animals were not treated; ROA = route of administration. ^a Dose volume: 0.2 mL. ^b Mixture contains isotype control antibodies with Fc domains of mIgG2a (10 mg/kg), mIgG2b (20 mg/kg), rat IgG2a (20 mg/kg) and rat IgG2b (20 mg/kg).

9.2.4 Tumor Measurements

[0374] Tumors were measured by caliper twice weekly and tumor volumes were calculated using the following formula:

$$\text{tumor volume (V)} = [\text{length (mm)} \times \text{width (mm)}^2] / 2$$

where length was defined as the larger side and width as the smaller side perpendicular to the length.

[0375] Antitumor effects of each group were expressed as tumor growth inhibition (TGI), which was calculated as follows:

$$\% \text{ TGI} = (1 - [\text{mean V of treatment group}] \div [\text{mean V of control group}]) \times 100$$

Tumor growth responses were categorized as a complete response (CR) if there was no measureable tumor.

9.3 Statistical Methods

[0376] One-way ANOVA was used to determine mean tumor volume differences. In the event of a significant F test a Dunnett's or Sidak's multiple comparison test was utilized (where appropriate). Where applicable, a log₁₀ transformation was applied to tumor volumes to account for heteroscedasticity. A p value < 0.05 was considered significant.

9.4 Results

[0377] Treatment of mice with the OX86 mIgG2a results in significantly reduced growth of CT26 and MCA205 tumor cells compared to untreated or negative, or isotype control antibody-treated mice control, (Table 9-6, Table 9-7 and Table 9-8; Figure 26A, Figure 27A, and Figure 28A). Treatment of mice with the OX86 mIgG2a results in delayed and reduced growth of 4T1 tumor cells compared to untreated or isotype control antibody (Table 9-9 and Figure 29A).

[0378] A mixed response is often shown in syngeneic models; however, the dramatic response of the treatment with OX86 mIgG2a is clearer from the individual animal tumor growth graphs (Figure 26B, Figure 27B, Figure 28B and Figure 29B). A dose response was not observed in mice treated with OX86 mIgG2a based on TGI (Table 9-6, Table 9-7, Table 9-8 and Table 9-9; Figure 26A, Figure 27A, Figure 28A and Figure 29A) or with respect to increasing the number of animals exhibiting complete responses (Table 9-6, Table 9-7, Table 9-8 and Table 9-9).

Table 9-6 Treatment Groups, Percent Tumor Growth Inhibition on Day 22, and number of Complete Responders in CT26 Syngeneic Model

Group ^a	Test/Control Antibody	Dose ^b (mg/kg)	% TGI ^c	Number of Complete Responders out of 12 mice ^d
1	None	NA	NA	0
2	OX86 mIgG2a	10	67	8
3	OX86 mIgG2a	3	67	6
4	OX86 mIgG2a	10	75	8
5	OX86 mIgG2a	0.3	65	8
6	OX86 mIgG2a	0.1	70	8

IP = intraperitoneal; NA = not applicable; TGI = tumor growth inhibition; V volume.

^a n = 12.

^b All animals received 200 µL of test antibody IP on Days 13 and 16.

^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of control group)] x 100.

^d Number of animals in a group with a tumor volume measurement recorded as zero at the end of the study.

Table 9-7 Treatment Groups, Percent Tumor Growth Inhibition on Day 26, and number of Complete Responders in CT26 Syngeneic Model

Group ^a	Test/Control Antibody	Dose ^b (mg/kg)	% TGI ^c	Number of Complete Responders out of 10 mice ^d
1	None	NA	NA	1

Group ^a	Test/Control Antibody	Dose ^b (mg/kg)	% TGI ^c	Number of Complete Responders out of 10 mice ^d
2	Negative control (OX86 mIgG1 D265A)	2.5	NA	1
3	OX86 mIgG2a	2.5	94	7
4	OX86 mIgG2a	1.0	97	8
5	OX86 mIgG2a	0.25	95	6
6	OX86 mIgG2a	0.1	92	7

IP = intraperitoneal; NA = not applicable; TGI = tumor growth inhibition; V volume.

^a n = 10.

^b All animals received 200 µL of test antibody IP on Days 9 and 12.

^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of control group)] x 100.

^d Number of animals in a group with a tumor volume measurement recorded as zero at the end of the study.

Table 9-8 Treatment Groups, Percent Tumor Growth Inhibition on Day 22, and Number of Complete Responders in MCA205 Syngeneic Model

Group ^a	Test/Control Antibody	Dose ^b (mg/kg)	% TGI ^c	Number of Complete Responders out of 14 mice ^d
1	None	NA	NA	0
2	Isotype control (mixture)	20	NA	0
3	OX86 mIgG2a	20	65	0
4	OX86 mIgG2a	10	76	4
5	OX86 mIgG2a	5	70	2

Table 9-9 Treatment Groups, Percent Tumor Growth Inhibition on Day 25, and number of Complete Responders in 4T1 Syngeneic Model

Group ^a	Test/Control Antibody	Dose ^b (mg/kg)	% TGI ^c	Number of Complete Responders out of 12 mice ^d
1	None	NA	NA	0
2	Isotype control (mixture)	70	NA	0
3	OX86 mIgG2a	10	5	0
4	OX86 mIgG2a	5	34	2
5	OX86 mIgG2a	2.5	37	0
6	OX86 mIgG2a	1.0	22	0
7	OX86 mIgG2a	0.25	ND	0

IP = intraperitoneal; NA = not applicable; TGI = tumor growth inhibition; V volume; ND = not determined.

^a n = 12.

^b All animals received 200 µL of test antibody IP on Days 13, 16, 20 and 23.

^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of control group)] x 100.

^d Number of animals in a group with a tumor volume measurement recorded as zero at the end of the study.

9-5 Conclusions

[0379] Single-agent treatment of tumor-bearing mice with OX86 mIgG2a results in anti-tumor activity that significantly reduces growth of multiple tumors as compared to untreated, negative control, and/or isotype control treated groups.

EXAMPLE 10: Characterization of the Epitope of OX40mAb24

[0380] This example examines the epitope of OX40mAb24 using a series of human/mouse OX40 chimeric variants.

10.1 Materials used in this study, and their source, are listed in Table 10-1.

Table 10-1 Materials

Item	Source
Anti-human IgG-Alexa480	Invitrogen (Carlsbad, CA)
Anti-sheep IgG-Alexa-480	Invitrogen (Carlsbad, CA)
Anti-goat IgG-Alexa480	Invitrogen (Carlsbad, CA)
FreeStyle 293F cells (HEK293F cells)	Invitrogen (Carlsbad, CA)
Goat anti-mouse OX40 polyclonal antibody	R&D Systems (Minneapolis, MN)
LSRII flow cytometer	BD Biosciences (San Jose, CA)
Phosphate buffered saline	Invitrogen (Carlsbad, CA)
Sheep anti-human OX40 polyclonal antibody	R&D Systems (Minneapolis, MN)
293fectin transfection reagent	Invitrogen (Carlsbad, CA)

10.2 Generation of Human/mouse Chimeric Variants

[0381] OX40mAb24 binds specifically to human OX40 (SEQ ID NO: 91) and does not recognize mouse OX40 (SEQ ID NO: 92), despite sharing 60% identity in their amino acid (aa) sequence (Figure 30). Human/mouse chimeric OX40 variants were engineered by swapping portions of the extracellular domain between human and mouse OX40. The cDNA constructs of human and mouse OX40 were used as templates in overlapping extension PCR to construct chimeric variants with a transmembrane domain for cell-surface expression of the recombinant proteins. OX40 is an approximately 45 kDa protein with three complete cysteine-rich domains (CRDs) and one truncated cysteine-rich domain. The structure is characteristic of the TNFR superfamily.

[0382] Thirteen chimeric knock-out (KO/loss-of-function) variants were constructed by replacing the following residues of the extracellular domain of human OX40 with the corresponding mouse OX40 aa or Alanine (Figure 31):

- •CRD1 (human OX40 aa 29 to 65 replaced with the mouse counterparts);

- •CRD2 (human OX40 aa 66 to 107 replaced with the mouse counterparts);
- •CRD3 (human OX40 aa 108 to 146 replaced with the mouse counterparts);
- •CRD4+linker (human OX40 aa 147 to 214 replaced with the mouse counterparts);
- •CRD3+4 (human OX40 aa 108 to 167 replaced with its mouse counterparts);
- •A¹¹¹ (human OX40 aa alanine 111 replaced with its mouse counterpart proline);
- •L¹¹⁶ (human OX40 aa leucine 116 replaced with its mouse counterpart glutamine);
- •P¹²¹ (human OX40 aa proline 121 replaced with its mouse counterpart leucine);
- •A¹²⁶ (human OX40 aa alanine 126 replaced with its mouse counterpart valine);
- •D¹³⁷ (human OX40 aa aspartic acid 137 replaced with its mouse counterpart asparagine);
- •A¹¹¹P¹²¹D¹³⁷ (human OX40 aa Ala111, Pro121 and Asp137 replaced with the mouse counterparts);
- •L¹¹⁶A¹²⁶ (human OX40 aa Leu116 and Ala126 replaced with the mouse counterparts);
- and
- •D¹¹⁷S¹¹⁸ (human OX40 aa Asp117 and Ser118 replaced with Ala mutations).

[0383] In addition, five knock-in (KI/gain-of-function) variants were constructed by grafting the following residues of human OX40 into mouse OX40 using the same method as described above for the KO constructs (Figure 31):

- CRD3 (human OX40 aa 108 to 146 grafted into the corresponding mouse regions);
- A¹¹¹ (human OX40 aa alanine 111 grafted into the corresponding mouse position);
- L¹¹⁶ (human OX40 aa leucine 116 grafted into the corresponding mouse position);
- A¹²⁶ (human OX40 aa alanine 126 grafted into the corresponding mouse position);
- L¹¹⁶A¹²⁶ (human OX40 aa leucine 116 and alanine 126 grafted into the corresponding mouse positions).

[0384] The resulting chimeric DNAs were closed into a mammalian expression vector pEBNA for transient mammalian expression. 293F cells were seeded at a density of 0.7×10^6 cells/mL one day prior to transfection. Three and a half micro grams of each expression vector were transfected into 5 mL of HEK293 F cells using 5 μ L of 293fectin transfection reagent following the manufacturer's instructions.

10.3 Characterization of Binding of OX40mAb24 to Chimeric OX40 Variants

[0385] Forty-eight hours after transfection, HEK293F cells were incubated with 1 $\mu\text{g/mL}$ of OX40mAb24 for 1 hour on ice in PBS. To detect bound OX40mAb24 by flow cytometry, the cells were washed 3-times with cold PBS, incubated with 1 $\mu\text{g/mL}$ of Alexa480-conjugated anti-human IgG antibody for 1 hr on ice, and then analyzed using the LSRII flow cytometer.

[0386] The expression levels of all chimeric OX40 variants were also monitored by flow cytometry; the cells were incubated with a mixture of sheep anti-human OX40 and goat anti-mouse OX40 polyclonal antibodies at 1 $\mu\text{g/mL}$ in PBS for 1 hour on ice. Cells were washed 3-times with cold PBS, and then incubated with a mixture of Alexa480-conjugated anti-goat and anti-sheep IgG polyclonal antibodies. After washing 3 times with cold PBS, cells were analyzed with the LSRII flow cytometer.

10.4 Results

[0387] The epitope of OX40mAb24 was characterized using chimeric human/mouse variants. Human OX40 and mouse OX40 share 60% identity in the aa sequence (Figure 30); yet, OX40mAb24 binds specifically to human OX40 and does not recognize mouse OX40. This specificity was employed to identify the epitope of OX40mAb24. Eighteen chimeric variants were constructed by swapping in or out various domains of the extracellular domain of mouse OX40 into human OX40 (KO/loss-of-function variants) or of human OX40 into mouse OX40 (KI/gain-of-function variants) (Figure 31). All variants encoded a transmembrane domain for cell-surface expression of the chimeric protein. The binding characteristics of OX40mAb24 to these variants were analyzed by flow cytometry.

[0388] The epitope of OX40mAb24 was mapped to the CRD3 domain of human OX40 by swapping individual domains between human and mouse OX40s. The binding of OX40mAb24 to human OX40 was abolished when replacing human CRD3 domain with the mouse counterpart (KO_CRD3 and KO_CRD3-4) (Figure 32A-C). Replacement of the other human CRD domains with mouse regions did not affect the binding of OX40mAb24 (KO_CRD1, KO_CRD2, and KO_CRD4+linker). Furthermore, grafting the human CRD3 domain into mouse OX40 led to the binding of OX40mAb24 (KI_CRD3). All variants were expressed as monitored by anti-human and mouse OX40s polyclonal antibodies (Figure 32A-C). The parental mAb of OX40mAb24,

9B12, was also characterized in the binding study. 9B12 shows the same binding profiles to these variants as OX40mAb24, suggesting both IgGs recognize the same epitope of the CRD3 domain.

[0389] Furthermore, critical epitope residues Leu¹¹⁶ and Ala¹²⁶ were identified in the CRD3 domain by mutating the amino acids that are different between human and mouse OX40s. Five amino acids, including Ala¹¹¹, Leu¹¹⁶, Pro¹²¹, Ala¹²⁶, and Asp¹³⁷ (Figure 30) in the human CRD3 domain, were mutated to corresponding mouse residues or Ala as individual amino acids or different combinations (Figure 31). The binding of OX40mAb24 was abolished when replacing human residues Leu¹¹⁶ and Ala¹²⁶ with the mouse counterparts (KO_L¹¹⁶+A¹²⁶). Furthermore, the KI/gain-of-function variants confirmed the importance of these two critical residues. Grafting Leu¹¹⁶, Ala¹²⁶, or the combination into mouse OX40 led to the binding of OX40mAb24.

10.5 Conclusions

[0390] The epitope OX40mAb24 was mapped to the CRD3 domain of human OX40 using human/mouse chimeric variants, with critical residues Leu¹¹⁶ and Ala¹²⁶. The binding profiles to all variants between OX40mAb24 and its parental 9B12 are identical, indicating they recognize the same epitope on human OX40.

EXAMPLE 11: Pharmacodynamic Activity of the Rat/Mouse Anti-Mouse OX40 IgG2a Chimera Antibody Clone OX86 in Naïve Mice and in Mice Bearing Syngeneic Tumors

[0391] OX86 is a rat anti-mOX40 IgG1 antibody that specifically binds to and triggers signaling of MOX40 (al-Shamkhani *et al*, 1996), and has anti-tumor activity in immunocompetent mouse models of cancer (Weinberg *et al*, 2000). To more fully study the effects of OX40 agonism using a mouse surrogate antibody with functional properties similar to OX40mAb24, this example utilizes the rat/mouse anti-mOX40 IgG2a chimera antibody OX86 described in Example 9. However, it is possible to draw some parallels between the species, for example mIgG2a and human IgG1 are often considered equivalent functionally as both isotypes are able to bind multiple Fcγ receptors, are capable of high affinity Fcγ receptor binding and are able to trigger ADCC and bind to human C1q, a prerequisite for complement-dependent cytotoxicity (CDC) by the classical complement pathway (Stewart *et al*. 2014; Dall'Acqua *et al*, 2006). OX86 mIgG2a binds to mouse OX40 (see Example 9) and was used as a surrogate mouse OX40 agonist antibody for OX40mAb24.

[0392] In this example, the ability of OX86 mIgG2a to induce T cells in naïve and tumor bearing mice, and to enter the cell cycle and proliferate. Further, K167 and ICOS were evaluated as a

potential biomarker of OX40 agonist activity, was investigated. T-cell proliferation and anti-tumor activity was determined in two syngeneic mouse models of cancer. Moreover, finally, the role of activating (Fc γ receptors I, III and IV) and/or inhibitory (Cf γ receptor IIb) Fc γ receptors play *in vivo* activity of OX86 mIgG2a was determined.

11.1 Materials and Methods

11.1.1 Animal Receipt and Identification

[0393] Wild-type Fcgr2b^{-/-} or Fcer1g^{-/-} BALB /c and Fcgr2b^{-/-} or Fcer1g^{-/-} C57BL/6 mice of 6-8 weeks of age were received at MedImmune from Harlan Laboratories, Inc. (Indianapolis, IN or Charles River Laboratories (UK)) and allowed to acclimatize for ≤ 15 days prior to study start. Thereafter, mice were implanted with microchip transponders to identify individual mice.

11.1.2 Housing

[0394] The animals were humanely treated and housed according to Institutional Animal Care and Use Committee (USA) and Home Office (UK) approved protocols in the Laboratory Animal Resources facility (USA) and Biological Sciences Unit (UK) at MedImmune, an Association for Animal Accreditation of Laboratory Animal Care and United States Department of Agriculture-licensed facility. The animals were kept in sterile micro-isolator units, provided with sterile bedding and food, and acidified drinking water (USA) or tap water (UK) ad libitum. Environmental conditions were standardized (room temperature: 20°C $\pm$ 1°C (USA) or 21°C $\pm$ 1°C (UK); relative humidity: 50% $\pm$ 10% (USA) or 55 $\pm$ 10% (UK); 12-hour light-dark cycle). The animals were monitored daily for adverse clinical signs and bi-weekly for body weight. If hind limb paralysis, respiratory distress, 20% body weight loss, or tumor volume greater than 2000 mm³ were noted, the animals were immediately sacrificed humanely by cervical dislocation or asphyxiation with CO₂.

11.1.3 Establishment of Syngeneic Tumors

[0395] The CT26 cell line (mouse colon carcinoma) was obtained from ATCC, Manassas, VA, and the cell line MCA 205 (chemically-induced mouse soft tissue sarcoma) was obtained from the Providence Cancer Center, Portland, OR. Both cell lines were maintained in RPMI 1640 medium +10% FBS at 37°C, 5% CO₂.

[0396] Allografts were established by subcutaneous (SC) injection of 5.0×10^5 CT26 cells, resuspended in 0.1 mL of PBS, into the right flank of 7- to 9-week-old wild-type, Fcgr2b^{-/-} or Fcer1g^{-/-} BALB/c mice. Allografts were also established by SC injection of 2.5×10^5 MCA205 cells, resuspended in 0.1 mL of PBS, into the right flank of 7- to 9-week-old wild-type, Fcgr2b^{-/-} or Fcer1g^{-/-} C57BL/6 mice.

11.1.4 Randomization, Group Designation and Dose Levels

[0397] Wild-type (n=70), Fcgr2b^{-/-} (n=36) or Fcer1g^{-/-} (n=36) BALB/c and Fcgr2b^{-/-} (n=36) or Fcer1g^{-/-} C57BL/6 female mice (n=36) were used in this study. All mice were randomly assigned into treatment groups. Group designations, number of animals, dose levels and dose schedule are presented in Table 11-1, Table 11-2 and Table 11-3. All test articles and control articles were administered intraperitoneally (IP). There were no animal substitutions.

[0398] Animals from each group were sacrificed when tumor volumes reached approximately 2000 mm³ or when tumors became ulcerated or necrotic.

Table 11-1 Group Designation and Dose Levels of Naïve Balb/c Mice

Group	Number of animals (M/F)	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1, 9	7 (F), 7 (F)	Saline	1	NA	IP
3, 11	7 (F), 7 (F)	Isotype control (NIP228 IgG2a)	1	20	IP
4, 12	7 (F)	OX86 mIgG2a	1	20	IP
5, 13	7 (F), 7 (F)	OX86 mIgG2a	1	2	IP
6, 14	7 (F), 7 (F)	OX86 mIgG2a	1	0.2	IP

F = female; M = male; NA = not applicable; IP = intraperitoneal; ROA = route of administration; OX86 mIgG2a = mouse anti-mouse OX40 IgG2a monoclonal antibody variant of OX86

^a Dose volume: 0.2 mL for saline or volume adjusted to body weight; 10 mL/kg for all other groups.

Table 11-2 Group Designation and Dose Levels in the CT26 Syngeneic Model

Group	Number of animals (M/F)	Mouse Strain	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1	8 (F)	Balb/c Fcgr2b ^{-/-}	None	NA	NA	IP
2	8 (F)		Control (OX86 mIgG1 D265A)	4, 7	2.5	IP
3	8 (F)		OX86 mIgG2a	4, 7	2.5	IP
4	8 (F)	Balb/c Fcer1g ^{-/-}	None	NA	NA	IP
5	8 (F)		Control (OX86 mIgG1 D265A)	4, 7	2.5	IP
6	8 (F)		OX86 mIgG2a	4, 7	2.5	IP

F = female; M = male; NA = not applicable because the animals were not treated; IP = intraperitoneal; ROA = route of administration; OX86 mIgG2a = rat anti-OX40 light and heavy chain variable regions of clone OX86 with mouse IgG2a constant regions.

^a Dose volume: 0.2 mL.

Table 11-3 Group Designation and Dose Levels in the MCA205 Syngeneic Model

Group	Number of animals (M/F)	Mouse Strain	Treatment	Dose schedule (study day)	Dose level (mg/kg) ^a	ROA
1	8 (F)	C57BL/6 Fcgr2b ^{-/-}	None	NA	NA	IP
2	8 (F)		Control (OX86 mIgG1 D265A)	4, 7	7.5	IP
3	8 (F)		OX86 mIgG2a	4, 7	7.5	IP
4	8 (F)	C57BL/6 Fcgr1g ^{-/-}	None	NA	NA	IP
5	8 (F)		Control (OX86 mIgG1 D265A)	4, 7	7.5	IP
6	8 (F)		OX86 mIgG2a	4, 7	7.5	IP

F = female; M = male; NA = not applicable because the animals were not treated; IP = intraperitoneal; ROA = route of administration; OX86 mIgG2a = rat anti-OX40 light and heavy chain variable regions of clone OX86 with mouse IgG2a constant regions.

^a Dose volume: 0.2 mL.

11.1.5 Tumor Measurements

[0399] Tumors were measured by caliper twice weekly and tumor volumes were calculated using the following formula:

$$\text{tumor volume} = [\text{length (mm)} \times \text{width (mm)}^2] / 2$$

where length was defined as the larger side, and width as the smaller side perpendicular to the length.

[0400] Antitumor effects of each group were expressed as tumor growth inhibition (TGI), which was calculated as follows:

$$\% \text{ TGI} = (1 - [\text{mean tumor V of treatment group}] \div [\text{mean tumor V of control group}]) \times 100$$

11.1.6 Tissue Collection and Single Cell Isolation

11.1.6.1 Preparation of Mouse Blood Cells for Flow Cytometry Analysis

[0401] Red blood cell lysis (RBCL) buffer (2 mL) was added to blood (50 µL) and incubated for 5 min. Volumes obtained from in-life bleeds ranged from 20 µL to 50 µL. Terminal bleeds were 50 µL. RPMI +10% fetal bovine serum (FBS) (8 mL) was added to each sample. Cells in each sample were pelleted (300 x g, 5 min) and then resuspended in 0.3 mL Flow Buffer. Cell suspensions (200 µL) were added to each well of a 96-well round-bottomed plate for staining with fluorescent antibodies. Pooled group samples were used for unstained, the single antibody staining, isotype staining and the fluorescence-minus-one (FMO) antibody staining controls.

11.1.6.2 Mouse Draining Lymph Node and Spleen Isolation and Generation of Single Cell Suspensions for Flow Cytometry Analysis

[0402] Spleens were placed in 10 mL of RPMI + 1X Pen/Strep solution and passed through a 40 μ m cell strainer. Samples were pelleted (300 x g, 5 min) and then resuspended in 1 mL RBCL buffer for 3 min. RPMI + FBS 10% (9 mL) was added to each sample. Cell suspensions were pelleted (300 x g, 5 min) and resuspended in 1 mL of Flow Buffer. Cell suspensions (100 μ L) were added to each well of a 96-well round-bottomed plate for staining with fluorescent antibodies. Pooled group samples were used for unstained, the single antibody staining, isotype staining and FMO antibody staining controls.

11.1.6.3 Preparation of Single-cell Suspension of Mouse Tumor for Flow Cytometry Analysis

[0403] Tumors were aseptically excised from euthanized mice, being careful to avoid collecting connective tissue or skin, and placed in collagenase diluted with Hanks balanced salt solution in a 6-well dish. To increase the efficiency of the enzymatic digestion process, each tumor was minced with a scalpel or a small pair of sharp scissors into smaller pieces. Minced tumors were transferred into 15 mL conical tubes and placed on a shaking platform to incubate at 37°C for 20-30 minutes. Five mL of RPMI + 10% FBS was added to each sample to deactivate the collagenase and maintain viability of the immune cells. Samples were passed through a 70 μ m cell strainer and placed into 50 mL conical tubes. An aliquot of each sample was removed for counting. The remaining amount sample was pelleted in a centrifuge at 330 x g and resuspended in FACS buffer (PBS +2% FBS) at a concentration of 1×10^7 cells per mL.

11.1.7 Flow Cytometry Analysis

11.1.7.1 Analysis of Tissues from Non-tumor-bearing Mice

[0404] Single-cell suspensions of blood or spleen cells were pelleted (300 x g, 5 min), resuspended in 50 μ L of Fc Block solution (1:50), diluted in eBiosciences Flow Buffer and incubated for 10 min on ice. Fifty microliters of fluorescently labelled antibodies (2X stock solution) were added to each sample (final volume 100 μ L). Single antibody staining solutions (extracellular) were added at 1 μ L per well and cell/antibody mixtures were incubated for 30 min on ice. Cells were pelleted (300 x g, 5 min), washed twice (200 μ L Flow Buffer per well, 300 x g, 5 min), resuspended in 50 μ L Fix/Penn buffer (one part concentrate to three part diluent) and then incubated overnight at 4°C in the dark.

[0405] Treated cells were washed twice in 1X Permeabilization Buffer (diluted in water). Intracellular labelling antibodies were diluted into Permeabilization Buffer (100 μ L per well) and added to the cells, which were then incubated at room temperature in the dark for 30 minutes. Antibodies for single marker staining (intracellular) were added to cells at 1 μ L per well into 100 μ L Permeabilization Buffer and incubated for 30 min on ice in the dark. Cells were washed once in Permeabilization Buffer and resuspended in 3.7% formaldehyde solution (100 μ L) before being analyzed on a Canto II flow cytometer (BD Biosciences, San Jose, CA).

11.1.7.2 Analysis of Tissues from Tumor-bearing Mice

11.1.7.2.1 Cell surface staining

[0406] Each sample of pelleted cells was resuspended in FACS Buffer. Fluorescent antibody mixtures in FACS Buffer were added to appropriate wells and incubated for 20-30 minutes at 4°C in the dark. Cells were washed twice with FACS Buffer, resuspended in FACS Buffer and analyzed on a LSRII flow cytometer (BD Biosciences, San Jose, CA).

11.1.7.2.2 Intracellular staining (fixed and permeabilized cells)

[0407] Each sample of pelleted cells was resuspended in 50 μ L of a 1:500 dilution of a fluorescence fixable blue dye for live/non-viable cell discrimination, and incubated for 15 minutes at 4°C in the dark. Cells were washed once with FACS Buffer, resuspended in 30 μ L PBS +4 % normal mouse serum containing Fc Block, and incubated for 15 minutes at room temperature. Fluorescent antibody mixtures in FACS Buffer were added to appropriate wells, and incubated for 20-30 minutes at 4°C in the dark. Cells were washed twice with FACS Buffer, resuspended in 150 μ L freshly prepared FoxP3 Fix/Penn working solution and incubated at room temperature for 30 minutes in the dark. Cells were washed twice with 1X Permeabilization Buffer, resuspended in 100 μ L 1X Permeabilization Buffer containing antibodies to stain intracellular antigens, and incubated at room temperature for 30 minutes in the dark. Cells were washed once with 1x Permeabilization Buffer, resuspended in FACS Buffer and analyzed on a LSRII flow cytometer.

11.1.8 Statistical Methods

[0408] One-way ANOVA was used to determine mean tumor volume differences and mean percentage of Ki67+ or ICOS+ cells. In the event of a significant F test, a Dunnett's or Sidak's multiple comparison test was utilized (where appropriate). Where applicable, a log10 transformation was applied to mean values to account for heteroscedasticity. A p value < 0.05 was

considered significant. GraphPad Prism 6.0 was employed for linear regression analysis which generated the best fit line that best predicted Y from X, the level of statistical significance of the line and the goodness of fit (r^2) of the determined best fit line.

11.1.9 MATERIALS

[0409] Materials used in this study, and their source, are listed in Table 11-4 and Table 11-5.

Table 11-4 Materials

Item	Source
0.25% Trypsin-EDTA (1X)	Life Technologies, Carlsbad, CA
10 x Permeabilization Buffer	eBioscience, UK
3.7% Formaldehyde solution	Sigma-Aldrich, UK
40 and 70 μ m cell strainers	Corning Life Sciences, Tewksbury, MA
Collagenase type 3	Worthington Biochem Corp., Lakewood, NJ
Fc Block	eBioscience, UK
Fetal bovine serum, heat inactivated	Life Technologies, Carlsbad, CA
Fixation/Permeabilization Concentrate	eBioscience, UK
Fixation/Permeabilization Diluent	eBioscience, UK
Flow Buffer	eBioscience, UK
FoxP3 Fix/Perm Kit	eBioscience, UK
Hanks buffered salt solution	Life Technologies USA
Heat Inactivated Gamma Irradiated FBS for RBCL Buffer neutralization	SASC Biosciences, KS, USA
LSRII and Canto II Flow Cytometers	BD Biosciences, San Jose, CA
Normal mouse serum	Jackson Labs, Bar harbor, MA
Pen/Strep solution	Life Technologies UK
Permeabilization Buffer	eBiosciences, USA
Phosphate buffered saline, pH 7.2	Life Technologies, Carlsbad, CA
Red Blood Cell Lysis Buffer	Sigma, UK
Roswell Park Memorial Institute 1640 medium	Life Technologies, Carlsbad, CA

Table 11-5 Fluorescent Antibodies for Flow Cytometry Studies

Item	Source
APC conjugated Rat IgG2a anti-CD4	eBiosciences, UK
FITC conjugated mIgG2a anti-MHC2	eBiosciences USA
APC-H7 conjugated Rat IgG2a anti-CD8	BD Biosciences, UK
EFluor 480 conjugated Rat IgG2a anti-Ki67	eBioscience, UK
APC conjugated isotype control Rat IgG2a	eBioscience, UK
APC-H7 conjugated isotype control Rat IgG2a	BD Biosciences, UK
eFluor conjugated isotype control Rat IgG2a	eBioscience, UK
PE conjugated isotype control Rat IgG2b	eBioscience, UK
PECy7 conjugated rIgG2a Ki67	eBioscience, USA
BV605 conjugated rIgG2a anti CD4	Biolegend USA

Item	Source
BV711 conjugated rlgG2a anti CD8	Biolegend USA
Fixable Blue	Life Technologies, USA
PeCy7 conjugated Isotype control rlgG2a	Biolegend USA

11.2 RESULTS

- [0410]** Intraperitoneal treatment of naïve mice with the anti-OX40 antibody OX86 mouse IgG2a (OX86 mIgG2a) resulted in a significant, dose-dependent and transient increase in the percentage of Ki67+ CD4+ and ICOS+CD4+ T cells in the blood over time, compared to mice treated with control articles (Figure 33A and C). The largest percentage of Ki67+ CD4+ and ICOS+CD4+ T cells was detected on Day 10. A significant increase in the percentage of Ki67+CD4+ and ICOS+CD4+ T cells was measured in the spleens of mice on Day 10 following the administration of OX86 mIgG2a, compared to mice treated with control articles (Figure 33B and D). A statistically significant and strong correlation between the percentage of Ki67+CD4+ T cells and ICOS+CD4+ T cells in the blood and spleen on Day 10 was identified (Figures 34A and 34B).
- [0411]** Treatment of naïve mice with the OX86 mIgG2a also resulted in a significant, transient increase in the percentage of Ki67+CD8+ T cells in the blood over time, when compared to mice treated with control articles (Figure 35A). Increases in the percentage of ICOS+CD8+ T cells in the blood over time (Figure 35C), and the percentage of Ki67+CD8+ T cells in the spleen on Day 10 (Figure 35B), were detected following treatment with OX86 mIgG2a, but were not statistically significant when compared to treatment with control articles. Treatment of naïve mice with OX86 mIgG2a did induce a significant, dose-dependent increase in the percentage of ICOS+CD8+ T cells in the spleen on Day 10, compared to mice treated with control articles (Figure 35D). Moderate but significant correlations between the percentage of Ki67+CD8+ T cells and ICOS+CD8+ T cells in the blood and spleen was determined (Figures 36A and 36B).
- [0412]** Single-agent treatment of tumor-bearing wild-type mice with OX86 mIgG2a results in an antitumor activity that reduces growth of two histologically different tumors as compared to untreated and isotype control treated groups (See Example 9). Treatment with OX86 mIgG2a resulted in significantly reduced growth of MCA205 tumors on Day 20 (Figure 38A, Table 11-7) when compared to treatment with control articles in C57BL/6 mice that were genetically engineered to lack expression of the inhibitory Fcγ receptor IIB (Fcgr2b^{-/-} mice). Identical treatment of mice with CT26 tumors resulted in a reduced growth of tumors that did not reach

statistical significance on Day 18 (Figure 37A, Table 11-6) when compared to treatment with control articles in Fcgr2b^{-/-} BALB/c mice. No inhibition of growth, by any agent, was observed in tumor bearing Balb/c and C57BL/6 mice genetically engineered to lack expression of the activating Fcγ receptors (Fcer1g^{-/-} mice; Figure 37C; Figure 38C). Mixed response is often observed in syngeneic models. However, the anti-tumor response following treatment with OX86 mIgG2a in the two different strains of Fcγ receptor knock-out mice can also be discerned from the individual animal tumor growth graphs (Figure 37B and D; Figure 38B and D); treatment with OX86 mIgG2a resulted in the inhibition of CT26 and MCA205 tumor growth and in some cases induced complete responses in Fcgr2b^{-/-} mice (Figure 37B; Figure 38B).

Table 11-6 Treatment Groups and Percent TGI and Number of Complete Responders in CT26 Syngeneic Mouse Model

Group ^a	Test/Control Article	Dose ^b (mg/kg)	Mouse Strain	% TGI ^c	Number of Complete Responders out of 8 mice ^d
1	None	NA	Balb/c Fcgr2b ^{-/-}	NA	0
2	Isotype control	2.5 mg/kg		NA	0
3	OX86 mIgG2a	2.5 mg/kg		15	0
4	None	NA	Balb/c Fcer1g ^{-/-}	NA	0
5	Isotype control	2.5 mg/kg		NA	0
6	OX86 mIgG2a	2.5 mg/kg		< 0	0

TGI = tumor growth inhibition; NA = not applicable; IP = intraperitoneal; V = volume

^a Number of animals per group: 8.

^b All animals received 200 μL of test article IP on Days 4 and 7.

^c % TGI = [1 - (mean tumor V of treatment group) ÷ (mean tumor V of control group)] × 100; calculated on Day 18 for Fcgr2b^{-/-} mice and Day 16 for Fcer1g^{-/-} mice

^d Number of animals in a group with a tumor volume measurement recorded as zero at the end of the study.

Table 11-7 Treatment Groups and Percent TGI on Day 20 and Number of Complete Responders in MCA205 Syngeneic Mouse Model

Group ^a	Test/Control Article	Dose ^b (mg/kg)	Mouse Strain	% TGI ^c	Number of Complete Responders out of 8 mice ^d
1	None	NA	C57BL/6 Fcgr2b ^{-/-}	NA	0
2	Isotype control	7.5 mg/kg		NA	0
3	OX86 mIgG2a	7.5 mg/kg		95	8
4	None	NA	C57BL/6 Fcer1g ^{-/-}	NA	0
5	Isotype control	7.5 mg/kg		NA	0

Group ^a	Test/Control Article	Dose ^b (mg/kg)	Mouse Strain	% TGI ^c	Number of Complete Responders out of 8 mice ^d
6	OX86 mIgG2a	7.5 mg/kg		35	1

TGI = tumor growth inhibition; NA = not applicable; IP = intraperitoneal; V = volume

^a Number of animals per group: 8.

^b All animals received 200 μ L of test article IP on Days 4 and 7.

^c % TGI = [1 - (mean tumor V of treatment group) $\div$ (mean tumor V of control group)] $\times$ 100

^d Number of animals in a group with a tumor volume measurement recorded as zero at the end of the study.

[0413] In parallel pharmacodynamics studies, treatment with OX86 mIgG2a compared to control articles caused a significant increase in the percentage of Ki67+CD4+ T cells in the spleen (Figure 39A) and Ki67+CD8+ T cells in the peripheral blood and the spleen, but not in the tumor (Figure 40A), of CT26 tumor bearing Fcgr2b^{-/-} Balb/c mice. No increase in Ki67+ CD4+ or CD8+ T cells was detected in the blood, spleen or tumor of CT26 tumor bearing Fcer1g^{-/-} Balb/c mice following treatment with OX86 mIgG2a, as compared to control articles (Figure 39B; Figure 40B).

[0414] Additionally, in parallel studies, treatment with OX86 mIgG2a compared to control articles resulted in a significant increase in the percentage of Ki67+CD4+ T cells in the draining lymph node, spleen and tumor (Figure 41A) and Ki67+CD8+ T cells in the draining lymph node and the spleen (Figure 42A) of MCA205 tumor bearing Fcgr2b^{-/-} C57BL/6 mice. Increases in the percentage of Ki67+CD8+ T cells in the tumor of MCA205 tumor bearing Fcgr2b^{-/-} C57BL/6 mice were not statistically significant (Figure 42A). Treatment with OX86 mIgG2a as compared to control articles also induced a significant increase in the percentage of Ki67+CD4+ T cells in the draining lymph node and spleen of MCA205 tumor bearing Fcer1g^{-/-} C57BL/6 mice (Figure 41B). A significant increase in the percentage of Ki67+CD8+ T cells in the spleen of these mice was also observed (Figure 42B). No significant increases in the percentage of Ki67+CD4+ T cells in the tumor (Figure 42B) or Ki67+CD8+ T cells in the draining lymph node or tumor of MCA205 tumor bearing Fcgr2b^{-/-} C57BL/6 mice were detected (Figure 42B).

11.3 CONCLUSIONS

[0415] A single dose of OX86 mIgG2a in naïve mice induced a transient increase in T-cell activation and proliferation as measured by increased expression of ICOS and Ki67, respectively. A significant linear correlation of the percentage of ICOS and Ki67 on CD4+ and CD8+ T cells

was found. Antitumor activity of OX86 mIgG2a, measured as inhibition of the growth of CT26 and MCA205 tumors, was dependent on the expression of activating Fc γ receptors I, III and IV, but not the inhibitory Fc γ receptor IIB. Increases in peripheral blood, draining lymph node and/or spleen T-cell proliferation (Ki67) were observed in parallel pharmacodynamic experiments in tumor-bearing mice that expressed the activating Fc γ receptors; some T-cell proliferation was observed in mice that expressed only the inhibitory Fc γ receptor. Together with the pharmacodynamic results from naïve mice and while not wishing to be bound by theory, a potential mechanism for OX86 mIgG2a antitumor activity is increased peripheral and intratumoral T-cell proliferation (Ki67). Moreover, OX86 mIgG2a *in vivo* antitumor activity occurs upon expression of activating Fc γ receptors.

EXAMPLE 12: T cell Pharmacodynamic Changes in Response to OX40mAb24 Therapy in Immunocompromised Mice Engrafted with Human Hematopoietic Stem Cells

[0416] This example investigates whether OX40mAb24 can activate and expand human CD4+ and CD8+ memory T cells or reduce human Tregs in immunocompromised mice with a reconstituted human immune system. OX40mAb24 was tested in an *in vivo* mouse model system reconstituted with human immune cells to determine if the drug has immunomodulatory effects on human T cells.

12.1 MATERIALS AND METHODS

12.1.1 Test Animals

[0417] Nod.Cg-*Prkdc*^{scid}*Il2rg*^{tm1W^y}/SzJ (NSG) Mice (n=14 ages 23-26 weeks) were obtained from the Jackson Laboratory. The animals were humanely treated and housed according to Institutional Animal Care and Use Committee approved protocols in the Laboratory Animal Resources facility at MedImmune, an Association for Animal Accreditation of Laboratory Animal Care and United States Department of Agriculture-licensed facility. The animals were kept in sterile micro-isolator units, provided with sterile bedding and food, and acidified drinking water ad libitum. Environmental conditions were standardized (room temperature: 20°C $\pm$ 1°C; relative humidity: 50% $\pm$ 10%; 12-hour light-dark cycle). The animals were monitored daily for adverse clinical signs during the course of the study.

12.1.2 Pharmacodynamics of OX40mAb24 in HSC Engrafted (Humanized) Mice

12.1.2.1 Establishment of Human HSC Engrafted Mice

[0418] Human CD34+ HSC engrafted mice were purchased from the Jackson Laboratory. Mice were generated as follows: pooled human CD34+ cells from multiple umbilical cord blood donors were isolated and injected intravenously into the tail vein of Nod.Cg-*Prkdc*^{scid}*112rg*^{tm1Wy}/SzJ (NSG) mice. At 12 weeks after engraftment, mouse peripheral blood was sampled and lysed with Pharm Lyse red blood cell lysis buffer and then analyzed by flow cytometry for human CD45, CD19 and CD3 positive cells to determine the degree of human immune cell engraftment in mice. Mice successfully engrafted with 25% or more CD45+ human immune cells of total viable blood cells were subsequently shipped to MedImmune.

12.1.2.2 Antigen Challenge and OX40mAb24 treatment of Human HSC Engrafted Mice

[0419] The sustained engraftment of human immune cells into NSG mice was confirmed by flow cytometry at 22 weeks post injection of human CD34 cells, and at 23 weeks mice were placed into 3 groups that were left untouched or received treatment (Table 12-1). There were no statistically significant differences in the mean percentage of human CD45+ cell engraftment between eventual treatment groups at week 22 prior to the start of the experiment. Group 1 consisted of 4 mice and did not receive subcutaneous (SC) keyhole limpet hemocyanin (KLH) immunization or antibody administration. Group 2 consisted of 5 mice and received 300 µg/50 µL of KLH and 50 µL of complete Freund's adjuvant (CFA) at two independent injection SC sites. This group of mice also received 2 mg/kg of hIgG1 isotype control antibody, administered intraperitoneally (IP), one day after KLH immunization. Group 3 consisted of 5 mice and received KLH/CFA SC immunization as described above. In addition, mice were also administered one day later a single IP dose of 2 mg/kg OX40mAb24. On the day of KLH/CFA SC immunization, prior to immunization (pre-treatment), and seven days later (post-treatment), whole blood was obtained through retro-orbital bleed and cells were immunophenotyped and counted by flow cytometry.

Table 12-1 Experimental Groups, Human CD45+ Cell Engraftment, and Treatment

Group	Mouse Number	Human CD45+ Cells as a Percentage of Total Viable Human Plus Mouse CD45 Cells ^a - Week 22 post HSC engraftment	Treatment ^b - Week 23 post HSC engraftment
1	1-1 4575	39.5	None
1	1-2 4576	68.3	None

Group	Mouse Number	Human CD45+ Cells as a Percentage of Total Viable Human Plus Mouse CD45 Cells ^a - Week 22 post HSC engraftment	Treatment ^b - Week 23 post HSC engraftment
1	1-3 4577	20.9	None
1	1-4 4578	23.8	None
2	2-1 4579	29.6	KLH immunization + NIP228 huIgG1
2	2-2 4580	21.7	KLH immunization + NIP228 huIgG1
2	2-3 4581	27.8	KLH immunization + NIP228 huIgG1
2	2-4 4582	50.3	KLH immunization + NIP228 huIgG1
2	2-5 4583	32.5	KLH immunization + NIP228 huIgG1
3	3-1 4584	15.4	KLH immunization + OX40mAb24
3	3-2 4585	44.3	KLH immunization + OX40mAb24
3	3-3 4586	20.3	KLH immunization + OX40mAb24
3	3-4 4587	16.3	KLH immunization + OX40mAb24
3	3-5 4588	25.6	KLH immunization + OX40mAb24

Hu = human; KLH = keyhole limpet hemocyanin; HSC = hematopoietic stem cells.

^a Human CD45+ cells determined by flow cytometry; ^b KLH immunization, keyhole limpet hemocyanin plus complete Freund's adjuvant.

12.1.2.3 Immune Cell Analysis by Flow Cytometry

[0420] Cells were immunophenotyped by flow cytometry using a whole blood antibody binding protocol. Briefly, EDTA anti-coagulated whole blood at a constant volume was added to wells of a deep well plate and a T cell staining master mix added to cells to bind cell surface antigens. After washes in FACS buffer (PBS, pH 7.2 plus 2% heat-inactivated newborn calf serum), red blood cells (RBC) were lysed using 1X RBC lysis buffer, and cells fixed and permeabilized using EBiosciences Fix and Perm kit according to the manufacturer's protocol. Subsequently, anti-FoxP3 and anti-Ki67 mAbs were bound to cells. Cells were washed using 1X fix and perm buffer, re-suspended in FACS buffer and events analyzed using an LSRII flow cytometer. Raw flow cytometry standard (FCS) data was analyzed using FlowJo software, and cell populations identified after live/dead cell discrimination and removal of doublets and cell debris. After gating for CD4+ and CD8+ T cell populations, memory T cells were determined by CD45RA and CCR7

expression profile, with naïve T cells defined as CD45RA+/CCR7+, effector T cells (Teff) as CD45RA+/CCR7-, effector memory T cells (Tem) as CD45RA-/CCR7-, and central memory T cells (Tcm) as CD45RA-/CCR7+. Tregs were defined as CD4+/FoxP3+ cells.

12.1.2.4 Statistical Methods

[0421] GraphPad Prism software for Windows (version 6.03) was used for graphing and statistical analysis of data.

[0422] One-way ANOVA multiple comparisons test with Turkey's post-test analysis was used to compare differences between experimental group means. In the event of a significant F test, a Sidak's multiple comparisons test was utilized. A p value of less than 0.05 was considered significant.

12.1.3 Materials

[0423] Materials used in this study, and their source, are listed in Table 12-2.

Table 12-2 Materials

Item	Source
Anti-human CCR7 APC clone 150503	R&D Systems, Minneapolis, MN
Anti-human CD14 APC clone M5E2	Biolegend, San Diego, CA
Anti-human CD16 PE clone 1243	Biolegend, San Diego, CA
Anti-human CD25 FITC clone M-A251	Biolegend, San Diego, CA
Anti-human CD3 FITC clone UCHT1	Biolegend, San Diego, CA
Anti-human CD4 BV605 clone RPA-T4	Becton Dickinson, San Jose, CA
Anti-human CD56 FITC clone HCD56	Biolegend, San Diego, CA
Anti-human CD8 PE-CF594 clone RPA-T8	Becton Dickinson, San Jose, CA
Anti-human CD19 APC clone HIB19	Biolegend, San Diego, CA
Anti-human CD19 PE-CF594 clone HIB19	Becton Dickinson, San Jose, CA
Anti-human CD45 PE clone HI30	Biolegend, San Diego, CA
Anti-human CD45 PE clone HI30	Biolegend, San Diego, CA
Anti-human FoxP3 PE clone PHC101	eBioscience, San Diego, CA
Anti-human HLA-DR BV421 clone 3G8	Biolegend, San Diego, CA
Anti-human Ki67 FITC clone B56	Becton Dickinson, San Jose, CA
Anti-mouse CD16/32 PE-Cy7	Biolegend, San Diego, CA
Anti-mouse CD45 PE-Cy7 clone 30F11	Biolegend, San Diego, CA
Complete Freund's adjuvant (CFA) Difco™	Becton Dickinson, San Jose, CA
EBiosciences Fix and Perm Kit	EBioscience, San Diego, CA
FlowJo Software	FlowJo, Ashland, OR
FoxP3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent	eBioscience, San Diego, CA
Graphpad Prism software, v 6.03	Graphpad Software, San Diego, CA
Heat-inactivated newborn calf serum	Life Technologies, Frederick, MD

Item	Source
Keyhole limpet hemocyanin	Thermo Fisher Scientific, Waltham, MA
Live/Dead® Fixable Blue Dye	Life Technologies, Frederick, MD
LSR II flow cytometer	BD Biosciences, San Jose, CA
Mouse IgG2a APC isotype control	eBioscience, San Diego, CA
Mouse IgG2b BV421 isotype control	Biolegend, San Diego, CA
Mouse IgG1 FITC isotype control	Biolegend, San Diego, CA
Phosphate-buffered saline (PBS), pH 7.2	Life Technologies, Frederick, MD
Rat IgG1 PE-CF594 isotype control	Becton Dickinson, San Jose, CA
Rat IgG2a PE isotype control	eBioscience, San Diego, CA
Rat IgG2a PE-Cy7 isotype control	Biolegend, San Diego, CA

12.2 RESULTS

[0424] OX40mAb24 or huIgG1 control antibody was administered one day after immunization with KLH. OX40mAb24 resulted in a statistically significant reduction in the percentage of Treg in peripheral blood after six days relative to the group administered a huIgG1 isotype control antibody ($p = 0.019$, one-way ANOVA; Figures 43A and 43B). No differences in the percentages of Tregs between treatment groups were observed prior to immunization and antibody administration.

[0425] In addition to a significant decrease in the percentage of peripheral blood Tregs, there was a statistically significant increase in the CD4⁺ Tem:Treg ratio following treatment with OX40mAb24 relative to huIgG1 isotype matched control ($p = 0.042$), and trends towards significant increases for total CD4⁺:Treg ($p = 0.051$), CD4⁺ Teff:Treg ($p = 0.10$), and CD4⁺ Tem:Treg ($p = 0.069$) ratios (Figures 44A-D). Likewise, a trend towards significance was observed for an increase in the CD8⁺ Teff:Treg ratio ($p = 0.064$) in peripheral blood of mice treated with OX40mAb24 relative to that of mice treated with huIgG1 isotype control (Figures 45A-B).

[0426] CD25 (IL-2 receptor) is up-regulated on T cells following activation, and is considered a marker of recently activated T cells. Increases in the percentage of CD25 positive CD8⁺ T cells were observed for the OX40mAb24 treatment group relative to the huIgG1 treatment group for total CD8⁺ ($p = 0.038$) effector CD8⁺ ($p = 0.076$), and for effector memory CD8⁺ ($p = 0.040$) T cells (Figures 46A-C). Therefore, agonism of OX40 by OX40mAb24 activated CD8⁺ T cells relative to the control antibody.

12.3 CONCLUSIONS

[0427] In mice engrafted with human immune cells, treatment with OX40mAb24 after antigen immunization resulted in decreased peripheral Treg cells relative to mice receiving a human IgG1 control antibody. Likewise, the ratio of total, effector, and memory CD4+ T cells, and effector CD8+ T cells, relative to Treg cells were also increased in the peripheral blood of mice receiving OX40mAb24 compared with those receiving huIgG1 control antibody. Finally, the percentage of CD8+ T cells expressing CD25 on the cell surface was increased after OX40mAb24 treatment, indicating an OX40 induction of peripheral CD8+ T-cell activation.

TABLE OF SEQUENCES

SEQ ID NO	Description	Sequence
1	9B12 VL	DIQMTQTTSSLSASLGDRVTISCRASQDISNYLNWYQQKPDGTVKLLIYYTSKLHSGVPSRFSGSGSR TDYSLTITDLQEDATYFCQQGSALPWTFGQGTKVEIK
2	LCDR1	RASQDISNYLN
3	LCDR2	YTSKLHS
4	LCDR3	QQGSALPWT
5	9B12 VH	EVQLQESGPSLVKPSQTLSTCSVTGDSFTSGYWNWIRKFPGNRLEYMGYISYNGITYHNPSLKS RISITRDTSKNHYYLQLNSVTEDTATYFCARYRYDYDGGHAMDYWGQGLTVTVSS
6	HFW1	QVQLQESGPGPLVKPSQTLSTCAVYGGSF
7	HFW1-variant	QVQLQESGPGPLVKPSQTLSTCAVYGDSF
8	HCDR1	SGYWN
9	HFW2-XXX	WIRX ₃₉ HPGKGLEX ₄₇ X ₄₈ G; where X ₃₉ is Q or K, X ₄₇ is W or Y, and X ₄₈ is I or M
10	HFW2-variant	WIRQHPGKGLEWIG
11	HFW2-variant	WIRKHPGKGLEYMG
12	HFW2-variant	WIRKHPGKGLEWIG
13	HFW2-variant	WIRKHPGKGLEYIG
14	HCDR2	YISYNGITYHNPSLKS
15	HCDR2-variant	YISYNAITYHNPSLKS
16	HCDR2-variant	YISYSGITYHNPSLKS
17	HFW3-XXX	RITINX ₇₁ DTSKNQX ₇₈ SLQLNSVTPEDTAVYX ₉₁ CAR; where X ₇₁ is P or R, X ₇₈ is F or Y, and X ₉₁ is Y or F
18	HFW3-variant	RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR
19	HFW3-variant	RITINRDTSKNQYSLQLNSVTPEDTAVYFCAR
20	HFW3-variant	RITINRDTSKNQFSLQLNSVTPEDTAVYYCAR
21	HFW3-variant	RITINRDTSKNQFSLQLNSVTPEDTAVYFCAR
22	HFW3-variant	RITINRDTSKNQYSLQLNSVTPEDTAVYYCAR
23	HFW3-variant	RITINPDTSKNQYSLQLNSVTPEDTAVYFCAR
24	HFW3-variant	RITINPDTSKNQYSLQLNSVTPEDTAVYYCAR
25	HCDR3	YRYDYDGGHAMDY
26	HCDR3-variant	YKYDYDAGHAMDY
27	HCDR3-variant	YKYDYDGGHAMDY
28	HFW4	WGQGLTVTVSS
29	OX40mAb VL	DIQMTQSPSSLSASVGRVTITCRASQDISNYLNWYQQKPGKAPKLLIYYTSKLHSGVPSRFSGSGS GTDYTLTISSLPEDFATYYCQQGSALPWTFGQGTKVEIK

SEQ ID NO	Description	Sequence
30	OX40mAb light chain	DIQMTQSPSSLSASVGDRVTITCRASQDISNYLNWYQQKPGKAPKLLIYYTSLKHSQVPSRFSGSGS GTDYTLTISSLQPEDFATYYCQQGSALPWTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCL LNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSLSTLTLSKADYEKHKVYACEVTHQGL SSPVTKSFNRGEC
31	OX40mAb light chain DNA	GACATCCAGATGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGTGGGCGACAGAGTGACCAT CACCTGTGCGGGCCAGCCAGGACATCAGCAACTACCTGAACTGGTATCAGCAGAAGCCCGGCAA GGCCCCCAAGCTGCTGATCTACTACACCAGCAAGCTGCACAGCGGCGTGCCAGCAGATTGAG CGGCAGCGGCTCCGGCACCAGCTACACCCTGACCATCAGCAGCCTGCAGCCCGAGGACTTCGC CACCTACTACTGCCAGCAGGGCTCCGCCCTGCCCTGGACCTTTGGCCAGGGCACCAAGGTGGA AATCAAGCGTACGGTGGCTGACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAA TCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAATAAATTCTATCCAGAGAGGCCAAAGTACAGT GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCAGGAGAGTGTACAGAGCAGGACAGC AAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAACA CAAAGTCTACGCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAA CAGGGGAGAGTGT
32	OX40mAb VL-hu2	DIQMTQSPSSLSASVGDRVTITCRASQDISNYLNWYQQKPGKAVKLLIYYTSLKHSQVPSRFSGSGS RTDYTLTISSLQPEDFATYYCQQGSALPWTFGQGTKVEIK
33	OX40mAb5 VH	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKGLEWIGYISYNGITYHNPSLKSRI TINPDTSKNQFSLQLNSVTPEDTAVYYCARYRYDYDGGHAMDYWGQGLTVTVSS
34	OX40mAb5 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGCAGCACCCCGG CAAGGGCCTGGAATGGATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCCCGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAG CGTGACCCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
35	OX40mAb8 VH	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKGLEWIGYISYNGITYHNPSLKSRI TINRDTSKNQFSLQLNSVTPEDTAVYYCARYRYDYDGGHAMDYWGQGLTVTVSS
36	OX40mAb8VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATGGATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCC TGAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACA GCGTGACCCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAGATACGACTACGACGGCG GCCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
37	OX40mAb13 VH	QVQLQESGPGLVKPSQTLSTLCAVYGDSFSSGYWNWIRKHPGKGLEWIGYISYNGITYHNPSLKSRI TINRDTSKNQYSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
38	OX40mAb13 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCGG CAAGGGCCTGGAATACATGGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCCGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
39	OX40mAb14 VH	QVQLQESGPGLVKPSQTLSTLCAVYGDSFSSGYWNWIRKHPGKGLEWIGYISYNGITYHNPSLKSRI INRDTSKNQYSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
40	OX40mAb14 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCGG CAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCTG AAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAGC GTGACCCCCGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGG GTGACCCCCGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGG

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		CACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
41	OX40mAb15 VH	QVQLQESGPGLVKPSQTLSTCAVYGDSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INRDTSKNQFSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
42	OX40mAb15 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCGG CAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCTG AAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAGC GTGACCCCGGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGGC CACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
43	OX40mAb16 VH	QVQLQESGPGLVKPSQTLSTCAVYGDSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INRDTSKNQFSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
44	OX40mAb16 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCGG CAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCTG AAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAGC GTGACCCCGGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGGC CACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
45	OX40mAb17 VH	QVQLQESGPGLVKPSQTLSTCAVYGDSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INRDTSKNQFSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
46	OX40mAb VH17 DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCGG CAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCTG AAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAGC GTGACCCCGGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGGC CACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
47	OX40mAb18 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INPDTSKNQFSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
48	OX40mAb18 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCCCGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAG CGTGACCCCGGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
49	OX40mAb19 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INRDTSKNQFSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
50	OX40mAb19 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAG CGTGACCCCGGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
51	OX40mAb20 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INRDTSKNQFSLQLNSVTPEDTAVYFCARYRYDYDGGHAMDYWGQGLTVTVSS
52	OX40mAb20 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGACAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTTCTCCCTGCAGCTGAACAG CGTGACCCCGGAGGACACCGCCGTGTACTTCTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT

SEQ ID NO	Description	Sequence
		CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
53	OX40mAb21 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRLT INPDTSKNQSLQLNSVTPEDTAVYYCARYRYDYDGGHAMDYWGQGLTVTVSS
54	OX40mAb21 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCCGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
55	OX40mAb22 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNAITYHNPSLKSRLT INRDTSKNQSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVTVSS
56	OX40mAb22 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
57	OX40mAb23 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNAITYHNPSLKSRLT INRDTSKNQSLQLNSVTPEDTAVYYCARYRYDYDGGHAMDYWGQGLTVTVSS
58	OX40mAb23 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAGATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
59	OX40mAb24 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRLT INRDTSKNQSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVTVSS
60	OX40mAb24 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
61	OX40mAb25 VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYSGITYHNPSLKSRLT NRDTSKNQSLQLNSVTPEDTAVYYCARYRYDYDGGHAMDYWGQGLTVTVSS
62	OX40mAb25 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAGCGGCATCACCTACCACAACCCAGCC TGAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACA GCGTGACCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAGATACGACTACGACGGCGG GCCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
63	OX40mAb25a VH	QVQLQESGPGLVKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYSGITYHNPSLKSRLT NRDTSKNQSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVTVSS
64	OX40mAb25a VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAGCGGCATCACCTACCACAACCCAGCC TGAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACA GCGTGACCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG GCCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT

SEQ ID NO	Description	Sequence
		GCCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
65	OX40mAb26 VH	EVQLQESGPSLVKPSQTLSTCSVTGDSFTSGYWNWIRKFPGNRLEYMGYISYNAITYHNPSLKSRI ITRDTSKNHYLQLNSVTEDTATYFCARYRYDYDGGHAMDYWGQGLTVTVSS
66	OX40mAb26 VH DNA	GAGGTGCAGCTGCAGGAAAGCGGCCAGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGCAGCGTGACCGGCGACAGCTTACCAGCGGCTACTGGAAGTGGATCCGGAAGTCCCGG CAACCGGCTCGAGTACATGGGCTACATCAGCTACAACGCCATCACCTACCACAACCCAGCCTG AAGTCCCGGATCAGCATCACCGGGACACCAGCAAGAACCAGTACTACCTGCAGCTGAACAGC GTGACCACCGAGGACACCGCCACTACTTTTGCGCCCGGTACAGATACGACTACGACGGCGGC CACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
67	OX40mAb27 VH	QVQLQESGPGVLKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNAITYHNPSLKSRI INRDTSKNQYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVTVSS
68	OX40mA27 VH DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTACGAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGCCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGAGGACACCGCGGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCT
69	Human IgG1 CH chain	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSKVHFTFPAVLQSSGLYSLSSV VTPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKHTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMI SRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESN GQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
70	Human IgG1 CH chain DNA	GCgTCgACCAAGGGCCCATCcGTCTCCCCCTGGCACCTCCTCCAAGAGCACCTCTGGGGGCA CAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTcTGGAAGT CAGGCGcCTGACCAGCGGCGTGACACCTTCCCGGCTGTCTACAGTCTCAGGACTCTACTC CCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGT GAATCACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAAAC TCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCTCTTCCCC CCAAAACCCAAGGACACCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGGTGAC GTGAGCCACGAAGACCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAA TGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTACAGCTCTCA CCGTCTGCACCAAGGACTGGCTGAATGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGCCC TCCAGCCCCCATCGAGAAAACCATCTCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTcT ACACCCTGCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTACGCTGACCTGCCTGGTCA AAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAAC TACAAGACCACGCCTCCCGTGTCTGGACTCCGACGGCTCCTTCTTCTCTATAGCAAGCTCACCGT GGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGC ACAACCACTACACGCAGAAGAGCttaagCCTGTCTCCGGGTAAA
71	OX40mAb24 heavy chain	QVQLQESGPGVLKPSQTLSTCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRI INRDTSKNQYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSKVHFTFPAVLQSSGLYSLSSVTVTPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCDKHTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTP PVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
72	OX40mAb24 heavy chain DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTACGAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGCCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG

SEQ ID NO	Description	Sequence
		CGTGACCCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCCTGGTCACCGTGTCTCTGCgTCgACCAAGGG CCCATCcGTCTTCCCCCTGGCACCCTCTCCAAGAGCACCTCTGGGGGACAGCGGCCCTGGGC TGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTcTGGAACCTAGGCGCtCTGACCA GCGGCGTGACACCTTCCCGGTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGT GACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCAG CAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGACAAAACCTCACATGCCCACC GTGCCAGCACCTGAACCTCTGGGGGGACCGTCAGTCTTCTCTTCCCCCAAACCCAAAGGAC ACCCTCATGATCTCCCGACCCCTGAGGTACATGCGTGGTGGTGGACGTGAGCCACGAAGAC CCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCC GCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTACGCGTCTCACCCTCTGCACCAGG ACTGGCTGAATGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCG AGAAAACCATCTCAAAGCCAAAGGGCAGCCCCGAGAACCAAGGTcTACACCCTGCCCCCAT CCCGGGAGGAGATGACCAAGAACCAGGTACGCTGACCTGCTGGTCAAAGGCTTCTATCCCA GCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGAGAACAACTACAAGACCACGCCT CCCGTGCTGGACTCCGACGGCTCTTCTCTCTATAGCAAGCTCACCCTGGACAAGAGCAGGT GGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGC AGAAGAGCttaagCCTGTCTCCGGGTAAA
73	OX40mAb28 heavy chain	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKLEYIGYISYNGITYHNPSLKSRI INRDTSKNQYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVSSASTKGPSVFPLA PCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGKT YTCNVDHKPSNTKVDKRVESKYGPPCPPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYCKVSNKGLPSSI EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSRLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSLGK
74	OX40mAb28 heavy chain DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAACGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCCGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCCTGGTCACCGTGTCTCTGCGTCGACCAAGGG CCCAGCGTGTTCCCCCTGGCCCTTGACAGCAGAAGCACCAGCGAGAGCACAGCCGCCCTGGG CTGCTGGTGAAGGACTACTTCCCCGAGCCCGTGACCGTGTCTGGAACAGCGGCGCTCTGAC CAGCGGCGTGACATACCTTCCCCGCCGTGCTCCAGAGCAGCGGACTGTACTCCCTGAGCAGCGT GGTGACCGTGCCTTCAGCAGCCTGGGCACCAAGACCTACACCTGCAACGTGGACCACAAGCC CAGCAACACCAAGGTGGACAAGAGAGTGGAGAGCAAGTACGGCCCTCCCTGCCCCCTTGCCC TGCCCCGAGTTCTGGGCGGACCTAGCGTGTTCTGTTCCCCCAAGCCCAAGGACACCCCTG ATGATCAGCAGAACCCCCGAGGTGACCTGCGTGGTGGTGGACGTGTCCAGGAGGACCCCGA GGTCCAGTTTAATTGGTACGTGGACGGCGTGGAAGTGCATAACGCCAAGACCAAGCCAGAG AGGAGCAGTTCAACAGCACCTACAGAGTGGTGTCCGTGCTGACCGTGCTGCACCAGGACTGGC TGAACGGCAAGGAATACAAGTGAAGGTCTCCAACAAGGGCCTGCCTAGCAGCATCGAGAAG ACCATCAGCAAGGCCAAGGGCCAGCCACGGGAGCCCCAGGTCTACACCTGCCACCTAGCCAA GAGGAGATGACCAAGAACCAGGTGTCCCTGACCTGTCTGGTGAAGGCTTCTATCCCAGCGAT ATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCCAGAACAACTACAAGACCACCCCCCTGT GCTGGACAGCGACGGCAGCTTCTTCTGTACTCCAGACTGACCGTGGACAAGTCCAGATGGCA GGAGGGCAACGTCTTCAGCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCAGAA GTCCTGAGCCTGAGCCTGGGCAAG
75	OX40mAb29 heavy chain	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKLEYIGYISYNGITYHNPSLKSRI INRDTSKNQYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVSSASTKGPSVFPLA

SEQ ID NO	Description	Sequence
		PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQT YICNVNHKPSNTKVDKRVEPKSCDKHTCTPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVTV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PASIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP PVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
76	OX40mAb29 heavy chain DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCCTGGTCACCGTGTCTCTGCGTCGACCAAGGG CCCATCCGTCTTCCCCCTGGCACCTCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGC TGCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGGAAGTCAAGCGCTCTGACCA GCGGCGTGCACACCTTCCCGGCTGTCTACAGTCTCAGGACTTACTCCCTCAGCAGCGTGGT GACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCAG CAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAACCTCACACATgCacCG TGCCACGACCTGAATTCGAGGGGGGACGTCAGTCTTCTTCCCCCAAAACCCaaGgACAC CCTCATGATCTCCCGACCCCTGAGGTACATGCGTGGTGGTGGACGTGAGCCACGAAGACCC TGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGC GGGAGGAGCAGTACAACAGCACGTACCGTGTGGTACGCTCCTACCGTCTGACACGAGTACT GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCTCCATCGAGA AAACCATCTCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTcTACACCCTGCCCCCATCCCG GGAGGAGATGACCAAGAACCAGGTGACGCTGACCTGGTCAAAGGCTTCTATCCAGCGA CATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCG TGCTGGACTCCGACGGCTCTTCTTCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCA GCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAA GAGCCTCTCCCTGTCTCCGGGTAAA
77	OX40mAb31 heavy chain	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNAITYHNPSLKSRT INRDTSKNQSLQLNSVTPEDTAVVYCARYKYDYDGGHAMDYWGQGLTVTVSSASTKGPSVFPLA PCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQT YTCNVNDHKPSNTKVDKRVEPKYPPCPAPEFLGGGPSVFLFPPKPKDTLMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSI EKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVL DSDGSFFLYSRLTVDKSRWQEGNVFSCFSVMHEALHNHYTQKSLSLSLGK
78	OX40mAb31 heavy chain DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGCCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCGGAGGACACCGCCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCCTGGTCACCGTGTCTCTGCGTCGACCAAGGG CCCCAGCGTGTTCCTTGGCCCTTGCAGCAGAAGCACCAGCGAGAGCACAGCCGCCCTGGG CTGCTGGTGAAGGACTACTTCCCCGAGCCCGTGACCGTGTCTGGAACAGCGGCGCTGTGAC CAGCGGCGTGCATACCTTCCCCGCCGTGCTCCAGAGCAGCGGACTGTACTCCCTGAGCAGCGT GGTGACCGTGCCTTCCAGCAGCCTGGGCACCAAGACCTACACCTGCAACGTGGACCACAAGCC CAGCAACACCAAGGTGGACAAGAGAGTGGAGAGCAAGTACGGCCCTCCCTGCCCCCTTGCCC TGCCCCGAGTTCTGGGCGGACCTAGCGTGTCTGTTCCCCCAAGCCCAAGGACACCCCTG ATGATCAGCAGAACCCCGAGGTGACCTGCGTGGTGGTGGACGTGTCCAGGAGGACCCCGA GGTCCAGTTTAATTGGTACGTGGACGGCGTGGAGTGCATAACGCCAAGACCAAGCCAGAG AGGAGCAGTTCAACAGCACCTACAGAGTGGTGTCCGTGCTGACCGTGTGACACGAGGACTGGC

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		TGAACGGCAAGGAATACAAGTGCAAGGTCTCCAACAAGGGCCTGCCTAGCAGCATCGAGAAG ACCATCAGCAAGGCCAAGGGCCAGCCACGGGAGCCCCAGGTCTACACCTGCCACCTAGCCAA GAGGAGATGACCAAGAACCAGGTGTCCCTGACCTGTCTGGTGAAAGGCTTCTATCCCAGCGAT ATCGCCGTGGAGTGGGAGAGCAACGGCCAGCCCAGAACAACTACAAGACCACCCCCCTGT GCTGGACAGCGACGGCAGCTTCTTCTGTACTCCAGACTGACCGTGGACAAGTCCAGATGGCA GGAGGGCAACGTCTTCAGCTGCTCCGTGATGCACGAGGGCCTGCACAACCACTACCCCAGAA GTCCTTGAGCCTGAGCCTGGGCAAG
79	OX40mAb32 heavy chain	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNAITYHNPSLKSRT INRDTSKNQYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVVSSASTKGPSVFLPA PSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQT YICNVNHPKSNTKVDRVEPKSCDKHTHTCPPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL PASIEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTP PVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
80	OX40mAb32 heavy chain DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGCCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCCGAGGACACCGCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCTGCGTGCACCAAGGG CCCATCCGTCTTCCCCCTGGCACCTCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGC TGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGGAAGTCAAGGCGCTCTGACCA GCGGCGTGACACCTTCCCGGTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGGT GACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAG CAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGACAAAACCTCACACATgcCCacCG TGCCCAGCACCTGAATTCGAGGGGGGAcCGTCAGTCTTCTCTTCCCCCAAAACCCaAGGACAC CCTCATGATCTCCCGGACCCCTGAGGTACATGCGTGGTGGTGGACGTGAGCCACGAAGACCC TGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAAGCCGC GGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAAGGTCTCACCCTCTGCACCAGGACT GGCTGAATGGCAAGGAGTACAAGTGAAGGTCTCCAACAAAGCCCTCCAGCCTCCATCGAGA AAACCATCTCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTcTACACCTGCCCCATCCCCG GGAGGAGATGACCAAGAACCAGGTGACCTGACCTGGTCAAAGGCTTCTATCCCAGCGA CATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCG TGCTGGACTCCGACGGCTCTTCTCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCA GCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAA GAGCCTCTCCCTGTCTCCGGGTAAA
81	OX40mAb37 heavy chain	QVQLQESGPGLVKPSQTLSTLCAVYGGSFSSGYWNWIRKHPGKGLYIGYISYNGITYHNPSLKSRT INRDTSKNQYSLQLNSVTPEDTAVYYCARYKYDYDGGHAMDYWGQGLTVVSSAKTTPPSVYPLA PGSAAQTNSMVLGLVKGYFPEPTVTVNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSE TVTCNVAHPASSTKVDDKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLITITLTPKVTCTVVVDISKDD PEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFKCRVNSAFAPIEKT SKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDT DGSYFVYSKLNQVQSNWEAGNTFTCSVLHEGLHNHHTKSLSHSPGK
82	OX40mAb37 heavy chain DNA	CAGGTGCAGCTGCAGGAAAGCGGCCCTGGCCTGGTCAAGCCCAGCCAGACCCTGAGCCTGAC CTGTGCCGTGTACGGCGGCAGCTTCAGCAGCGGCTACTGGAAGTGGATCCGGAAGCACCCCCG GCAAGGGCCTGGAATACATCGGCTACATCAGCTACAACGGCATCACCTACCACAACCCAGCCT GAAGTCCCGGATCACCATCAACCGGGACACCAGCAAGAACCAGTACTCCCTGCAGCTGAACAG CGTGACCCCCGAGGACACCGCGTGTACTACTGCGCCCGGTACAAATACGACTACGACGGCGG CCACGCCATGGACTACTGGGGCCAGGGCACCTGGTCACCGTGTCTCTGCGaAGACGACACC

SEQ ID NO	Description	Sequence
		CCCATCTGTCTATCCACTGGCCCCTGGATCTGCTGCCCAAACCTAACTCCATGGTGACCCTGGGAT GCCTGGTCAAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACCTCTGGATCCCTGTCCAG CGGTGTGCACACCTTCCCAGCTGTCTGCAGTCTGACCTCTACACTCTGAGCAGCTCAGTGACT GTCCCCTCCAGCACCTGGCCCAGCGAGACCGTCACCTGCAACGTTGCCACCCGGCCAGCAGC ACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTGGTTGTAAGCCTTGATATGTACcGTC CCAGAAGTATCATCTGTCTTCATCTTCCCCCAAAGCCCAAGGATGTGCTCACCATTACTCTGAC TCCTAAGGTCACGTGTGTTGTGGTAGACATCAGCAAGGATGATCCCGAGGTCCAGTTCAGCTG GTTTGTAGATGATGTGGAGGTGCACACAGCTCAGACGCAACCCCGGAGGAGCAGTTCAACA GCACTTTCGGCTCAGTCAGTGAACCTTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTT CAAATGCAGGGTCAACAGTGCAGCTTTCCTGCCCCATCGAGAAAACCTCTCCAAAACCAAA GGCAGACCGAAGGCTCCACAGGTGTaACCATTCCACCTCCAAGGAGCAGATGGCCAAGGAT AAAGTCAGTCTGACCTGCATGATAACAGACTTCTTCCCTGAAGACATTACTGTGGAGTGGCAGT GGAATGGGCAGCCAGCGGAGAACTACAAGAACAACCTAGCCCATCATGGACACAGATGGCTCTT ACTTCGTCTACAGCAAGCTCAATGTGCAGAAGAGCAACTGGGAGGCAGGAAATACTTTACCT GCTCTGTGTACATGAGGGCCTGCACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCCTGG TAAA
83	OX40mAb37 light chain	DIQMTQSPSSLSASVGRVTITCRASQDISNYLNWYQQKPGKAPKLLIYYTSLKHSVPSRFSGSGS GTDYTLTISLQPEDFATYYCQQGSALPWTFGQGTKVEIKRADAAPTVSIFPPSSEQLTSGGASVVCF LNNFYPKDINVKWKIDGSRQNGVLNSWTDQDSKSTYSMSSTLTLTKDEYERHNSYCEATHKTS TSPIVKSFNRECE
84	OX40mAb37 light chain DNA	GACATCCAGATGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGTGGGCGACAGAGTGACCAT CACCTGTGCGGGCCAGCCAGGACATCAGCAACTACCTGAACTGGTATCAGCAGAAGCCCGCAA GGCCCCAAGCTGCTGATCTACTACACAGCAAGCTGCACAGCGGCGTGGCCAGCAGATTGAG CGGCAGCGGCTCCGGCACCGACTACACCCTGACCATCAGCAGCCTGCAGCCCGAGGACTTCGC CACCTACTACTGCCAGCAGGGCTCCGCCCTGCCCTGGACCTTTGGCCAGGGCACCAAGGTGGA AATCAAGCGGGCTGATGCGGCGCCAACCTGTATCCATCTTCCACCATTCCAGTGAGCAGTTAACA TCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACCTTACCCCAAAGACATCAATGTCAAGT GGAAGATTGATGGCAGTGAACGACAAAATGGCGTCTTGAACAGTTGGACTGATCAGGACAGC AAAGACAGCACCTACAGCATGAGCAGCACCTCACGTTGACCAAGGACGAGTATGAACGACAT AACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTACCCATTGTCAAGAGCTTCAACA GGAATGAGTGT
85	OX86 VH	QVQLKESGPGLVQPSQTLSTCTVSGFSLTGYNLHWVRQPPGKGLEWMGRMRYDGDYYNSVLK SRLSISRDTSKNQVFLKMNSLQTDITAIYYCTRDGRGDSFDYWGGQVMVTVSS
86	OX86 heavy chain	QVQLKESGPGLVQPSQTLSTCTVSGFSLTGYNLHWVRQPPGKGLEWMGRMRYDGDYYNSVLK SRLSISRDTSKNQVFLKMNSLQTDITAIYYCTRDGRGDSFDYWGGQVMVTVSSASTPPSVYPLAP GSAAQTNSMVLGLCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSTWPSET VTCNVAHPASSTKVDKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLITITLTPKVTVCVVDISKDDP EVQFSWFVDDDEVHTAQTQPREEQFNSTFRVSSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTIS KTKGRPKAPQVYTIPTPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTD GSYFVYSKLNQKSNWEAGNTFTCSVLHEGLHNHTEKSLSHSPGK
87	OX86 heavy chain DNA	CAGGTGCAGCTGAAGGAGTCAGGACCTGGTCTGGTGCAGCCCTCACAGACCCTGTCCCTCACC TGCACTGTCTCTGGGTTCTACTAACCGGTTACAATTTACTCTGGGTTCCGACGCTCCAGGAA AGGGTCTGGAGTGGATGGGAAGAATGAGGTATGATGGAGACACATATTATAATTCAGTTCTCA AATCCCGACTGAGCATCAGCAGGGACACCTCCAAGAACCAAGTTTCTTGAAAAATGAACAGTCT GCAAACGGATGACACAGCCATTTACTATTGTACCAGAGACGGGCGTGGTGACTCCTTTGATTAC TGGGGCCAAGGAGTCATGGTCACAGTCTCCTCCGCGTCGACGACACCCCATCTGTCTATCCAC TGCCCCCTGGATCTGCTGCCAACTAACTCCATGGTGACCTGGGATGCCTGGTCAAGGGCT ATTTCCCTGAGCCAGTGACAGTGACCTGGAACCTCTGGATCCCTGTCCAGCGGTGTGCACACCTT CCCAGCTGTCTGCAGTCTGACCTCTACACTCTGAGCAGCTCAGTGACTGTCCCCTCCAGCACCT

SEQ ID NO	Description	Sequence
		GGCCCAGCGAGACCGTCACCTGCAACGTTGCCACCCGGCCAGCAGCACCAAGGTGGACAAG AAAATTGTGCCAGGGATTGTGGTTGTAAGCCTTGCATATGTACCGTCCCAGAAGTATCATCTG TCTTCATCTTCCCCCAAAGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCACGTGT GTTGTGGTAGACATCAGCAAGGATGATCCCAGGTCCAGTTCAGCTGGTTTGTAGATGATGTG GAGGTGCACACAGCTCAGACGCAACCCCGGGAGGAGCAGTTCAACAGCACTTTCGCTCAGTC AGTGAACCTCCCATCATGCACCAGGACTGGCTCAATGGCAAGGAGTTCAAATGCAGGGTCAAC AGTGCAGCTTTCCTGCCCCATCGAGAAAACCATCTCCAAAACCAAAGGCAGACCGAAGGCT CCACAGGTGTATACCATTCCACCTCCCAAGGAGCAGATGGCCAAGGATAAAGTCAGTCTGACC TGCATGATAACAGACTTCTTCCCTGAAGACATTACTGTGGAGTGGCAGTGGAAATGGGCAGCCA GCGGAGAACTACAAGAACAATCAGCCCATCATGGACACAGATGGCTCTTACTTCGTCTACAGC AAGCTCAATGTGCAGAAGAGCAACTGGGAGGCAGGAAATACTTTCACCTGCTCTGTGTTACAT GAGGGCCTGCACAACCACCATACTGAGAAGAGCCTCTCCCACTCTCTGGTAAA
88	OX86 VL	DIVMTQGALPNPVPSPGESASITCRSSQSLVYKDGQTYLWNFLQRPQSQPQLLTYWMSTRASGVSD RFSGSGSGTYFTLKISRVRADAGVYYCQQVREYPFTFGSGTKLEIK
89	OX86 light chain	DIVMTQGALPNPVPSPGESASITCRSSQSLVYKDGQTYLWNFLQRPQSQPQLLTYWMSTRASGVSD RFSGSGSGTYFTLKISRVRADAGVYYCQQVREYPFTFGSGTKLEIKRADAAPTVISIFPPSEQLTSGG ASVVCFLNNFYPKIDINVKWKIDGSEKQNGVLNSWTDQDSKDYMSSTLTLTDEYERHNSYTCE ATHKTSTSPIVKSFNRNEC
90	OX86 light chain DNA	GATATTGTGATGACCCAGGGTGCACTCCCAATCCTGTCCCTTCTGGAGAGTCAGCTTCCATCA CCTGCAGGTCTAGTCAGAGTCTGGTATACAAAGACGGCCAGACATACTTGAATTGGTTTCTGCA GAGGCCAGGACAGTCTCCTCAGCTTCTGACCTATTGGATGTCTACCCGTGCATCAGGAGTCTCA GACAGGTTCAAGTGGCAGTGGGTGAGGAACATATTTCACTGAAAATCAGTAGAGTGAGGGC TGAGGATGCGGGTGTGTATTACTGTGCAAGTTCGAGAGTATCCTTTCACTTTCGGCTCAGGG ACGAAGTTGGAAATAAAACGGGCTGATGCGGCGCCAAGTGTATCCATCTTCCACCATCCAGT GAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACA TCAATGTCAAGTGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTG ATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCTCACGTTGACCAAGGACGAGT ATGAACGACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTACCCATTGTCAA GAGCTTCAACAGGAATGAGTGT
91	Human OX40	MCVGARRLGRGPCAALLLLGLGLSTVTGLHCVGDTYPSNDRCCHECRPGNGMVSRCRSQNTVC RPCGPGFYNDVVSSKPKPCTWCNLRSGSERKQLCTATQDTCRCRAGTQPLDSYKPGVDCAPCP PGHFSPGDNQACKPWTNCTLAGKHTLQPASNSSDAICEDRDPPATQPQETQGPPARPITVQPTEA WPRTSQGPSTRPVEVPGGRAVAAILGLGLVLGLLPLAILLALYLLRRDQRLPPDAHKKPPGGGSFRT PIQEEQADAHSTLAKI
92	Mouse OX40	MYVWVQQPTALLLLGLTLGVTARRLNCVKHTYPSGHKCCRECPGHGMVSRCDHTRDTLCHPCE TGFYNEAVNYDTCKQCTQCNHRSGSELKQNCTPTQDTCRCRPGTQPRQDSGYKLGVDVCPCP GHFSPGNQACKPWTNCTLSGKQTRHPASDSLDAVCEDRSLLATLLWETQRPTFRPTTVQSTTVW PRTSELPSPPTLVTPEGPAFAVLLGLGLLAPLTVLLALYLLRKAWRLPNTPKPCWGNFSRFTPIQEE HTDAHFTLAKI

WHAT IS CLAIMED IS:

1. An isolated antibody or fragment thereof comprising a humanized heavy chain variable region (VH) and a humanized light chain variable region (VL);

wherein the VH comprises:

- (a) a CDR1 comprising the amino acid sequence of SEQ ID NO: 8,
- (b) a CDR2 comprising the amino acid sequence of SEQ ID NO: 14, and
- (c) a CDR3 comprising the amino acid sequence of SEQ ID NO: 27, and

wherein the VL comprises:

- (d) a CDR1 comprising the amino acid sequence of residues 24-34 of SEQ ID NO: 29,
- (e) a CDR2 comprising the amino acid sequence of residues 50-56 of SEQ ID NO: 29, and
- (f) a CDR3 comprising the amino acid sequence of residues 89-97 of SEQ ID NO: 29,

wherein the antibody or fragment thereof can specifically bind to human OX40.

2. The antibody or fragment of claim 1, wherein the antigen-binding fragment is an Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, or an sc(Fv)₂ fragment, or any combination thereof.

3. The antibody or fragment thereof of claim 1, which can induce dose-dependent proliferation of activated CD4⁺ T cells and dose-dependent cytokine release in primary activated human CD4⁺ T cells in a plate-based assay.

4. The antibody or fragment thereof of claim 3, wherein a 20% maximal proliferation response (EC₂₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 14 pM to about 28 pM, a 50% maximal proliferation response (EC₅₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 0.3 pM to about 130 pM, and a 90% maximal proliferation response (EC₉₀) can be achieved in primary activated human CD4⁺ T cells at an antibody concentration of about 50 pM to about 90 pM, all as measured by flow cytometry.

5. The antibody or fragment thereof of claim 4, wherein EC₂₀ is about 21 pM, EC₅₀ is about 28 pM, and EC₉₀ is about 72 pM.

6. The antibody or fragment thereof of claim 3, wherein the cytokine is IFN γ , TNF α , IL-5, IL-10, IL-2, IL-4, IL-13, IL-8, IL-12 p70, IL-1 β , or any combination thereof.

7. The antibody or fragment thereof of claim 6 wherein the cytokine is IFN γ , TNF α , IL-5, IL-10, IL-13, or any combination thereof.
8. The antibody or fragment thereof of claim 1, which can achieve CD4⁺ T cell proliferation and cytokine release in primary activated cynomolgus monkey CD4⁺ T cells and in primary activated rhesus monkey CD4⁺ T cells.
9. The antibody or fragment thereof of claim 1, which can activate the NF κ B pathway in OX40 expressing T cells in the presence of Fc γ R-expressing cells.
10. The antibody or fragment thereof of claim 9, wherein the OX40-expressing T cells are OX40-expressing Jurkat NF κ B-luciferase reporter cells that produce luciferase in response to stimulation of the NF κ B signaling pathway.
11. The antibody or fragment thereof of claim 1, which can trigger complement-dependent or antibody-dependent cellular cytotoxicity against OX40-expressing cells.
12. The antibody or fragment thereof of claim 11, which can bind to C1q and trigger NK-mediated antibody-dependent cellular cytotoxicity against the OX40-expressing cells.
13. A composition comprising the antibody or fragment thereof of claim 1, and a carrier.
14. An antibody or antigen-binding fragment thereof comprising a humanized heavy chain variable region (VH) and a humanized light chain variable region (VL), wherein the VH comprises the amino acid sequence SEQ ID NO: 59, wherein the VL comprises the amino acid sequence SEQ ID NO: 29, and wherein the antibody or fragment thereof can specifically bind to human OX40.
15. The antibody or fragment thereof of claim 14 comprising the heavy chain amino acid sequence SEQ ID NO: 71 and the light chain amino acid sequence SEQ ID NO: 30.
16. A polynucleotide comprising a nucleic acid that encodes the antibody or fragment thereof, of claim 14.

17. The polynucleotide of claim 16, comprising the nucleic acid of SEQ ID NO: 60, the nucleic acid of SEQ ID NO: 31, the nucleic acid of SEQ ID NO: 72, or any combination thereof.
18. A vector comprising the polynucleotide of claim 16.
19. A host cell comprising the polynucleotide of claim 16.
20. A method of producing an antibody or fragment thereof, comprising culturing the host cell of claim 19 under conditions in which the antibody or fragment thereof encoded by the polynucleotide is expressed, and recovering the antibody or fragment thereof.
21. A host cell comprising the vector of claim 18.
22. The antibody or fragment of claim 1 for use in therapy.
23. The antibody or fragment of claim 1 for use as a medicament.
24. The antibody or fragment of claim 1 for use in the treatment of cancer with a T cell infiltrate in a subject, wherein an effective amount of the antibody or fragment is to be administered to the subject.
25. The antibody or fragment for use of claim 24, wherein the cancer is a solid tumor.
26. The antibody or fragment for use of claim 24, wherein administration of the antibody or fragment can inhibit tumor growth, can promote tumor reduction, or both.
27. The antibody or fragment for use of claim 26, wherein tumor growth inhibition is achieved in the presence of T cells.
28. The antibody or fragment for use of claim 27, wherein tumor growth is inhibited by at least 10%, at least 20%, at least 30%, at least 40%, and least 50%, at least 60%, or at least 70% compared to administration of an isotype-matched control antibody or fragment thereof.
29. The antibody or fragment for use of claim 24, wherein the antibody or fragment comprises a humanized

heavy chain variable region (VH) and a humanized light chain variable region (VL), wherein the VH comprises the amino acid sequence SEQ ID NO: 59, wherein the VL comprises the amino acid sequence SEQ ID NO: 29, and wherein the antibody or fragment can specifically bind to human OX40.

30. The antibody or fragment for use of claim 29, wherein the antibody or fragment comprises the heavy chain amino acid sequence SEQ ID NO: 71 and the light chain amino acid sequence SEQ ID NO: 30.

31. The antibody or fragment for use of claim 24, wherein the antigen-binding fragment is an Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, or an sc(Fv)₂ fragment, or any combination thereof.

32. A composition comprising the antibody or fragment of claim 1 for use in the treatment of cancer with a T cell infiltrate in a subject, wherein an effective amount of the composition is to be administered to the subject.

33. Use of the antibody or fragment of claim 1 in the manufacture of a medicament for the treatment of cancer with a T cell infiltrate in a subject, wherein an effective amount of the medicament is to be administered to the subject.

34. The use of claim 33, wherein the cancer is a solid tumor.

35. The use of claim 33, wherein administration of the antibody or fragment can inhibit tumor growth, can promote tumor reduction, or both.

36. The use of claim 35, wherein tumor growth inhibition is achieved in the presence of T cells.

37. The use of claim 36, wherein tumor growth is inhibited by at least 10%, at least 20%, at least 30%, at least 40%, and least 50%, at least 60%, or at least 70% compared to administration of an isotype-matched control antibody or fragment thereof.

38. The use of claim 33, wherein the antibody or fragment comprises a humanized heavy chain variable region (VH) and a humanized light chain variable region (VL), wherein the VH comprises the amino acid sequence SEQ ID NO: 59, wherein the VL comprises the amino acid sequence SEQ ID NO: 29, and wherein the antibody or fragment can specifically bind to human OX40.

39. The use of claim 38, wherein the antibody or fragment comprises the heavy chain amino acid sequence SEQ ID NO: 71 and the light chain amino acid sequence SEQ ID NO: 30.
40. The use of claim 33, wherein the antigen-binding fragment is an Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, or an sc(Fv)₂ fragment, or any combination thereof.
41. Use of a composition comprising the antibody or fragment of claim 1 in the manufacture of a medicament in the treatment of cancer with a T cell infiltrate in a subject, wherein an effective amount of the medicament is to be administered to the subject.