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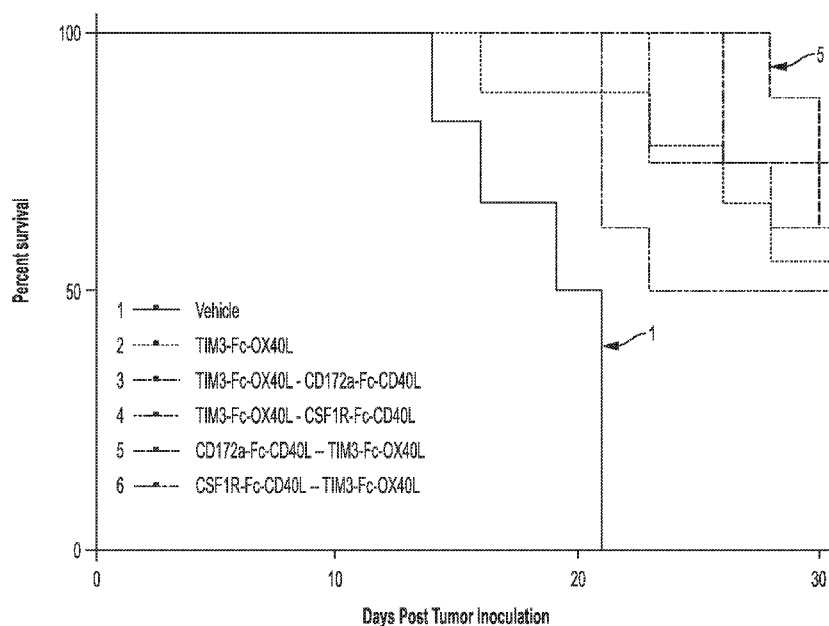
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(54) Title: METHODS OF MAKING AND USING EXTRACELLULAR DOMAIN-BASED CHIMERIC PROTEINS

FIG. 15B

(57) Abstract: The present invention relates, *inter alia*, to compositions and methods, including chimeric proteins and combination therapies that find use in the treatment of disease, such as cancer and/or an inflammatory disease.

**METHODS OF MAKING AND USING EXTRACELLULAR DOMAIN-BASED CHIMERIC PROTEINS****PRIORITY**

This application claims the benefit of, and priority to, U.S. Provisional Application No. 62/464,002, filed February 27, 2017, the contents of which are hereby incorporated by reference in its entirety.

**DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY**

This application contains a sequence listing. It has been submitted electronically *via* EFS-Web as an ASCII text file entitled "SHK-001PC2\_SequenceListing\_ST25". The sequence listing is 149,084 bytes in size, and was created on or about February 27, 2018. The sequence listing is hereby incorporated by reference in its entirety.

**TECHNICAL FIELD**

The present invention relates to, *inter alia*, compositions and methods, including chimeric proteins that find use in the treatment of disease, such as immunotherapies for cancer and autoimmunity.

**BACKGROUND**

Exceptional treatment for cancer have been evasive despite years of efforts. This is so, in part, because many cancers have developed mechanisms to avoid the immune system. Thus, there remains a need to develop therapeutics and treatment regimens that adequately engage multiple arms of the immune system to generate an anti-cancer immune response.

**SUMMARY**

Accordingly, in various aspects, the present invention provides for compositions and methods that are useful for cancer immunotherapy. For instance, the present invention, in part, relates to specific chimeric proteins that reverse or suppresses immune inhibitory signals while providing immune activating or co-stimulatory signals. Importantly, *inter alia*, the present invention provides for improved chimeric proteins that can maintain a stable and producible multimeric state based on, without wishing to be bound by theory, stabilization in a linker region including one or more disulfide bonds. Accordingly, the present compositions and methods overcome various deficiencies in producing bi-specific agents.

In some aspects, the chimeric protein is of a general structure of: N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond (including without limitation, hinge-CH2-CH3 Fc domain is derived from human IgG4), and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, where the linker connects the first domain and the second domain and optionally comprises one or more joining linkers as described herein.

Further, in various aspects, the present invention relates to methods of treating cancer by using a combination of the present chimeric proteins. For example, the present methods allow for regimens that modulate specific arms of the immune system, such as innate and adaptive immune responses, optionally in order.

In some aspects, there is provided a method of treating cancer, comprising administering to a subject in need thereof: (i) a first chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where: (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of a Type II transmembrane protein, and the first chimeric protein modulates the innate immune system; and (ii) a second chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and the second chimeric protein modulates the adaptive immune system.

In some aspects, there is provided a method of treating cancer, comprising administering to a subject in need thereof: a second chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and the second chimeric protein modulates the adaptive immune system, where the subject is undergoing or has undergone treatment with a first chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and the first chimeric protein modulates the innate immune system.

In embodiments, first chimeric protein is administered before the second chimeric protein.

In embodiments, the first chimeric protein is administered after the second chimeric protein.

In embodiments, the first chimeric protein comprises at least one of: TIGIT, CSF1R, CD172a(SIRP1 $\alpha$ ), VSIG8, TIM3, 41BBL, CD40L, SIGLEC7, SIGLEC9, and LIGHT.

In embodiments, the second chimeric protein comprises at least one of: PD-1, TIM3, VSIG8, CD172a(SIRP1 $\alpha$ ), OX40L, GITRL, TL1A, and IL-2

In embodiments, the first chimeric protein and the second chimeric protein are independently selected from TIM3-Fc-OX40L, CD172a(SIRP1 $\alpha$ )-Fc-CD40L, and CSF1R-Fc-CD40L.

In embodiments, TIM3-Fc-OX40L is administered before CD172a(SIRP1 $\alpha$ )-Fc-CD40L. In embodiments, TIM3-Fc-OX40L is administered before CSF1R-Fc-CD40L. In embodiments, CD172a(SIRP1 $\alpha$ )-Fc-CD40L is administered before TIM3-Fc-OX40L. In embodiments, CSF1R-Fc-CD40L is administered before TIM3-Fc-OX40L.

Any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein.

## BRIEF DESCRIPTION OF THE DRAWINGS

**FIG. 1A to FIG. 1D** show schematic illustrations of how a Type I and Type II membrane protein (**FIG. 1A** and **FIG. 1C**) may be engineered with transmembrane and intracellular domains removed and adjoined using a linker sequence (**FIG. 1B**) to generate a single chimeric protein wherein the extracellular domains of the Type I and Type II membrane proteins each face outward in a single chimeric protein. **FIG. 1B** depicts the linkage of a Type I and Type II membrane protein by removal of the transmembrane and intracellular domains of each protein, and where the liberated extracellular domains (ECD) from each protein have been adjoined by a linker sequence. The ECD in this depiction may include the entire amino acid sequence of a candidate Type I or Type II protein which is typically localized outside the cell membrane, or any portion thereof which retains binding to the intended receptor or ligand. **FIG. 1D** depicts adjoined extracellular domains in a linear construct wherein the extracellular domain of the Type I membrane protein faces the “left” side of the construct and the extracellular domain of the Type II membrane protein faces the “right” side of the construct.

**FIG. 2** shows, without wishing to be bound by theory, an *in silico* predicted structure of a monomeric CD172a(SIRPα)-Fc-CD40L chimeric protein (SL-172154).

**FIG. 3A** shows, without wishing to be bound by theory, a schematic diagram illustrating a mechanism of action of the hCD172a(SIRPα)-Fc-CD40L chimeric protein for stimulating active tumor destruction. The chimeric protein may then “dangle” from the surface of the tumor cell, and the CD40L portion of the chimeric protein may then bind to CD40 expressed on the surface of the T cell. This would result in replacement of an inhibitory hCD172a(SIRPα) signal with a co-stimulatory CD40L signal to enhance the anti-tumor activity of T cells. **FIG. 3B** shows a synapse that has formed by a chimeric protein between a tumor cell and a T cell.

**FIG. 4** shows characterization of human CD172a(SIRPα)-Fc-CD40L chimeric protein (SL-172154) by Western blot. Specifically, each individual domain of the chimeric protein was probed using an anti-CD172a, anti -Fc, or anti-CD40L antibody. Untreated samples of the hCD172a(SIRPα)-Fc-CD40L chimeric protein, e.g., control, were loaded into lane 2 in all the blots (no β-mercaptoethanol or PNGase). Samples in lane 3 were treated with the reducing agent, β-mercaptoethanol, while samples in lane 4 were treated with PNGase.

**FIG. 5A to FIG. 5C** are tables of results showing the identified binding partners of human PD1-Fc-OX40L (**FIG. 5A**), of human CSF1R-Fc-CD40L (**FIG. 5B**), or of human CD172a(SIRPα)-Fc-CD40L (**FIG. 5C**) from a microarray containing about 6,000 human membrane proteins. In each case, the expected binding partners for each candidate molecule were identified by the screen. There was no evidence of non-specific binding to other human proteins, and binding to Galectin-1 is seen in the screen for all Fc-containing fusion proteins.

**FIG. 6** shows ELISA assays demonstrating the binding affinity of the different domains of human CD172a(SIRPα)-Fc-CD40L chimeric protein for their respective binding partners. The first (left-most) panel shows the binding and detection of hCD172a(SIRPα)-Fc-CD40L chimeric protein to human IgG, the binding partner for Fc. Human Ig (hIg) was used as a standard. The second panel from the left shows the binding and detection of hCD172a(SIRPα)-Fc-CD40L chimeric protein to CD47, the binding partner for CD172a. The third panel from the left shows the binding

and detection of hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to the receptor CD40, the binding partner for CD40L. The last (right-most) panel demonstrates a dual-binding functional ELISA assay where recombinant human CD40 was used to capture the CD40L domain and recombinant human CD47 was used to detect the CD172a(SIRP $\alpha$ ) domain, demonstrating contemporaneous binding of CD172a(SIRP $\alpha$ )-Fc-CD40L to both of its binding partners.

**FIG. 7A** and **FIG. 7B** show *ex vivo* cell binding assays demonstrating the ability of different domains of the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to bind their respective binding partners (e.g., receptor or ligand) on the surface of a mammalian cell membrane. **FIG. 7A** shows binding of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to hCD47 (top curve is HeLa/hCD47, bottom is HeLa Parental). **FIG. 7B** shows binding of the murine CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to mCD40 (top curve is CHOK1/mCD40, bottom curve is CHOK1 Parental).

**FIG. 8A** to **FIG. 8E** show the binding affinity of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein by surface plasmon resonance (SPR). **FIG. 8A** shows binding of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to hCD47 (top curve is CD172A-Fc-CD40L (250 nM), bottom curve is CD172A-Fc-Cntl (250 nM)). **FIG. 8B** shows binding of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to hFc $\gamma$ R1A (bottom curve is CD172a-Fc-CD40L (250 nM), top curve is CD172a-Fc-Cntl (250 nM)). **FIG. 8C** shows binding of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to hFc $\gamma$ Rn (bottom curve is CD172a-Fc-CD40L (250 nM), top curve is CD172A-Fc-Cntl (250 nM)). **FIG. 8D** summarizes the affinity results in **FIG. 8A** to **FIG. 8C**. **FIG. 8E** shows binding affinity of the murine CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to mCD40.

**FIG. 9A** to **FIG. 9C** show *ex vivo* functional assays of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein. **FIG. 9A** shows an ELISA-based blocking assay demonstrating the binding of hCD172a(SIRP $\alpha$ )-Fc-CD40L to cells overexpressing hCD47. **FIG. 9B** shows a schematic representation of the mode of action of a macrophage engulfment assay. **FIG. 9C** shows increased levels of double positive cells (phagocytosis) in response to CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein in a concentration dependent manner.

**FIG. 10A** and **FIG. 10B** show characterization of murine CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein by Western blot analysis and ELISA assays. **FIG. 10A** shows the detection of each individual domain of the mCD172a(SIRP $\alpha$ )-Fc-CD40L fusion construct using an anti-CD172a, anti-Fc, or anti-CD40L antibody. Untreated samples of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein, e.g., control, were loaded into lane 2 in all the blots (no  $\beta$ -mercaptoethanol or PNGase). Samples in lane 3 were treated with the reducing agent,  $\beta$ -mercaptoethanol, while samples in lane 4 were treated with PNGase. **FIG. 10B** shows ELISA assays to demonstrate the binding affinity of the different domains of mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein for their respective binding partners. In binding to CD47 (**FIG. 10B**, left side) the binding and detection of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to CD47, the binding partner for CD172a (square symbols) were demonstrated. Recombinant mCD172a-mFc was used to generate a standard curve (circle symbols). In binding to CD40 (**FIG. 10B**, right side) the binding and detection of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to the receptor CD40, the binding partner for CD40L

(square symbols) were demonstrated. Recombinant mCD40L was used to generate a standard curve (circle symbols).

**FIG. 11A** to **FIG. 11C** shows results from *in vivo* tumor studies demonstrating the anti-tumor efficacy of mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein. A CT26 tumor was implanted into Balb/c mice prior to treatment with anti-CD47, anti-CD40, a combination of the two antibodies, with mCD172a(SIRP $\alpha$ )-Fc-CD40L, or with control antibodies. **FIG. 11A** shows the evolution of tumor size over forty-five days after tumor inoculation for each group. **FIG. 11B** shows the overall survival percentage of mice through fifty days after tumor inoculation (at day 50, the curves top to bottom are: CD172a-Fc-CD40L (150  $\mu$ g x 2), CD172a-Fc-CD40L (300  $\mu$ g x 2),  $\alpha$ CD47/CD40,  $\alpha$ CD40,  $\alpha$ CD47 (0% survival by between days 30-35) and untreated (0% survival by between days 20-25). **FIG. 11C** summarizes the group sizes and treatment outcomes for each group.

**FIG. 12A** to **FIG. 12F** show *in vivo* functional assays of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein. Immune profiling was performed on tumor-bearing mice treated with the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein. **FIG. 12A** shows changes in the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations in the spleen in mice treated with the chimeric protein. **FIG. 12B** shows changes in the CD4<sup>+</sup>CD25<sup>-</sup> effector T cells or CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in the spleen of mice treated with the chimeric protein. **FIG. 12C** and **FIG. 12D** show changes in the CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations in the peripheral lymph nodes and tumor, respectively, in mice treated with the chimeric protein. **FIG. 12E** shows tetramer staining for determining the fraction of CD8<sup>+</sup> T cells within splenocytes or tumor infiltrated lymphocytes (TIL) that recognized the AH1 tumor antigen natively expressed by CT26 tumors. **FIG. 12F** shows changes in the proportion of cells which upregulate IL-15 receptor alpha (IL15R $\alpha$ ), which is an indicator of CD40 activation. In each of **FIG. 12A** to **FIG. 12F**, the conditions from left to right are: untreated,  $\alpha$ CD40/CD47, mCD172a(SIRP $\alpha$ )-Fc-CD40L (150  $\mu$ g x 2), mCD172a(SIRP $\alpha$ )-Fc-CD40L (300  $\mu$ g x 2).

**FIG. 13** shows data from cynomolgus macaques treated with human CD172a(SIRP $\alpha$ )-Fc-CD40L. 1 male and 1 female cynomolgus macaque were treated with a single dose of hCD172a(SIRP $\alpha$ )-Fc-CD40L at 1 mg/kg. Serum was collected at multiple time points from pre-treatment to fourteen days after treatment to evaluate pharmacokinetics and safety. CBC/CMPs were performed at five time points for safety and specific evaluation of hemolysis and thrombocytopenia. No evidence of red blood cell lysis or platelet depletion were observed following treatment with hCD172a(SIRP $\alpha$ )-Fc-CD40L. Gross safety assessments were made multiple times daily, and no additional safety signals were observed.

**FIG. 14** shows *in vivo* synergy in reducing tumor volumes of treatments with antibodies (as monotherapies or in combinations) directed to checkpoint proteins or treatments with chimeric proteins. The conditions are, left to right: vehicle, anti-OX40 (OX86), anti-PD-1 (RMP1-14), anti-PD-1 (29F.1A12), anti-TIM3 (RMT3-23), anti-TIM3 + OX40, anti-TIM3 + OX40 +PD-1 (RMP1-14), anti-TIM3 + OX40 +PD-1 (29F.1A12), ARC 300 $\mu$ g x 2 ("ARC" is the TIM3-Fc-OX40L chimeric protein).

**FIG. 15A** shows *in vivo* reduction in tumor volume from sequential treatments of chimeric proteins, either two sequential treatments with the same chimeric protein or treatments with two different chimeric proteins. **FIG. 15B**

shows percent survival over time for the mice shown in **FIG. 15A**. For clarity, in **FIG. 15A**, the conditions at point 20 days on the x axis are (top to bottom): vehicle, CSF1R-Fc-CD40L -- TIM3-Fc-OX40L, TIM3-Fc-OX40L (150  $\mu$ g x 2), TIM3-Fc-OX40L -- CSF1R-Fc-CD40L, TIM3-Fc-OX40L -- CD172a-Fc-CD40L, and CD172a-Fc-CD40L -- TIM3-Fc-OX40L. For clarity, in **FIG. 15B**, the conditions are identified as: vehicle: "1"; TIM3-Fc-OX40L (150  $\mu$ g x 2): "2"; TIM3-Fc-OX40L -- CD172a-Fc-CD40L: "3"; TIM3-Fc-OX40L -- CSF1R-Fc-CD40L: "4"; CD172a-Fc-CD40L -- TIM3-Fc-OX40L: "5"; and CSF1R-Fc-CD40L -- TIM3-Fc-OX40L: "6".

**FIG. 16** shows, without wishing to be bound by theory, four potential configurations of PD-1-Fc-OX40L chimeric proteins.

**FIG. 17** shows Western blots of PD-1-Fc-OX40L chimeric proteins run on SDS-PAGE under a non-reducing condition, a reducing condition, and a reducing condition and following treatment with Peptide-N-Glycosidase F (PNGaseF).

**FIG. 18** shows a chromatograph for PD-1-Fc-OX40L chimeric proteins run on Size Exclusion Chromatography (SEC).

**FIG. 19** shows SDS-PAGE and native (non-SDS) PAGE gels for PD-1-Fc-OX40L chimeric proteins run under a non-reducing condition ("−") or a reducing condition ("+").

**FIG. 20** shows a native (non-SDS) PAGE gel for PD-1-No Fc-OX40L chimeric proteins which lack a Fc domain.

**FIG. 21** shows, without wishing to be bound by theory, a model for how a hexamer and concatemers form from chimeric proteins of the present invention.

**FIG. 22A** to **FIG. 22Q** show characterization of PD-1-Fc-OX40L chimeric proteins with different joining linker sequences by Western blot analysis. Sequences of the different joining linkers are provided below in the Examples section. Specifically, each individual domain of the fusion construct was probed using an  $\alpha$ -PD-1,  $\alpha$ -Fc, or  $\alpha$ -OX40L antibody. In each figure, untreated samples of the PD-1-Fc-OX40L chimeric protein, e.g., control, were loaded into lane 1 in all the blots (no  $\beta$ -mercaptoethanol or PNGase). Samples in lane 2 were treated with the reducing agent,  $\beta$ -mercaptoethanol, while samples in lane 3 were treated with PNGase.

**FIG. 23** shows characterization of PD-1-Fc-OX40L chimeric proteins with different joining linker sequences by ELISA-based capture and detection assay against the central Fc region of the protein. The protein concentration of each PD-1-Fc-OX40L chimeric protein with different joining linker sequence (#1 to #17) was determined.

**FIG. 24A** to **FIG. 24P** show the flow cytometry profiles of PD-1-Fc-OX40L chimeric proteins with different joining linker sequences by FACS analysis to PD-L1 or OX40. The  $EC_{50}$  values were calculated for each PD-1-Fc-OX40L chimeric protein with different joining linker sequence (#2 to #17).

**FIG. 25A**, using an PD1-Fc-OX40L chimeric protein as a non-limiting example, shows that tumor cells may express PD-L1 on the cell surface, which can bind to PD-1 expressed by a T cell (**FIG. 25B**). This interaction suppresses activation of T cells. A chimeric protein comprising the extracellular domain of PD-1, adjoined to the extracellular

domain of OX40L may bind to PD-L1 on the surface of a tumor cell, preventing binding to PD-1 on the surface of a T cell (**FIG. 25C**). The chimeric protein may then “dangle” from the surface of the tumor cell, and the OX40L portion of the chimeric protein may then bind to OX40 expressed on the surface of the T cell. This would result in replacement of an inhibitory PD-L1 signal with a co-stimulatory OX40L signal to enhance the anti-tumor activity of T cells.

**FIG. 26A** shows cell lines generated to overexpress murine PD-L1 (CHOK1/mPD-L1), PD-L2 (CHOK1/mPD-L2), or OX40 (CHOK1/mOX40). **FIG. 26B** shows *in vitro* cell binding to demonstrate the ability of the murine PD-1-Fc-OX40L chimeric protein to bind to the engineered cell lines. **FIG. 26C** and **FIG. 26D** show *Staphylococcus aureus*, Enterotoxin Type B (SEB) superantigen cytokine release assays which demonstrate the effects of the mPD-1-Fc-OX40L chimeric protein on IL-2 (**FIG. 26C**) and TNF $\alpha$  (**FIG. 26D**) secretion. In **FIG. 26C** and **FIG. 26D**, curves top to bottom at x axis point 2.5 are: PD1-Fc-OX40L, PD1-Fc/OX40L, OX40L-Fc, PD1-Fc/OX40L-Fc, PD1-Fc, and IgG control.

**FIG. 27A** shows the evolution of *in vivo* tumor size after CT26 tumor inoculation for each group of mice described in the figure. **FIG. 27B** and **FIG. 27C** show the overall survival percentage, and statistics, of mice and tumor rejection through forty days after tumor inoculation. In **FIG. 27B**, the different treatment conditions are identified as: untreated: “a”, “e”, and “h”;  $\alpha$ PD-L1 (10F.9G2): “b”;  $\alpha$ PD-1 (RMP1-14): “c”;  $\alpha$ OX40 (OX86): “d”; PD-L1/OX40: “f”;  $\alpha$ PD-1 (RMP1-14)/OX40: “g”; PD-1-Fc-OX40L (100  $\mu$ g x 2): “i”; PD-1-Fc-OX40L (150  $\mu$ g x 2): “j”; and PD-1-Fc-OX40L (300  $\mu$ g x 2): “k”. **FIG. 27D** and **FIG. 27E** show immune profiling performed on CT26-tumor bearing mice treated with the murine PD-1-Fc-OX40L chimeric protein or antibodies (as monotherapies of  $\alpha$ PD-1,  $\alpha$  PD-L1, or  $\alpha$ OX40 or as combination therapy of  $\alpha$ PD-L1 and  $\alpha$ OX40 or  $\alpha$ PD-1 and  $\alpha$ OX40). In both **FIG. 27D** and **FIG. 27E**, the order of test articles from left to right is: untreated,  $\alpha$ PD-1 (RMP1-14),  $\alpha$ PD-L1 (10F.9G2),  $\alpha$ OX40 (OX86),  $\alpha$ PD-1 (RMP1-14)/OX40, PD-L1/OX40, PD-1-Fc-OX40L (150  $\mu$ g x 2), and PD-1-Fc-OX40L (300  $\mu$ g x 2). **FIG. 27F** summarizes the treatment outcomes for each experimental group. **FIG. 27G** and **FIG. 27H** show *in vivo* anti-tumor activity of the mPD-1-Fc-OX40L chimeric protein in the CT26 tumor model. In **FIG. 27G**, left two panels: untreated is the top curve. In **FIG. 27G**, third panel from left: curves top to bottom are untreated,  $\alpha$ PD1 (2x100  $\mu$ g),  $\alpha$ PD-L1 (1x100  $\mu$ g), and  $\alpha$ PD-L1 (2x100  $\mu$ g). In **FIG. 27G**, rightmost panel: curves top to bottom are untreated,  $\alpha$ OX40/ $\alpha$ PD-L1 (1x100  $\mu$ g),  $\alpha$ OX40/ $\alpha$ PD-L1 (2x100  $\mu$ g),  $\alpha$ OX40/ $\alpha$ PD1 (2x100  $\mu$ g). In **FIG. 27H**, panel labelled “ARC Fusion Protein,” curves are, top to bottom: untreated, PD1-Fc-OX40L (300  $\mu$ g x 2), and PD1-Fc-OX40L (150  $\mu$ g x 2). In **FIG. 27H**, panel labelled “OX40 Agonist,” curves are, top to bottom: untreated and  $\alpha$ OX86. In **FIG. 27H**, panel labelled “PD-1/L1 Blockade,” curves are, top to bottom: untreated,  $\alpha$ PD1,  $\alpha$ PD-L1. In **FIG. 27H**, panel labelled “Antibody Combinations,” curves are, top to bottom: untreated,  $\alpha$ PD1/ $\alpha$ OX86,  $\alpha$ PD-L1/ $\alpha$ OX86.

**FIG. 28A** to **FIG. 28C** show ELISA assays demonstrating binding affinity of the different domains of human PD-1-Fc-OX40L chimeric protein (also referred to as SL-279252) for their respective binding histograms partners. In **FIG. 28C**, for each concentration, left is OX40-His and right is HVEM-His.



**FIG. 29A** shows the characterization of human cell lines used for *in vitro* binding to human PD-1-Fc-OX40L. **FIG. 29B** to **FIG. 29D** show binding of hPD-1-Fc-OX40L to cells expressing, respectively, PD-L1, PD-L2, or OX40. In each, the right panel shows the titration curve for increasing concentrations of the chimeric protein.

**FIG. 30** shows characterization of human PD-1-Fc-OX40L fusion protein (SL-279252) by Western blot analysis. Each of the three domains of the chimeric protein was probed, respectively, with an anti-PD-1, anti -Fc, or anti -OX40L antibody. Untreated samples of the hPD-1-Fc-OX40L fusion protein, *e.g.*, control, were loaded into lane 1 in all the blots (no  $\beta$ -mercaptoethanol or Peptide:N-Glycosidase (PNGase)).

**FIG. 31A** and **FIG. 31B** show functional ELISA (**FIG. 31A**) and cell binding assays (**FIG. 31B**) which determine whether glycosylation of the PD-1-Fc-OX40L fusion protein (SL-279252) impacts its function.

**FIG. 32A** to **32G** show *in vitro* functional assays of human PD-1-Fc-OX40L fusion protein. In **FIG. 32C**, the hPD-1-Fc-OX40L induced higher levels of secreted IL2 in PC3 cells (**FIG. 32C**, left bundle) than in HCC827 cells (**FIG. 32C**, right bundle). **FIG. 32D** is a flow cytometry analysis of cells taken from the T cell/tumor cell co-culture assays outlined in **FIG. 32C**. The left-most bars indicate the proportion of CD4+ or CD8+ cells expressing Ki67 (as an indicator of proliferation) in the absence of tumor cells. The second from left bars indicates the proportion of CD4+ or CD8+ cells expressing Ki67 in the presence of tumor cells but without ARC, whereas the third-from left and right-most bars indicate the proportion of CD4+ or CD8+ cells expressing Ki67 in the presence of 500 ng or 5 ug of ARC, respectively. **FIG. 32F** and **FIG. 32G**, the order of test articles in the inset histograms, left to right: media control, IgG control, PD1-Fc, OX40L-Fc, PD-1-Fc/OX40L-Fc, and PD-1-Fc-Ox40L.

**FIG. 33A** to **FIG. 33C** show ELISA assays demonstrating binding affinity and cross-reactivity of the human PD-1-Fc-OX40L fusion protein to PD-L1 (**FIG. 33A**) and PD-L2 (**FIG. 33A**) of cynomolgus macaque or to OX40 (**FIG. 33C**) of rhesus macaque.

**FIG. 34** shows, without wishing to be bound by theory, an *in silico* predicted structure of monomeric TIM3-Fc-OX40L chimeric protein (SL-366252).

**FIG. 35** shows characterization of murine TIM3-Fc-OX40L chimeric protein (SL-366252) by Western blot analysis. Specifically, each individual domain of the fusion construct was probed using an anti-TIM3, anti-Fc, or anti-OX40L antibody. Untreated samples of the mTIM3-Fc-OX40L chimeric protein, *e.g.*, control, were loaded into lane 2 in all the blots (no  $\beta$ -mercaptoethanol or PNGase). Samples in lane 3 were treated with the reducing agent,  $\beta$ -mercaptoethanol, while samples in lane 4 were treated with PNGase.

**FIG. 36** shows *ex vivo* cell binding assays demonstrating the ability of the TIM3-Fc-OX40L chimeric protein to bind its binding partners (*e.g.*, receptor or ligand) on the surface of a mammalian cell membrane. Specifically, the graph shows the binding of the TIM3-Fc-OX40L chimeric protein to mOX40 (CHOK1-mOX40 is top curve, CHOK1 parental is bottom).

**FIG. 37A** to **FIG. 37C** show *in vivo* functional assays of the murine TIM3-Fc-OX40L chimeric protein. Immune profiling was performed on tumor-bearing mice treated with mTIM3-Fc-OX40L chimeric protein. **FIG. 37A** shows

changes in the CD4+ and CD8+ T-cell populations in mice treated with the chimeric protein. **FIG. 37B** shows changes in the CD4+CD25- effector T cells or CD4+CD25+ regulatory T cells in mice treated with the chimeric protein. **FIG. 37C** shows tetramer staining to analyze the fraction of CD8+ T cells within splenocytes or tumor infiltrated lymphocytes (TIL) that recognized the AH1 tumor antigen natively expressed by CT26 tumors. In all of the panels, the left condition is untreated and the right condition is mTIM3-Fc-OX40L (150 µg x 2).

**FIG. 38A** to **FIG. 38C** show results from *in vivo* tumor studies demonstrating that mTIM3-Fc-OX40L chimeric protein had significant anti-tumor activity in the CT26 mouse model. Mice were inoculated with CT26 tumors. When tumors reached 4-5 mm, mice were treated twice with 150 µg of mTIM3-Fc-OX40L chimeric protein or with control antibodies. **FIG. 38A** shows the evolution of tumor size over forty-five days after tumor inoculation for each group. **FIG. 38B** shows the overall survival percentage of mice through fifty days after tumor inoculation (TIM3-Fc-OX40L is the top curve). **FIG. 38C** summarizes the group sizes and treatment outcomes for each group.

**FIG. 39** is a table showing joining linkers and Fc linkers that can be combined into exemplary modular linkers. The exemplary modular linkers shown can be combined with any herein-described Type I and Type II proteins and/or extracellular domains of a herein described Type I and Type II proteins to form a chimeric protein of the present invention.

## DETAILED DESCRIPTION

The present invention is based, in part, on the discovery that chimeric proteins can be engineered from the extracellular, or effector, regions of immune-modulating transmembrane proteins in a manner that exploits the orientations of these proteins (*e.g.*, Type I versus Type II) and therefore allows the delivery of immune stimulatory and/or immune inhibitory signals, including, for example, masking an immune inhibitory signal and replacing it with an immune stimulatory signal in the treatment of cancer.

### Chimeric Proteins

In some aspects, the chimeric protein is of a general structure of: N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker having at least one cysteine residue capable of forming a disulfide bond (including without limitation, hinge-CH2-CH3 Fc domain is derived from human IgG4), and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, where the linker connects the first domain and the second domain and optionally comprises one or more joining linkers as described herein, where one of the first and second extracellular domains is an immune inhibitory signal and one of the first and second extracellular domains is an immune stimulatory signal.

In embodiments, chimeric protein refers to a recombinant fusion protein, *e.g.*, a single polypeptide having the extracellular domains described herein. For example, in embodiments, the chimeric protein is translated as a single unit in a cell. In embodiments, chimeric protein refers to a recombinant protein of multiple polypeptides, *e.g.*, multiple extracellular domains described herein, that are linked to yield a single unit, *e.g.*, *in vitro* (*e.g.*, with one or

more synthetic linkers described herein). In embodiments, the chimeric protein is chemically synthesized as one polypeptide or each domain may be chemically synthesized separately and then combined. In embodiments, a portion of the chimeric protein is translated and a portion is chemically synthesized.

In embodiments, an extracellular domain refers to a portion of a transmembrane protein which is capable of interacting with the extracellular environment. In embodiments, an extracellular domain refers to a portion of a transmembrane protein which is sufficient to bind to a ligand or receptor and effectively transmit a signal to a cell. In embodiments, an extracellular domain is the entire amino acid sequence of a transmembrane protein which is external of a cell or the cell membrane. In embodiments, an extracellular domain is the that portion of an amino acid sequence of a transmembrane protein which is external of a cell or the cell membrane and is needed for signal transduction and/or ligand binding as may be assayed using methods known in the art (*e.g.*, *in vitro* ligand binding and/or cellular activation assays).

In embodiments, an immune inhibitory signal refers to a signal that diminishes or eliminates an immune response. For example, in the context of oncology, such signals may diminish or eliminate antitumor immunity. Under normal physiological conditions, inhibitory signals are useful in the maintenance of self-tolerance (*e.g.*, prevention of autoimmunity) and also to protect tissues from damage when the immune system is responding to pathogenic infection. For instance, without limitation, immune inhibitory signal may be identified by detecting an increase in cellular proliferation, cytokine production, cell killing activity or phagocytic activity when such an inhibitory signal is blocked.

In embodiments, an immune stimulatory signal refers to a signal that enhances an immune response. For example, in the context of oncology, such signals may enhance antitumor immunity. For instance, without limitation, immune stimulatory signal may be identified by directly stimulating proliferation, cytokine production, killing activity or phagocytic activity of leukocytes. Specific examples include direct stimulation of TNF superfamily receptors such as OX40, LT $\beta$ R, 4-1BB or TNFRSF25 using either receptor agonist antibodies or using chimeric proteins encoding the ligands for such receptors (OX40L, LIGHT, 4-1BBL, TL1A, respectively). Stimulation from any one of these receptors may directly stimulate the proliferation and cytokine production of individual T cell subsets. Another example includes direct stimulation of an immune inhibitory cell with through a receptor that inhibits the activity of such an immune suppressor cell. This would include, for example, stimulation of CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells with a GITR agonist antibody or GITRL containing chimeric protein, which would reduce the ability of those regulatory T cells to suppress the proliferation of conventional CD4<sup>+</sup> or CD8<sup>+</sup> T cells. In another example, this would include stimulation of CD40 on the surface of an antigen presenting cell using a CD40 agonist antibody or a chimeric protein containing CD40L, causing activation of antigen presenting cells including enhanced ability of those cells to present antigen in the context of appropriate native costimulatory molecules, including those in the B7 or TNF superfamily. In another example, this would include stimulation of LTBR on the surface of a lymphoid or stromal cell using a LIGHT containing chimeric protein, causing activation of the lymphoid cell and/or production of pro-inflammatory cytokines or chemokines to further stimulate an immune response, optionally within a tumor.

Membrane proteins typically consist of an extracellular domain, one or a series of trans-membrane domains, and an intracellular domain. Without wishing to be bound by theory, the extracellular domain of a membrane protein is responsible for interacting with a soluble or membrane bound receptor or ligand. Without wishing to be bound by theory, the trans-membrane domain(s) are responsible for localizing a protein to the plasma membrane. Without wishing to be bound by theory, the intracellular domain of a membrane protein is responsible for coordinating interactions with cellular signaling molecules to coordinate intracellular responses with the extracellular environment (or visa-versa). There are two types of single-pass membrane proteins, those with an extracellular amino terminus and intracellular carboxy terminus (Type I) and those with an extracellular carboxy terminus and intracellular amino terminus (Type II). Both Type I and Type II membrane proteins can be either receptors or ligands. For Type I membrane proteins, the amino terminus of the protein faces outside the cell, and therefore contains the functional domains that are responsible for interacting with other binding partners (either ligands or receptors) in the extracellular environment. For Type II membrane proteins, the carboxy terminus of the protein faces outside the cell, and therefore contains the functional domains that are responsible for interacting with other binding partners (either ligands or receptors) in the extracellular environment. Thus, these two types of proteins have opposite orientations to each other.

Because the outward facing domains of Type I and Type II membrane proteins are opposite, it is possible to link the extracellular domains of a Type I and Type II membrane protein such that the 'outward facing' domains of the molecules are also in opposing orientation to each other (**FIG. 1D**). The resulting construct would therefore consist of the extracellular domain of a Type I membrane protein on the 'left' side of the molecule, connected to the extracellular domain of a Type II membrane protein on the 'right' side of the molecule using a linker sequence. This construct could be produced by cloning of these three fragments (the extracellular domain of a Type I protein, followed by a linker sequence, followed by the extracellular domain of a Type II protein) into a vector (plasmid, viral or other) wherein the amino terminus of the complete sequence corresponded to the 'left' side of the molecule containing the Type I protein and the carboxy terminus of the complete sequence corresponded to the 'right' side of the molecule containing the Type II protein. Accordingly, in embodiments, the present chimeric proteins are engineered as such.

In embodiments, the extracellular domain may be used to produce a soluble protein to competitively inhibit signaling by that receptor's ligand. In embodiments, the extracellular domain may be used to provide artificial signaling.

In embodiments, the extracellular domain of a Type I transmembrane protein is an immune inhibitory signal. In embodiments, the extracellular domain of a Type II transmembrane protein is an immune stimulatory signal.

In embodiments, the present chimeric proteins comprise an extracellular domain of a Type I transmembrane protein, or a functional fragment thereof. In embodiments, the present chimeric proteins comprise an extracellular domain of a Type II transmembrane protein, or a functional fragment thereof. In embodiments, the present chimeric

proteins comprise an extracellular domain of a Type I transmembrane protein, or a functional fragment thereof, and an extracellular domain of a Type II transmembrane protein, or a functional fragment thereof.

In embodiments, the present chimeric proteins may be engineered to target one or more molecules that reside on human leukocytes including, without limitation, the extracellular domains (where applicable) of SLAMF4, IL-2 R  $\alpha$ , 4-1BB/TNFRSF9, IL-2 R  $\beta$ , ALCAM, BTLA, B7-1, IL-4 R, B7-H3, BLAME/SLAMFS, CEACAM1, IL-6 R, IL-7 R $\alpha$ , IL-10R  $\alpha$ , IL-10 R  $\beta$ , IL-12 R  $\beta$  1, IL-12 R  $\beta$  2, CD2, IL-13 R  $\alpha$  1, IL-13, CD3, CD4, ILT2/CDS5j, ILT3/CDS5k, ILT4/CDS5d, ILT5/CDS5a, Integrin  $\alpha$  4/CD49d, CDS, Integrin  $\alpha$  E/CD103, CD6, Integrin  $\alpha$  M/CD 11 b, CDS, Integrin  $\alpha$  X/CD11c, Integrin  $\beta$  2/CDIS, KIR/CD15S, CD27/TNFRSF7, KIR2DL1, CD2S, KIR2DL3, CD30/TNFRSFS, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, CD40 Ligand/TNFSF5, LAG-3, CD43, LAIR1, CD45, LAIR2, CDS3, Leukotriene B4-R1, CDS4/SLAMF5, NCAM-L1, CD94, NKG2A, CD97, NKG2C, CD229/SLAMF3, NKG2D, CD2F-10/SLAMF9, NT-4, CD69, NTB-A/SLAMF6, Common  $\gamma$  Chain/IL-2 R  $\gamma$ , Osteopontin, CRACC/SLAMF7, PD-1, CRTAM, PSGL-1, CTLA-4, RANK/TNFRSF11A, CX3CR1, CX3CL1, L-Selectin, SIRP  $\beta$  1, SLAM, TCCR/WSX-1, DNAM-1, Thymopoietin, EMMPRIN/CD147, TIM-1, EphB6, TIM-2, Fas/TNFRSF6, TIM-3, Fas Ligand/TNFSF6, TIM-4, Fc $\gamma$  RIII/CD16, TIM-6, TNFR1/TNFRSF1A, Granulysin, TNF RIII/TNFRSF1B, TRAIL RI/TNFRSF10A, ICAM-1/CD54, TRAIL R2/TNFRSF10B, ICAM-2/CD102, TRAILR3/TNFRSF10C, IFN- $\gamma$ R1, TRAILR4/TNFRSF10D, IFN- $\gamma$  R2, TSLP, IL-1 R1, LIGHT, LTBR (TNFRSF3) and TSLP R.

The activation of regulatory T cells is critically influenced by costimulatory and coinhibitory signals. Two major families of costimulatory molecules include the B7 and the tumor necrosis factor (TNF) families. These molecules bind to receptors on T cells belonging to the CD28 or TNF receptor families, respectively. Many well-defined coinhibitors and their receptors belong to the B7 and CD28 families.

In embodiments, the present chimeric proteins may be engineered to target one or more molecules involved in immune inhibition, including for example: CSF1R, CTLA-4, PD-L1, PD-L2, PD-1, BTLA, HVEM, TIM3, GAL9, VISTA/SIG8, KIR, 2B4, TIGIT, CD160 (also referred to as BY55), CHK 1 and CHK2 kinases, A2aR, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), and various B-7 family ligands (including, but are not limited to, B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of an immune inhibitory agent, including without limitation, one or more of TIM-3, BTLA, PD-1, CSF1R, CTLA-4, CD244, CD160, TIGIT, CD172a(SIRP1 $\alpha$ ), 2B4, VISTA, VSIG8, CD200 and TMIGD2.

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of a Type I membrane protein which has immune inhibitory properties. In embodiments, the chimeric protein is engineered to disrupt, block, reduce, and/or inhibit the transmission of an immune inhibitory signal.

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of an immune stimulatory signal is one or more of 4-1BBL, OX-40 ligand (OX-40L), LIGHT (CD258), GITR ligand (GITRL), CD70, CD30 ligand, CD40 ligand (CD40L), CD137 ligand, TRAIL, and TL1A.

In embodiments, the chimeric protein simulates binding of an inhibitory signal ligand to its cognate receptor (e.g., PD-1 to PD-L1 or PD-L2; e.g., CD172a(SIRP1 $\alpha$ ) to CD47; e.g., CD115 to CSF1; e.g., TIM-3 to galectin-9 or phosphatidylserine) but inhibits the inhibitory signal transmission to an immune cell (e.g., a T cell, macrophage or other leukocyte).

In embodiments, the chimeric protein comprises an immune inhibitory receptor extracellular domain and an immune stimulatory ligand extracellular domain which can, without limitation, deliver an immune stimulation to a T cell while masking a tumor cell's immune inhibitory signals. In embodiments, the chimeric protein delivers a signal that has the net result of T cell activation.

In embodiments, the chimeric protein comprises an immune inhibitory signal which is an ECD of a receptor of an immune inhibitory signal and this acts on a tumor cell that bears a cognate ligand of the immune inhibitory signal. In embodiments, the chimeric protein comprises an immune stimulatory signal which is an ECD of a ligand of an immune stimulatory signal and this acts on a T cell that bears a cognate receptor of the immune stimulatory signal. In embodiments, the chimeric protein comprises both (i) an immune inhibitory signal which is a receptor of an immune inhibitory signal and this acts on a tumor cell that bears a cognate ligand of the immune inhibitory signal and (ii) an immune stimulatory signal which is a ligand of an immune stimulatory signal and this acts on a T cell that bears a cognate receptor of the immune stimulatory signal.

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of one or more of the immune-modulating agents described in Mahoney, *Nature Reviews Drug Discovery* 2015;14:561-585, the entire contents of which are hereby incorporated by reference.

In embodiments, a chimeric protein is capable of binding murine ligand(s)/receptor(s).

In embodiments, a chimeric protein is capable of binding human ligand(s)/receptor(s)

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of a Type II membrane protein which has immune stimulatory properties. In embodiments, the chimeric protein is engineered to enhance, increase, and/or stimulate the transmission of an immune stimulatory signal.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent PD-1 and is paired with an immune stimulatory agent as follows: PD-1/4-1BBL; PD-1/OX-40L; PD-1/LIGHT; PD-1/GITRL; PD-1/CD70; PD-1/CD30L; PD-1/CD40L; and PD-1/TL1A. In embodiments the chimeric protein is PD-1-Fc-LIGHT or PD-1-Fc-OX40L, in which the Fc represents a linker that comprises at least a portion of an Fc domain of an antibody and which comprises at least one cysteine residue capable of forming a disulfide bond.

In an embodiment, the chimeric protein comprises the extracellular domain of the immune inhibitory agent PD-1 and is paired with the immune stimulatory agent OX-40L. In embodiments, the chimeric protein binds to human PD-L1 or PD-L2 with a  $K_D$  of about 1 nM to about 5 nM, for example, about 1 nM, about 1.5 nM, about 2 nM, about 2.5 nM, about 3 nM, about 3.5 nM, about 4 nM, about 4.5 nM, or about 5 nM. In embodiments, the chimeric protein binds to human PD-L1 with a  $K_D$  of about 5 nM to about 15 nM, for example, about 5 nM, about 5.5 nM, about 6

nM, about 6.5 nM, about 7 nM, about 7.5 nM, about 8 nM, about 8.5 nM, about 9 nM, about 9.5 nM, about 10 nM, about 10.5 nM, about 11 nM, about 11.5 nM, about 12 nM, about 12.5 nM, about 13 nM, about 13.5 nM, about 14 nM, about 14.5 nM, or about 15 nM.

In embodiments, the chimeric protein exhibits enhanced stability and protein half-life. In embodiments, the chimeric protein binds to FcRn with high affinity. In embodiments, the chimeric protein may bind to FcRn with a  $K_D$  of about 1 nM to about 80 nM. For example, the chimeric protein may bind to FcRn with a  $K_D$  of about 1 nM, about 2 nM, about 3 nM, about 4 nM, about 5 nM, about 6 nM, about 7 nM, about 8 nM, about 9 nM, about 10 nM, about 15 nM, about 20 nM, about 25 nM, about 30 nM, about 35 nM, about 40 nM, about 45 nM, about 50 nM, about 55 nM, about 60 nM, about 65 nM, about 70 nM, about 71 nM, about 72 nM, about 73 nM, about 74 nM, about 75 nM, about 76 nM, about 77 nM, about 78 nM, about 79 nM, or about 80 nM. In an embodiment, the chimeric protein may bind to FcRn with a  $K_D$  of about 9 nM. In embodiments, the chimeric protein does not substantially bind to other Fc receptors (*i.e.* other than FcRn) with effector function.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent PD-L1 or PD-L2 and is paired with an immune stimulatory receptor as follows: PD-L1/4-1BB; PD-L1/OX-40; PD-L1/HVEM; PD-L1/GITR; PD-L1/CD27; PD-L1/CD28; PD-L1/CD30; PD-L1/CD40 and PD-L1/CD137.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent PD-L2 and is paired with an immune stimulatory receptor as follows: PD-L2/4-1BB; PD-L2/OX-40; PD-L2/HVEM; PD-L2/GITR; PD-L2/CD27; PD-L2/CD28; PD-L2/CD30; PD-L2/CD40 and PD-L2/CD137.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent TIM-3 and is paired with an immune stimulatory agent as follows: TIM-3/OX-40L; TIM-3/LIGHT; TIM-3/GITRL; TIM-3/CD70; TIM-3/CD30L; TIM-3/CD40L; TIM-3/CD137L; TIM-3/TL1A; and TIM-3/OX40L. In embodiments the chimeric protein is TIM3-Fc-OX40L, in which the Fc represents a linker that comprises at least a portion of an Fc domain of an antibody and which comprises at least one cysteine residue capable of forming a disulfide bond.

In embodiments, there is provided a method of treating a cancer or an inflammatory disease (*e.g.*, any one of those described elsewhere herein) by administering to a subject a TIM3-Fc-OX40L chimeric protein, in which the Fc represents a linker that comprises at least a portion of an Fc domain of an antibody and which comprises at least one cysteine residue capable of forming a disulfide bond. In embodiments, the method generates a memory response which may, *e.g.*, be capable of preventing relapse.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent BTLA and is paired with an immune stimulatory agent as follows: BTLA/OX-40L; BTLA/LIGHT; BTLA/GITRL; BTLA/CD70; BTLA/CD30L; BTLA/CD40L; BTLA/CD137L; BTLA/TL1A; and BTLA/OX40L.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent CD172a(SIRP1 $\alpha$ ) and is paired with an immune stimulatory agent as follows: CD172a(SIRP1 $\alpha$ )/OX-40L; CD172a(SIRP1 $\alpha$ )/LIGHT; CD172a(SIRP1 $\alpha$ )/CD70; CD172a(SIRP1 $\alpha$ )/CD30L; CD172a(SIRP1 $\alpha$ )/CD40L;

CD172a(SIRP1 $\alpha$ )/CD137L; CD172a(SIRP1 $\alpha$ )/TL1A; and CD172a(SIRP1 $\alpha$ )/OX40L. In embodiments the chimeric protein is CD172a(SIRP1 $\alpha$ )-Fc-CD40L or CD172a(SIRP1 $\alpha$ )-Fc-LIGHT, in which the Fc represents a linker that comprises at least a portion of an Fc domain of an antibody and which comprises at least one cysteine residue capable of forming a disulfide bond.

In embodiments, there is provided a method of treating a cancer or an inflammatory disease (e.g., any one of those described elsewhere herein) by administering to a subject a CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein, in which the Fc represents a linker that comprises at least a portion of an Fc domain of an antibody and which comprises at least one cysteine residue capable of forming a disulfide bond. In embodiments, the method generates a memory response which may, e.g., be capable of preventing relapse. In embodiments, the method includes a sustained therapeutic effect of the CD172a(SIRP $\alpha$ )-Fc-CD40L, e.g., due to binding of the extracellular domain components to their respective binding partners with slow off rates ( $K_d$  or  $K_{off}$ ) to optionally provide sustained negative signal masking effect and/or a longer positive signal effect, e.g., to allow an effector cell to be adequately stimulated for an anti-tumor effect.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent CD115 and is paired with an immune stimulatory agent as follows: CD115/OX-40L; CD115/LIGHT; CD115/CD70; CD115/CD30L; CD115/CD40L; CD115/CD137L; CD115/TL1A; and CD115/OX40L.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent TMIGD2 and is paired with an immune stimulatory agent as follows: TMIGD2/OX-40L; TMIGD2/LIGHT; TMIGD2/GITRL; TMIGD2/CD70; TMIGD2/CD30L; TMIGD2/CD40L; TMIGD2/CD137L; TMIGD2/TL1A; and TMIGD2/OX40L.

In embodiments, the chimeric protein comprises the extracellular domain of the immune inhibitory agent CD200 and is paired with an immune stimulatory agent as follows: CD200/OX-40L; CD200/LIGHT; CD200/GITRL; CD200/CD70; CD200/CD30L; CD200/CD40L; CD200/CD137L; CD200/TL1A; and CD200/OX40L.

In embodiments, the present chimeric proteins may comprises variants of the extracellular domains described herein, for instance, a sequence having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99%) sequence identity with the known amino acid or nucleic acid sequence of the extracellular domains, e.g., human extracellular domains, e.g., one or more of SEQ IDs NOs: 2, 4, 7, 10, 12, 15, 18, 21, 24, 29, 32, 34, 36, 42, and 44.



In embodiments, the chimeric protein of the present invention comprises an extracellular domain of TIM3 (SEQ ID NO: 2).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of PD-1 (SEQ ID NO: 7).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CD172a(SIRP1 $\alpha$ ) (SEQ ID NO: 10).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of OX40L (SEQ ID NO: 4).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CD40L (SEQ ID NO: 12).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of TIM3 (SEQ ID NO: 2) and the extracellular domain of OX40L (SEQ ID NO: 4).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of PD-1 (SEQ ID NO: 7) and the extracellular domain of OX40L (SEQ ID NO: 4).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CD172a(SIRP1 $\alpha$ ) (SEQ ID NO: 10) and the extracellular domain of CD40L (SEQ ID NO: 12).

In embodiments, the chimeric protein of the present invention comprises the hinge-CH2-CH3 domain from a human IgG4 antibody sequence (SEQ ID NO: 45, 46, or 47).

In embodiments, a chimeric protein comprises a modular linker as shown in **FIG. 39**.

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of TIM3 and the extracellular domain of OX40L, using the hinge-CH2-CH3 domain from a human IgG4 antibody sequence as a linker (this TIM3-Fc-OX40L chimera is SEQ ID NO: 5).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of PD-1 (SEQ ID NO: 7) and the extracellular domain of OX40L, using the hinge-CH2-CH3 domain from a human IgG4 antibody sequence as a linker (this PD-1 -Fc-OX40L chimera is SEQ ID NO: 8).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CD172a(SIRP1 $\alpha$ ) (SEQ ID NO: 10) and the extracellular domain of CD40L, using the hinge-CH2-CH3 domain from a human IgG4 antibody sequence as a linker (this CD172a(SIRP1 $\alpha$ )-Fc-CD40L chimera is SEQ ID NO: 13).

In another embodiment, the chimeric protein of the present invention comprises the extracellular domain of PD-1 and the extracellular domain of TL1A (SEQ ID NO: 15).

Additional examples include a chimeric protein encoding the extracellular domain of BTLA, linked through an Fc to OX40L (SEQ ID NO: 19).

Another example is a chimeric protein incorporating the extracellular domain of TMIGD2 adjoined with an Fc linker sequence to the extracellular domain of human OX40L (SEQ ID NO: 22).

Another example is a chimeric protein incorporating the extracellular domain of CD172a(SIRPa) adjoined with an Fc linker sequence to the extracellular domain of human OX40L (SEQ ID NO: 26).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R (SEQ ID NO: 29).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R (SEQ ID NO: 29) and an extracellular domain of CD40L (SEQ ID NO: 12).

In embodiments, the chimeric protein of the present invention comprises an extracellular domain of CSF1R (SEQ ID NO: 29) and an extracellular domain of CD40L (SEQ ID NO: 12), using the hinge-CH2-CH3 domain from a human IgG4 antibody sequence as a linker (this CSF1R-Fc-CD40L chimera is SEQ ID NO: 30)

In embodiments, a chimeric protein can comprise an extracellular domain from a sequence identified herein combined with an extracellular domain from another sequence identified herein.

In embodiments, the present chimeric proteins may be variants described herein, for instance, the present chimeric proteins may have a sequence having at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99%) sequence identity with the amino acid sequence of the present chimeric proteins, e.g. one or more of SEQ IDs Nos 5, 5, 8, 13, 16, 19, 22, 25, 26, 27, or 30.

In embodiments, the present chimeric proteins comprise an extracellular domain of a human Type I transmembrane protein as recited in TABLE 1 of PCT/US2016/054598, or a functional fragment thereof. In embodiments, the present chimeric proteins comprise an extracellular domain of a human Type II transmembrane protein as recited in TABLE 2 of PCT/US2016/054598, or a functional fragment thereof. In embodiments, the present chimeric proteins comprise an extracellular domain of a Type I transmembrane protein as recited in TABLE 1 of PCT/US2016/054598, or a functional fragment thereof, and an extracellular domain of a Type II transmembrane protein as recited in TABLE 2 of PCT/US2016/054598, or a functional fragment thereof. The entire contents of PCT/US2016/054598 are hereby incorporated by reference.

In embodiments, the chimeric protein may comprise an amino acid sequence having one or more amino acid mutations relative to any of the protein sequences described herein. In embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

In embodiments, the amino acid mutations are amino acid substitutions, and may include conservative and/or non-conservative substitutions.

"Conservative substitutions" may be made, for instance, on the basis of similarity in polarity, charge, size, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the amino acid residues involved. The 20 naturally occurring amino acids can be grouped into the following six standard amino acid groups: (1) hydrophobic: Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr; Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe.

As used herein, "conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed within the same group of the six standard amino acid groups shown above. For example, the exchange of Asp by Glu retains one negative charge in the so modified polypeptide. In addition, glycine and proline may be substituted for one another based on their ability to disrupt  $\alpha$ -helices.

As used herein, "non-conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed in a different group of the six standard amino acid groups (1) to (6) shown above.

In embodiments, the substitutions may also include non-classical amino acids (e.g., selenocysteine, pyrrolysine, *N*-formylmethionine  $\beta$ -alanine, GABA and  $\delta$ -Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteine, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$  methyl amino acids, C  $\alpha$ -methyl amino acids, N  $\alpha$ -methyl amino acids, and amino acid analogs in general).

Mutations may also be made to the nucleotide sequences of the chimeric proteins by reference to the genetic code, including taking into account codon degeneracy.

In embodiments, the chimeric protein comprises a linker. In embodiments, the linker comprising at least one cysteine residue capable of forming a disulfide bond. As described elsewhere herein, such at least one cysteine residue capable of forming a disulfide bond is, without wishing to be bound by theory, responsible for maintain a proper multimeric state of the chimeric protein and allowing for efficient production.

In embodiments, there is provided a method of making a stable chimeric protein comprising adjoining a Type I and Type II transmembrane protein extracellular domain with a linker comprising at least one cysteine residue capable of forming a disulfide bond such that the resultant chimeric protein is properly folded and/or forms into a stable multimeric state.

In embodiments, the linker may be derived from naturally-occurring multi-domain proteins or are empirical linkers as described, for example, in Chichili *et al.*, (2013), Protein Sci. 22(2):153-167, Chen *et al.*, (2013), Adv Drug Deliv Rev. 65(10):1357-1369, the entire contents of which are hereby incorporated by reference. In embodiments, the linker may be designed using linker designing databases and computer programs such as those described in Chen *et al.*, (2013), Adv Drug Deliv Rev. 65(10):1357-1369 and Crasto *et. al.*, (2000), Protein Eng. 13(5):309-312, the entire contents of which are hereby incorporated by reference.

In embodiments, the linker is a synthetic linker such as PEG.

In embodiments, the linker is a polypeptide. In embodiments, the linker is less than about 500 amino acids long, about 450 amino acids long, about 400 amino acids long, about 350 amino acids long, about 300 amino acids long, about 250 amino acids long, about 200 amino acids long, about 150 amino acids long, or about 100 amino acids long. For example, the linker may be less than about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids long. In embodiments, the linker is flexible. In another embodiment, the linker is rigid.

In embodiments, the linker is substantially comprised of glycine and serine residues (*e.g.*, about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97%, or about 98%, or about 99%, or about 100% glycines and serines).

In embodiments, the linker is a hinge region of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.*, IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region, found in IgG, IgA, IgD, and IgE class antibodies, acts as a flexible spacer, allowing the Fab portion to move freely in space. In contrast to the constant regions, the hinge domains are structurally diverse, varying in both sequence and length among immunoglobulin classes and subclasses. For example, the length and flexibility of the hinge region varies among the IgG subclasses. The hinge region of IgG1 encompasses amino acids 216-231 and, because it is freely flexible, the Fab fragments can rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges. IgG2 has a shorter hinge than IgG1, with 12 amino acid residues and four disulfide bridges. The hinge region of IgG2 lacks a glycine residue, is relatively short, and contains a rigid poly-proline double helix, stabilized by extra inter-heavy chain disulfide bridges. These properties restrict the flexibility of the IgG2 molecule. IgG3 differs from the other subclasses by its unique extended hinge region (about four times as long as the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible poly-proline double helix. In IgG3, the Fab fragments are relatively far away from the Fc fragment, giving the molecule a greater flexibility. The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the other subclasses. The hinge region of IgG4 is shorter than that of IgG1 and its flexibility is intermediate between that of IgG1 and IgG2. The flexibility of the hinge regions reportedly decreases in the order

IgG3>IgG1>IgG4>IgG2. In embodiments, the linker may be derived from human IgG4 and contain one or more mutations to enhance dimerization (including S228P) or FcRn binding.

According to crystallographic studies, the immunoglobulin hinge region can be further subdivided functionally into three regions: the upper hinge region, the core region, and the lower hinge region. See Shin *et al.*, 1992 *Immunological Reviews* 130:87. The upper hinge region includes amino acids from the carboxyl end of C<sub>H1</sub> to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the C<sub>H2</sub> domain and includes residues in C<sub>H2</sub>. *Id.* The core hinge region of wild-type human IgG1 contains the sequence Cys-Pro-Pro-Cys which, when dimerized by disulfide bond formation, results in a cyclic octapeptide believed to act as a pivot, thus conferring flexibility. In embodiments, the present linker comprises, one, or two, or three of the upper hinge region, the core region, and the lower hinge region of any antibody (e.g., of IgG, IgA, IgD, and IgE, inclusive of subclasses (e.g., IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region may also contain one or more glycosylation sites, which include a number of structurally distinct types of sites for carbohydrate attachment. For example, IgA1 contains five glycosylation sites within a 17-amino-acid segment of the hinge region, conferring resistance of the hinge region polypeptide to intestinal proteases, considered an advantageous property for a secretory immunoglobulin. In embodiments, the linker of the present invention comprises one or more glycosylation sites.

In embodiments, the linker comprises an Fc domain of an antibody (e.g., of IgG, IgA, IgD, and IgE, inclusive of subclasses (e.g., IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from a human IgG4 antibody. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from a human IgG1 antibody. In embodiments, the Fc domain exhibits increased affinity for and enhanced binding to the neonatal Fc receptor (FcRn). In embodiments, the Fc domain includes one or more mutations that increases the affinity and enhances binding to FcRn. Without wishing to be bound by theory, it is believed that increased affinity and enhanced binding to FcRn increases the *in vivo* half-life of the present chimeric proteins.

In embodiments, the Fc domain linker contains one or more amino acid substitutions at amino acid residue 250, 252, 254, 256, 308, 309, 311, 416, 428, 433 or 434 (in accordance with Kabat numbering, as in as in Kabat, *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference), or equivalents thereof. In an embodiment, the amino acid substitution at amino acid residue 250 is a substitution with glutamine. In an embodiment, the amino acid substitution at amino acid residue 252 is a substitution with tyrosine, phenylalanine, tryptophan or threonine. In an embodiment, the amino acid substitution at amino acid residue 254 is a substitution with threonine. In an embodiment, the amino acid substitution at amino acid residue 256 is a substitution with serine, arginine, glutamine, glutamic acid, aspartic acid, or threonine. In an embodiment, the amino acid substitution at amino acid residue 308 is a substitution with threonine. In an embodiment, the amino acid substitution at amino acid residue

309 is a substitution with proline. In an embodiment, the amino acid substitution at amino acid residue 311 is a substitution with serine. In an embodiment, the amino acid substitution at amino acid residue 385 is a substitution with arginine, aspartic acid, serine, threonine, histidine, lysine, alanine or glycine. In an embodiment, the amino acid substitution at amino acid residue 386 is a substitution with threonine, proline, aspartic acid, serine, lysine, arginine, isoleucine, or methionine. In an embodiment, the amino acid substitution at amino acid residue 387 is a substitution with arginine, proline, histidine, serine, threonine, or alanine. In an embodiment, the amino acid substitution at amino acid residue 389 is a substitution with proline, serine or asparagine. In an embodiment, the amino acid substitution at amino acid residue 416 is a substitution with serine. In an embodiment, the amino acid substitution at amino acid residue 428 is a substitution with leucine. In an embodiment, the amino acid substitution at amino acid residue 433 is a substitution with arginine, serine, isoleucine, proline, or glutamine. In an embodiment, the amino acid substitution at amino acid residue 434 is a substitution with histidine, phenylalanine, or tyrosine.

In embodiments, the Fc domain linker (e.g., comprising an IgG constant region) comprises one or more mutations such as substitutions at amino acid residue 252, 254, 256, 433, 434, or 436 (in accordance with Kabat numbering, as in as in Kabat, *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference). In an embodiment, the IgG constant region includes a triple M252Y/S254T/T256E mutation or YTE mutation. In another embodiment, the IgG constant region includes a triple H433K/N434F/Y436H mutation or KFH mutation. In a further embodiment, the IgG constant region includes an YTE and KFH mutation in combination.

In embodiments, the modified humanized antibodies of the invention comprise an IgG constant region that contains one or more mutations at amino acid residues 250, 253, 307, 310, 380, 416, 428, 433, 434, and 435. Illustrative mutations include T250Q, M428L, T307A, E380A, I253A, H310A, R416S, M428L, H433K, N434A, N434F, N434S, and H435A. In an embodiment, the IgG constant region comprises a M428L/N434S mutation or LS mutation. In another embodiment, the IgG constant region comprises a T250Q/M428L mutation or QL mutation. In another embodiment, the IgG constant region comprises an N434A mutation. In another embodiment, the IgG constant region comprises a T307A/E380A/N434A mutation or AAA mutation. In another embodiment, the IgG constant region comprises an I253A/H310A/H435A mutation or IHH mutation. In another embodiment, the IgG constant region comprises a H433K/N434F mutation. In another embodiment, the IgG constant region comprises a M252Y/S254T/T256E and a H433K/N434F mutation in combination.

Additional illustrative mutations in the IgG constant region are described, for example, in Robbie, *et al.*, Antimicrobial Agents and Chemotherapy (2013), 57(12):6147-6153, Dall'Acqua *et al.*, JBC (2006), 281(33):23514-24, Dall'Acqua *et al.*, Journal of Immunology (2002), 169:5171-80, Ko *et al.*, Nature (2014) 514:642-645, Grevys *et al.*, Journal of Immunology. (2015), 194(11):5497-508, and U.S. Patent No. 7,083,784, the entire contents of which are hereby incorporated by reference.

In embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 45, or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto. In embodiments, mutations are made to SEQ ID NO: 45 to increase stability and/or half-life. For instance, in embodiments, the linker has the amino acid sequence of SEQ ID NO: 46, or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto. In embodiments, the linker comprises the amino acid sequence of SEQ ID NO: 47, or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto.

Without wishing to be bound by theory, including a linker comprising at least a part of an Fc domain in a chimeric protein, helps avoid formation of insoluble and, likely, non-functional protein concatamers and/or aggregates. This is in part due to the presence of cysteines in the Fc domain which are capable of forming disulfide bonds between chimeric proteins.

An illustrative Fc stabilizing mutant is S228P. Illustrative Fc half-life extending mutants are T250Q, M428L, V308T, L309P, and Q311S and the present linkers may comprise 1, or 2, or 3, or 4, or 5 of these mutants.

Further, one or more joining linkers may be employed to connect an Fc domain in a linker (e.g., one of SEQ ID NO: 45, SEQ ID NO: 46, or SEQ ID NO: 47 or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto) and the extracellular domains. For example, any one of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, or variants thereof may connect an extracellular domain as described herein and a linker as described herein. Optionally, any one of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, or variants thereof are displaced between an extracellular domain as described herein and a linker as described herein. Optionally, any one of SEQ ID NOs: 45 to 94, or variants thereof are located between an extracellular domain as described herein and an Fc domain as described herein. In embodiments, a chimeric protein comprises one joining linker preceding an Fc domain and a second joining linker following the Fc domain; thus, a chimeric protein may comprise the following structure:

ECD 1 – Joining Linker 1 – Fc Domain – Joining Linker 2 – ECD 2.

In embodiments, the first and second joining linkers may be different or they may be the same.

The amino acid sequences of illustrative linkers are provided in Table 1 below:

**Table 1: Illustrative linkers (Fc domain linkers and joining linkers)**

SEQ ID NO.	Sequence
45	APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTVLHQDWLSGKEYCKVSSKGLPSSIEKTISNATGQPREPQVY TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYS RLTVDKSSWQEGNVFSCVMHEALHNHYTQKSLSLGLGK
46	APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTPHSDWLSGKEYCKVSSKGLPSSIEKTISNATGQPREPQVY

	TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSGDGSFFLYS RLTVDKSSWQEGNVFSCSVLHEALHNHYTQKSLSLGLK
47	APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVWVDVSQEDPEVQFNWYVDGVEVHNAKT KPREEQFNSTYRVVSVLTVLHQDWLMSGKEYCKVSSKGLPSSIEKTISNATGQPREPQVY TLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSGDGSFFLYS RLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLGLK
48	SKYGPPCPSCP
49	SKYGPPCPPCP
50	SKYGPP
51	IEGRMD
52	GGGVPRDCG
53	IEGRMDGGGGAGGGG
54	GGSGGGGS
55	GGSGGGGGSGGG
56	EGKSSGSGSESKST
57	GGSG
58	GGSGGGSGGGSG
59	EAAAKEAAAKEAAAK
60	EAAAREAAAREAAAREAAAR
61	GGGSGGGGSGGGGSAS
62	GGGGAGGGG
63	GS or GGS or LE
64	GSGSGS
65	GSGSGSGSGS
66	GGGGSAS
67	APAPAPAPAPAPAPAPAP
68	CPPC
69	GGGS
70	GGGSGGGGS
71	GGGSGGGGSGGGGS
72	GGGSGGGGSGGGGSGGGGS
73	GGGSGGGGSGGGGSGGGGSGGGGS
74	GGGSGGGGSGGGGSGGGGSGGGGSGGGGS
75	GGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
76	GGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS



77	GGSGGSGGGGSGGGGS
78	GGGGGGGG
79	GGGGGG
80	EAAAK
81	EAAAKEAAAK
82	EAAAKEAAAKEAAAK
83	AEAAAKEAAAKA
84	AEAAAKEAAAKEAAAKA
85	AEAAAKEAAAKEAAAKEAAAKA
86	AEAAAKEAAAKEAAAKEAAAKEAAAKA
87	AEAAAKEAAAKEAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKA
88	PAPAP
89	KESGSVSSEQLAQFRSLD
90	GSAGSAAGSGEF
91	GGGSE
92	GSESG
93	GSEGS
94	GEGGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGGS

Additional illustrative joining linkers include, but are not limited to, linkers having the sequence LE, GGGGS (SEQ ID NO: 69), (GGGGS)<sub>n</sub> (n=1-4) (SEQ ID NO: 69-72), (Gly)<sub>8</sub> (SEQ ID NO: 78), (Gly)<sub>6</sub> (SEQ ID NO: 79), (EAAAK)<sub>n</sub> (n=1-3) (SEQ ID NO: 80-82), A(EAAAK)<sub>n</sub>A (n = 2-5) (SEQ ID NO: 83-86), AEAAAKEAAAKA (SEQ ID NO: 83), A(EAAAK)<sub>4</sub>AEAE(AAAK)<sub>4</sub>A (SEQ ID NO: 87), PAPAP (SEQ ID NO: 88), KESGSVSSEQLAQFRSLD (SEQ ID NO: 89), EGKSSGSGSESKST (SEQ ID NO: 56), GSAGSAAGSGEF (SEQ ID NO: 90), and (XP)<sub>n</sub>, with X designating any amino acid, *e.g.*, Ala, Lys, or Glu.

In embodiments, the joining linker is substantially comprised of glycine and serine residues (*e.g.*, about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97%, or about 98%, or about 99%, or about 100%) glycines and serines). For example, in embodiments, the joining linker is (Gly<sub>4</sub>Ser)<sub>n</sub>, where n is from about 1 to about 8, *e.g.*, 1, 2, 3, 4, 5, 6, 7, or 8 (SEQ ID NO: 69 to SEQ ID NO: 76, respectively). In embodiments, the joining linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO: 77). Additional illustrative joining linkers include, but are not limited to, linkers having the sequence LE, (Gly)<sub>8</sub> (SEQ ID NO: 78), (Gly)<sub>6</sub> (SEQ ID NO: 79), (EAAAK)<sub>n</sub> (n=1-3) (SEQ ID NO: 80 - SEQ ID NO: 82), A(EAAAK)<sub>n</sub>A (n = 2-5) (SEQ ID NO: 83 – SEQ ID NO: 86), A(EAAAK)<sub>4</sub>AEAE(AAAK)<sub>4</sub>A (SEQ ID NO: 43), PAPAP (SEQ ID NO: 44), KESGSVSSEQLAQFRSLD (SEQ ID NO: 45), GSAGSAAGSGEF (SEQ ID NO: 87), and (XP)<sub>n</sub>, with X designating any amino acid, *e.g.*, Ala, Lys, or Glu. In embodiments, the joining linker is GGS.

[illegible]

In embodiments, a chimeric protein comprises a modular linker as shown in **FIG. 39**.

In embodiments, the linker may be flexible, including without limitation highly flexible. In embodiments, the linker may be rigid, including without limitation a rigid alpha helix.

In embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present chimeric protein. In another example, the linker may function to target the chimeric protein to a particular cell type or location.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, promoting immune activation (e.g., against tumors). In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, suppressing immune inhibition (e.g., that allows tumors to survive). In embodiments, the present chimeric proteins provide improved immune activation and/or improved suppression of immune inhibition due to the proximity of signaling that is provided by the chimeric nature of the constructs.

In embodiments, the present chimeric proteins are capable of, or can be used in methods comprising, modulating the amplitude of an immune response, *e.g.*, modulating the level of effector output. In embodiments, *e.g.*, when used for the treatment of cancer, the present chimeric proteins alter the extent of immune stimulation as compared to immune inhibition to increase the amplitude of a T cell response, including, without limitation, stimulating increased levels of cytokine production, proliferation or target killing potential.

In embodiments the present chimeric proteins, in embodiments are capable of, or find use in methods involving, masking an inhibitory ligand on the surface of a tumor cell and replacing that immune inhibitory ligand with an immune stimulatory ligand. Accordingly, the present chimeric proteins, in embodiments are capable of, or find use in methods involving, reducing or eliminating an inhibitory immune signal and/or increasing or activating an immune stimulatory signal. For example, a tumor cell bearing an inhibitory signal (and thus evading an immune response) may be substituted for a positive signal binding on a T cell that can then attack a tumor cell. Accordingly, in embodiments, an inhibitory immune signal is masked by the present constructs and a stimulatory immune signal is activated. Such beneficial properties are enhanced by the single construct approach of the present chimeric proteins. For instance, the signal replacement can be effected nearly simultaneously and the signal replacement is tailored to be local at a site of clinical importance (*e.g.*, the tumor microenvironment). Further embodiments apply the same principle to other chimeric protein constructs, such as, for example, (i) the extracellular domain of PD-1 and (ii) extracellular domain of GITRL; (i) the extracellular domain of BTLA and (ii) extracellular domain of OX40L; (i) the extracellular domain of TIGIT and (ii) extracellular domain of OX40L; (i) the extracellular domain of TIM3 and (ii) extracellular domain of OX40L; and (i) the extracellular domain of CD172a(SIRP1 $\alpha$ ) and (ii) extracellular domain of CD40L; and (i) the extracellular domain of CD115 and (ii) extracellular domain of CD40L; and (i) the

extracellular domain of TIM3 and (ii) extracellular domain of OX40L; and (i) the extracellular domain of TIGIT and (ii) extracellular domain of OX40L; among others.

In embodiments, the present chimeric proteins are capable of, or find use in methods comprising, stimulating or enhancing the binding of immune stimulatory receptor/ligand pairs. Illustrative T cell costimulatory receptors and their ligands include OX-40:OX40-L, CD27:CD70, CD30:CD30-L, CD40:CD40-L; CD137:CD137-L, HVEM:LIGHT, GITR:GITR-L, TNFRSF25:TL1A, DR5:TRAIL, and BTLA:HVEM. In embodiments, the present chimeric proteins are capable of, or find use in methods comprising, inhibiting or reducing the binding of immune inhibitory receptor/ligand pairs. Illustrative T cell coinhibitory receptors and their ligands include, for example, CTLA-4:CD80/CD86, PD-1:PD-L1/PD-L2, BTLA:HVEM, TIM-3:galectin-9/phosphatidylserine, TIGIT/CD155 or CD112, VISTA/SIG8, CD172a(SIRP $\alpha$ )/CD47, B7H3R/B7H3, B7H4R/B7H4, CD244/CD48, TMIGD2/HHLA2, among others.

In embodiments, the present chimeric protein blocks, reduces and/or inhibits PD-1 and PD-L1 or PD-L2 and/or the binding of PD-1 with PD-L1 or PD-L2. In embodiments, the present chimeric protein blocks, reduces and/or inhibits the activity of CTLA-4 and/or the binding of CTLA-4 with one or more of AP2M1, CD80, CD86, SHP-2, and PPP2R5A. In embodiments, the present chimeric protein increases and/or stimulates GITR and/or the binding of GITR with one or more of GITR ligand. In embodiments, the present chimeric protein increases and/or stimulates OX40 and/or the binding of OX40 with one or more of OX40 ligand.

In embodiments, the present chimeric proteins are capable of, or find use in methods involving, enhancing, restoring, promoting and/or stimulating immune modulation. In embodiments, the present chimeric proteins described herein, restore, promote and/or stimulate the activity or activation of one or more immune cells against tumor cells including, but not limited to: T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, anti-tumor macrophages (e.g., M1 macrophages), B cells, and dendritic cells. In embodiments, the present chimeric proteins enhance, restore, promote and/or stimulate the activity and/or activation of T cells, including, by way of a non-limiting example, activating and/or stimulating one or more T-cell intrinsic signals, including a pro-survival signal; an autocrine or paracrine growth signal; a p38 MAPK-, ERK-, STAT-, JAK-, AKT- or PI3K-mediated signal; an anti-apoptotic signal; and/or a signal promoting and/or necessary for one or more of: proinflammatory cytokine production or T cell migration or T cell tumor infiltration.

In embodiments, the present chimeric proteins are capable of, or find use in methods involving, causing an increase of one or more of T cells (including without limitation cytotoxic T lymphocytes, T helper cells, natural killer T (NKT) cells), B cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells, monocytes, and macrophages (e.g., one or more of M1 and M2) into a tumor or the tumor microenvironment. In embodiments, the present chimeric proteins are capable of, or find use in methods involving, inhibiting and/or causing a decrease in recruitment of immunosuppressive cells (e.g., myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor associated neutrophils (TANs), M2 macrophages, and tumor associated macrophages (TAMs)) to

the tumor and/or tumor microenvironment (TME). In embodiments, the present therapies may alter the ratio of M1 versus M2 macrophages in the tumor site and/or TME to favor M1 macrophages.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, inhibiting and/or reducing T cell inactivation and/or immune tolerance to a tumor, comprising administering an effective amount of a chimeric protein described herein to a subject. In embodiments, the present chimeric proteins are able to increase the serum levels of various cytokines including, but not limited to, one or more of IFN $\gamma$ , TNF $\alpha$ , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, and IL-22. In embodiments, the present chimeric proteins are capable of enhancing IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-22, TNF $\alpha$  or IFN $\gamma$  in the serum of a treated subject. In embodiments, administration of the present chimeric protein is capable of enhancing TNF $\alpha$  secretion. In a specific embodiment, administration of the present chimeric protein is capable of enhancing superantigen mediated TNF $\alpha$  secretion by leukocytes. Detection of such a cytokine response may provide a method to determine the optimal dosing regimen for the indicated chimeric protein.

In embodiments, the present chimeric proteins inhibit, block and/or reduce cell death of an anti-tumor CD8 $^{+}$  and/or CD4 $^{+}$  T cell; or stimulate, induce, and/or increase cell death of a pro-tumor T cell. T cell exhaustion is a state of T cell dysfunction characterized by progressive loss of proliferative and effector functions, culminating in clonal deletion. Accordingly, a pro-tumor T cell refers to a state of T cell dysfunction that arises during many chronic infections and cancer. This dysfunction is defined by poor proliferative and/or effector functions, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells. Exhaustion prevents optimal control of infection and tumors. In addition, an anti-tumor CD8 $^{+}$  and/or CD4 $^{+}$  T cell refers to T cells that can mount an immune response to a tumor. Illustrative pro-tumor T cells include, but are not limited to, Tregs, CD4 $^{+}$  and/or CD8 $^{+}$  T cells expressing one or more checkpoint inhibitory receptors, Th2 cells and Th17 cells. Checkpoint inhibitory receptors refers to receptors (e.g., CTLA-4, B7-H3, B7-H4, TIM-3) expressed on immune cells that prevent or inhibit uncontrolled immune responses.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, increasing a ratio of effector T cells to regulatory T cells. Illustrative effector T cells include ICOS $^{+}$  effector T cells; cytotoxic T cells (e.g.,  $\alpha\beta$  TCR, CD3 $^{+}$ , CD8 $^{+}$ , CD45RO $^{+}$ ); CD4 $^{+}$  effector T cells (e.g.,  $\alpha\beta$  TCR, CD3 $^{+}$ , CD4 $^{+}$ , CCR7 $^{+}$ , CD62L $^{hi}$ , IL-7R/CD127 $^{+}$ ); CD8 $^{+}$  effector T cells (e.g.,  $\alpha\beta$  TCR, CD3 $^{+}$ , CD8 $^{+}$ , CCR7 $^{+}$ , CD62L $^{hi}$ , IL-7R/CD127 $^{+}$ ); effector memory T cells (e.g., CD62L $^{low}$ , CD44 $^{+}$ , TCR, CD3 $^{+}$ , IL-7R/CD127 $^{+}$ , IL-15R $^{+}$ , CCR7 $^{low}$ ); central memory T cells (e.g., CCR7 $^{+}$ , CD62L $^{+}$ , CD27 $^{+}$ ; or CCR7 $^{hi}$ , CD44 $^{+}$ , CD62L $^{hi}$ , TCR, CD3 $^{+}$ , IL-7R/CD127 $^{+}$ , IL-15R $^{+}$ ); CD62L $^{+}$  effector T cells; CD8 $^{+}$  effector memory T cells (TEM) including early effector memory T cells (CD27 $^{+}$  CD62L $^{-}$ ) and late effector memory T cells (CD27 $^{-}$  CD62L $^{-}$ ) (TemE and TemL, respectively); CD127 $^{+}$ CD25 $^{low/-}$  effector T cells; CD127 $^{-}$ CD25 $^{-}$  effector T cells; CD8 $^{+}$  stem cell memory effector cells (TSCM) (e.g., CD44 $^{low}$ CD62L $^{high}$ CD122 $^{high}$ sca $^{+}$ ); TH1 effector T-cells (e.g., CXCR3 $^{+}$ , CXCR6 $^{+}$  and CCR5 $^{+}$ ; or  $\alpha\beta$  TCR, CD3 $^{+}$ , CD4 $^{+}$ , IL-12R $^{+}$ , IFN $\gamma$ R $^{+}$ , CXCR3 $^{+}$ ); TH2 effector T cells (e.g., CCR3 $^{+}$ , CCR4 $^{+}$  and CCR8 $^{+}$ ; or  $\alpha\beta$  TCR, CD3 $^{+}$ , CD4 $^{+}$ , IL-4R $^{+}$ , IL-33R $^{+}$ , CCR4 $^{+}$ , IL-17RB $^{+}$ , CRT2 $^{+}$ ); TH9 effector T cells (e.g.,  $\alpha\beta$  TCR, CD3 $^{+}$ , CD4 $^{+}$ ); TH17 effector

T cells (e.g.,  $\alpha\beta$  TCR, CD3<sup>+</sup>, CD4<sup>+</sup>, IL-23R<sup>+</sup>, CCR6<sup>+</sup>, IL-1R<sup>+</sup>); CD4<sup>+</sup>CD45RO<sup>+</sup>CCR7<sup>+</sup> effector T cells, CD4<sup>+</sup>CD45RO<sup>+</sup>CCR7<sup>(-)</sup> effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN- $\gamma$ . Illustrative regulatory T cells include ICOS<sup>+</sup> regulatory T cells, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, CD4<sup>+</sup>CD25<sup>-</sup> regulatory T cells, CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells, TIM-3<sup>+</sup>PD-1<sup>+</sup> regulatory T cells, lymphocyte activation gene-3 (LAG-3)<sup>+</sup> regulatory T cells, CTLA-4/CD152<sup>+</sup> regulatory T cells, neuropilin-1 (Nrp-1)<sup>+</sup> regulatory T cells, CCR4<sup>+</sup>CCR8<sup>+</sup> regulatory T cells, CD62L (L-selectin)<sup>+</sup> regulatory T cells, CD45RB<sup>low</sup> regulatory T cells, CD127<sup>low</sup> regulatory T cells, LRRC32/GARP<sup>+</sup> regulatory T cells, CD39<sup>+</sup> regulatory T cells, GITR<sup>+</sup> regulatory T cells, LAP<sup>+</sup> regulatory T cells, 1B11<sup>+</sup> regulatory T cells, BTLA<sup>+</sup> regulatory T cells, type 1 regulatory T cells (Tr1 cells), T helper type 3 (Th3) cells, regulatory cell of natural killer T cell phenotype (NKTregs), CD8<sup>+</sup> regulatory T cells, CD8<sup>+</sup>CD28<sup>-</sup> regulatory T cells and/or regulatory T-cells secreting IL-10, IL-35, TGF- $\beta$ , TNF- $\alpha$ , Galectin-1, IFN- $\gamma$  and/or MCP1.

In embodiments, the chimeric protein generates a memory response which may, e.g., be capable of preventing relapse or protecting the animal from a recurrence and/or preventing, or reducing the likelihood of, metastasis. Thus, an animal treated with the chimeric protein is later able to attack tumor cells and/or prevent development of tumors when exposed to the relevant antigen after an initial treatment with the chimeric protein. Accordingly, a chimeric protein of the present invention stimulates both active tumor destruction and also immune recognition of tumor antigens, which are essential in programming a memory response capable of preventing relapse.

In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, transiently stimulating effector T cells for no longer than about 12 hours, about 24 hours, about 48 hours, about 72 hours or about 96 hours or about 1 week or about 2 weeks. In embodiments, the present chimeric proteins are capable of, and can be used in methods comprising, transiently depleting or inhibiting regulatory T cells for no longer than about 12 hours, about 24 hours, about 48 hours, about 72 hours or about 96 hours or about 1 week or about 2 weeks. In embodiments, the transient stimulation of effector T cells and/or transient depletion or inhibition of regulatory T cells occurs substantially in a patient's bloodstream or in a particular tissue/location including lymphoid tissues such as for example, the bone marrow, lymph-node, spleen, thymus, mucosa-associated lymphoid tissue (MALT), non-lymphoid tissues, or in the tumor microenvironment.

In embodiments, the present chimeric proteins provide advantages including, without limitation, ease of use and ease of production. This is because two distinct immunotherapy agents are combined into a single product which allows for a single manufacturing process instead of two independent manufacturing processes. In addition, administration of a single agent instead of two separate agents allows for easier administration and greater patient compliance. Further, in contrast to, for example, monoclonal antibodies, which are large multimeric proteins containing numerous disulfide bonds and post-translational modifications such as glycosylation, the present chimeric proteins are easier and more cost effective to manufacture.

In embodiments, the present chimeric protein is producible in a mammalian host cell as a secretable and fully functional single polypeptide chain.

In embodiments, the present chimeric protein unexpectedly provides binding of the extracellular domain components to their respective binding partners with slow off rates ( $K_d$  or  $K_{off}$ ). In embodiments, this provides an unexpectedly long interaction of the receptor to ligand and vice versa. Such an effect allows for a sustained negative signal masking effect. Further, in embodiments, this delivers a longer positive signal effect, *e.g.*, to allow an effector cell to be adequately stimulated for an anti-tumor effect. For example, the present chimeric protein, *e.g.*, via the long off rate binding allows sufficient signal transmission to provide T cell proliferation and allow for anti-tumor attack. By way of further example, the present chimeric protein, *e.g.*, via the long off rate binding allows sufficient signal transmission to provide release of stimulatory signals, such as, for example, cytokines.

The stable synapse of cells promoted by the present agents (*e.g.*, a tumor cell bearing negative signals and a T cell which could attack the tumor) provides spatial orientation to favor tumor reduction - such as positioning the T cells to attack tumor cells and/or sterically preventing the tumor cell from delivering negative signals, including negative signals beyond those masked by the chimeric protein of the invention.

In embodiments, this provides longer on-target (*e.g.*, intra-tumoral) half-life ( $t_{1/2}$ ) as compared to serum  $t_{1/2}$  of the chimeric proteins. Such properties could have the combined advantage of reducing off-target toxicities which may be associated with systemic distribution of the chimeric proteins.

Further, in embodiments, the present chimeric proteins provide synergistic therapeutic effects as it allows for improved site-specific interplay of two immunotherapy agents.

In embodiments, the present chimeric proteins provide the potential for reducing off-site and/or systemic toxicity.

In embodiments, the present chimeric proteins provide reduced side-effects, *e.g.*, GI complications, relative to current immunotherapies, *e.g.*, antibodies directed to checkpoint molecules as described herein. Illustrative GI complications include abdominal pain, appetite loss, autoimmune effects, constipation, cramping, dehydration, diarrhea, eating problems, fatigue, flatulence, fluid in the abdomen or ascites, gastrointestinal (GI) dysbiosis, GI mucositis, inflammatory bowel disease, irritable bowel syndrome (IBS-D and IBS-C), nausea, pain, stool or urine changes, ulcerative colitis, vomiting, weight gain from retaining fluid, and/or weakness

#### ***Diseases; Methods of Treatment, and Patient Selections***

In embodiments, the present invention pertains to cancers and/or tumors; for example, the treatment or prevention of cancers and/or tumors. As described elsewhere herein, the treatment of cancer may involve in embodiments, modulating the immune system with the present chimeric proteins to favor immune stimulation over immune inhibition.

Cancers or tumors refer to an uncontrolled growth of cells and/or abnormal increased cell survival and/or inhibition of apoptosis which interferes with the normal functioning of the bodily organs and systems. Included are benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Also, included are cells having abnormal proliferation that is not impeded by the immune system (*e.g.*, virus infected cells). The cancer may be a primary cancer or a metastatic cancer. The primary cancer may be an area of cancer cells at an

originating site that becomes clinically detectable, and may be a primary tumor. In contrast, the metastatic cancer may be the spread of a disease from one organ or part to another non-adjacent organ or part. The metastatic cancer may be caused by a cancer cell that acquires the ability to penetrate and infiltrate surrounding normal tissues in a local area, forming a new tumor, which may be a local metastasis. The cancer may also be caused by a cancer cell that acquires the ability to penetrate the walls of lymphatic and/or blood vessels, after which the cancer cell is able to circulate through the bloodstream (thereby being a circulating tumor cell) to other sites and tissues in the body. The cancer may be due to a process such as lymphatic or hematogenous spread. The cancer may also be caused by a tumor cell that comes to rest at another site, re-penetrates through the vessel or walls, continues to multiply, and eventually forms another clinically detectable tumor. The cancer may be this new tumor, which may be a metastatic (or secondary) tumor.

The cancer may be caused by tumor cells that have metastasized, which may be a secondary or metastatic tumor. The cells of the tumor may be like those in the original tumor. As an example, if a breast cancer or colon cancer metastasizes to the liver, the secondary tumor, while present in the liver, is made up of abnormal breast or colon cells, not of abnormal liver cells. The tumor in the liver may thus be a metastatic breast cancer or a metastatic colon cancer, not liver cancer.

The cancer may have an origin from any tissue. The cancer may originate from melanoma, colon, breast, or prostate, and thus may be made up of cells that were originally skin, colon, breast, or prostate, respectively. The cancer may also be a hematological malignancy, which may be leukemia or lymphoma. The cancer may invade a tissue such as liver, lung, bladder, or intestinal.

Representative cancers and/or tumors of the present invention include, but are not limited to, a basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal

vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

In embodiments, the chimeric protein is used to treat a subject that has a treatment-refractory cancer. In embodiments, the chimeric protein is used to treat a subject that is refractory to one or more immune-modulating agents. For example, in embodiments, the chimeric protein is used to treat a subject that presents no response to treatment, or even progress, after 12 weeks or so of treatment. For instance, in embodiments, the subject is refractory to a PD-1 and/or PD-L1 and/or PD-L2 agent, including, for example, nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and/or MPDL3280A (ROCHE)-refractory patients. For instance, in embodiments, the subject is refractory to an anti-CTLA-4 agent, *e.g.*, ipilimumab (YERVOY)-refractory patients (*e.g.*, melanoma patients). Accordingly, in embodiments the present invention provides methods of cancer treatment that rescue patients that are non-responsive to various therapies, including monotherapy of one or more immune-modulating agents.

In embodiments, the present invention provides chimeric proteins which target a cell or tissue within the tumor microenvironment. In embodiments, the cell or tissue within the tumor microenvironment expresses one or more targets or binding partners of the chimeric protein. The tumor microenvironment refers to the cellular milieu, including cells, secreted proteins, physiological small molecules, and blood vessels in which the tumor exists. In embodiments, the cells or tissue within the tumor microenvironment are one or more of: tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T-cells; regulatory T cells; macrophages; neutrophils; and other immune cells located proximal to a tumor. In embodiments, the present chimeric protein targets a cancer cell. In embodiments, the cancer cell expresses one or more of targets or binding partners of the chimeric protein.

In an illustrative embodiment, the chimeric protein of the invention may target a cell (*e.g.*, cancer cell or immune cell) that expresses PD-L1 and/or PD-L2. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses OX-40. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses GITR. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses 4-1BB. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses CD40. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses VISTA. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses CSF1. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses IL-34. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell or immune cell) that expresses CD47. In an illustrative embodiment, the chimeric protein may target a cell (*e.g.*, cancer cell, stromal cell or immune cell) that expresses galectin-9 and/or phosphatidylserine.



In embodiments, the present methods provide treatment with the chimeric protein in a patient who is refractory to an additional agent, such "additional agents" being described elsewhere herein, inclusive, without limitation, of the various chemotherapeutic agents described herein.

In embodiments, the chimeric proteins are used to treat, control or prevent one or more inflammatory diseases or conditions. Non-limiting examples of inflammatory diseases include acne vulgaris, acute inflammation, allergic rhinitis, asthma, atherosclerosis, atopic dermatitis, autoimmune disease, autoinflammatory diseases, autosomal recessive spastic ataxia, bronchiectasis, celiac disease, chronic cholecystitis, chronic inflammation, chronic prostatitis, colitis, diverticulitis, familial eosinophilia (fe), glomerulonephritis, glycerol kinase deficiency, hidradenitis suppurativa, hypersensitivities, inflammation, inflammatory bowel diseases, inflammatory pelvic disease, interstitial cystitis, laryngeal inflammatory disease, Leigh syndrome, lichen planus, mast cell activation syndrome, mastocytosis, ocular inflammatory disease, otitis, pain, pelvic inflammatory disease, reperfusion injury, respiratory disease, restenosis, rheumatic fever, rheumatoid arthritis, rhinitis, sarcoidosis, septic shock, silicosis and other pneumoconioses, transplant rejection, tuberculosis, and vasculitis.

In embodiments, the inflammatory disease is an autoimmune disease or condition, such as multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (e.g., prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, Reiter's syndrome, Grave's disease, and other autoimmune diseases.

In some aspects, the present chimeric agents are used to eliminate intracellular pathogens. In some aspects, the present chimeric agents are used to treat one or more infections. In embodiments, the present chimeric proteins are used in methods of treating viral infections (including, for example, HIV and HCV), parasitic infections (including, for example, malaria), and bacterial infections. In embodiments, the infections induce immunosuppression. For example, HIV infections often result in immunosuppression in the infected subjects. Accordingly, as described elsewhere herein, the treatment of such infections may involve, in embodiments, modulating the immune system with the present chimeric proteins to favor immune stimulation over immune inhibition. Alternatively, the present invention provides methods for treating infections that induce immunoactivation. For example, intestinal helminth infections have been associated with chronic immune activation. In these embodiments, the treatment of such infections may involve modulating the immune system with the present chimeric proteins to favor immune inhibition over immune stimulation.

In embodiments, the present invention provides methods of treating viral infections including, without limitation, acute or chronic viral infections, for example, of the respiratory tract, of papilloma virus infections, of herpes simplex virus (HSV) infection, of human immunodeficiency virus (HIV) infection, and of viral infection of internal organs

such as infection with hepatitis viruses. In embodiments, the viral infection is caused by a virus of family Flaviviridae. In embodiments, the virus of family Flaviviridae is selected from Yellow Fever Virus, West Nile virus, Dengue virus, Japanese Encephalitis Virus, St. Louis Encephalitis Virus, and Hepatitis C Virus. In embodiments, the viral infection is caused by a virus of family Picornaviridae, e.g., poliovirus, rhinovirus, coxsackievirus. In embodiments, the viral infection is caused by a member of Orthomyxoviridae, e.g., an influenza virus. In embodiments, the viral infection is caused by a member of Retroviridae, e.g., a lentivirus. In embodiments, the viral infection is caused by a member of Paramyxoviridae, e.g., respiratory syncytial virus, a human parainfluenza virus, rubulavirus (e.g., mumps virus), measles virus, and human metapneumovirus. In embodiments, the viral infection is caused by a member of Bunyaviridae, e.g., hantavirus. In embodiments, the viral infection is caused by a member of Reoviridae, e.g., a rotavirus.

In embodiments, the present invention provides methods of treating parasitic infections such as protozoan or helminths infections. In embodiments, the parasitic infection is by a protozoan parasite. In embodiments, the oritziab parasite is selected from intestinal protozoa, tissue protozoa, or blood protozoa. Illustrative protozoan parasites include, but are not limited to, *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium muris*, *Trypanosomatida gambiense*, *Trypanosomatida rhodesiense*, *Trypanosomatida cruzi*, *Leishmania mexicana*, *Leishmania braziliensis*, *Leishmania tropica*, *Leishmania donovani*, *Toxoplasma gondii*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium falciparum*, *Trichomonas vaginalis*, and *Histomonas meleagridis*. In embodiments, the parasitic infection is by a helminthic parasite such as nematodes (e.g., Adenophorea). In embodiments, the parasite is selected from Secementea (e.g., *Trichuris trichiura*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *Ancylostoma duodenale*, *Necator americanus*, *Strongyloides stercoralis*, *Wuchereria bancrofti*, *Dracunculus medinensis*). In embodiments, the parasite is selected from trematodes (e.g., blood flukes, liver flukes, intestinal flukes, and lung flukes). In embodiments, the parasite is selected from: *Schistosoma mansoni*, *Schistosoma haematobium*, *Schistosoma japonicum*, *Fasciola hepatica*, *Fasciola gigantica*, *Heterophyes heterophyes*, *Paragonimus westermani*. In embodiments, the parasite is selected from cestodes (e.g., *Taenia solium*, *Taenia saginata*, *Hymenolepis nana*, *Echinococcus granulosus*).

In embodiments, the present invention provides methods of treating bacterial infections. In embodiments, the bacterial infection is by gram-positive bacteria, gram-negative bacteria, aerobic and/or anaerobic bacteria. In embodiments, the bacteria is selected from, but not limited to, *Staphylococcus*, *Lactobacillus*, *Streptococcus*, *Sarcina*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Mycobacterium*, *Proteus*, *Campylobacter*, *Citrobacter*, *Nisseria*, *Baccillus*, *Bacteroides*, *Peptococcus*, *Clostridium*, *Salmonella*, *Shigella*, *Serratia*, *Haemophilus*, *Brucella* and other organisms. In embodiments, the bacteria is selected from, but not limited to, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas acidovorans*, *Pseudomonas alcaligenes*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Aeromonas hydrophilia*, *Escherichia coli*, *Citrobacter freundii*, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella enteritidis*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, *Francisella tularensis*, *Morganella morganii*, *Proteus*

*mirabilis*, *Proteus vulgaris*, *Providencia alcalifaciens*, *Providencia rettgeri*, *Providencia stuartii*, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Yersinia intermedia*, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Haemophilus haemolyticus*, *Haemophilus parahaemolyticus*, *Haemophilus ducreyi*, *Pasteurella multocida*, *Pasteurella haemolytica*, *Branhamella catarrhalis*, *Helicobacter pylori*, *Campylobacter fetus*, *Campylobacter jejuni*, *Campylobacter coli*, *Borrelia burgdorferi*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Legionella pneumophila*, *Listeria monocytogenes*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Kingella*, *Moraxella*, *Gardnerella vaginalis*, *Bacteroides fragilis*, *Bacteroides distasonis*, *Bacteroides* 3452A homology group, *Bacteroides vulgatus*, *Bacteroides ovalus*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Bacteroides eggerthii*, *Bacteroides splanchnicus*, *Clostridium difficile*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium leprae*, *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus intermedius*, *Staphylococcus hyicus* subsp. *hyicus*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, or *Staphylococcus saccharolyticus*.

In some aspects, the present chimeric agents are used to treat one or more autoimmune diseases or disorders. In embodiments, the treatment of an autoimmune disease or disorder may involve modulating the immune system with the present chimeric proteins to favor immune inhibition over immune stimulation. Illustrative autoimmune diseases or disorders treatable with the present chimeric proteins include those in which the body's own antigens become targets for an immune response, such as, for example, rheumatoid arthritis, systemic lupus erythematosus, diabetes mellitus, ankylosing spondylitis, Sjögren's syndrome, inflammatory bowel diseases (e.g., colitis ulcerosa, Crohn's disease), multiple sclerosis, sarcoidosis, psoriasis, Grave's disease, Hashimoto's thyroiditis, psoriasis, hypersensitivity reactions (e.g., allergies, hay fever, asthma, and acute edema cause Type I hypersensitivity reactions), and vasculitis.

In still another other aspect, the present invention is directed toward methods of treating and preventing T cell-mediated diseases and disorders, such as, but not limited to diseases or disorders described elsewhere herein and inflammatory disease or disorder, graft-versus-host disease (GVHD), transplant rejection, and T cell proliferative disorder. Specific examples of Type I ECD domains with utility in this method of use include but are not limited to: TNFRSF1b, BTNL2, PD-L1, PD-L2, CTLA-4, B7-H3, B7-H4, CD40, OX40, CD137, among others.

In some aspects, the present chimeric agents are used in methods of activating a T cell, e.g., via the extracellular domain having an immune stimulatory signal.

In some aspects, the present chimeric agents are used in methods of preventing the cellular transmission of an immunosuppressive signal.

### **Combination Therapies and Conjugation**

In embodiments, the invention provides for chimeric proteins and methods that further comprise administering an additional agent to a subject. In embodiments, the invention pertains to co-administration and/or co-formulation. Any of the compositions described herein may be co-formulated and/or co-administered.

In embodiments, any chimeric protein described herein acts synergistically when co-administered with another agent and is administered at doses that are lower than the doses commonly employed when such agents are used as monotherapy. In embodiments, any agent referenced herein may be used in combination with any of the chimeric proteins described herein.

In embodiments, any of the chimeric proteins disclosed herein may be co-administered with another chimeric protein disclosed herein. Without wishing to be bound by theory, it is believed that a combined regimen involving the administration of one or more chimeric proteins which induce an innate immune response and one or more chimeric proteins which induce an adaptive immune response may provide synergistic effects (e.g., synergistic anti-tumor effects).

In some aspects, there is provided a method of treating cancer, comprising administering to a subject in need thereof: (i) a first chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where: (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of a Type II transmembrane protein, and the first chimeric protein modulates the innate immune system; and (ii) a second chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and the second chimeric protein modulates the adaptive immune system.

In some aspects, there is provided a method of treating cancer, comprising administering to a subject in need thereof: a second chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and the second chimeric protein modulates the adaptive immune system, where the subject is undergoing or has undergone treatment with a first chimeric protein comprising a general structure of N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and the first chimeric protein modulates the innate immune system.

In embodiments, first chimeric protein is administered before the second chimeric protein.

In embodiments, the first chimeric protein is administered after the second chimeric protein.

In embodiments, the first chimeric protein comprises at least one of: TIGIT, CSF1R, CD172a(SIRP1 $\alpha$ ), VSIG8, TIM3, 41BBL, CD40L, SIGLEC7, SIGLEC9 LIGHT.

In embodiments, the second chimeric protein comprises at least one of: PD-1, TIM3, VSIG8, CD172a(SIRP1 $\alpha$ ), OX40L, GITRL, TL1A, IL-2

In embodiments, the first chimeric protein and the second chimeric protein are independently selected from TIM3-Fc-OX40L, CD172a(SIRP1 $\alpha$ )-Fc-CD40L, and CSF1R-Fc-CD40L.

In embodiments, TIM3-Fc-OX40L is administered before CD172a(SIRP1 $\alpha$ )-Fc-CD40L. In embodiments, TIM3-Fc-OX40L is administered before CSF1R-Fc-CD40L. In embodiments, CD172a(SIRP1 $\alpha$ )-Fc-CD40L is administered before TIM3-Fc-OX40L. In embodiments, CSF1R-Fc-CD40L is administered before TIM3-Fc-OX40L.

In embodiments, the first chimeric protein and/or the second chimeric protein causes activation of antigen presenting cells.

In embodiments, the first chimeric protein and/or the second chimeric protein enhances the ability of antigen presenting cells to present antigen.

In embodiments, the first chimeric protein and/or the second chimeric protein provides a sustained immunomodulatory effect.

In embodiments, the first chimeric protein and/or the second chimeric protein prevents a tumor cell from transmitting an immunosuppressive signal.

In embodiments, the second chimeric protein enhances tumor killing activity by T cells.

In embodiments, any chimeric protein which induces an innate immune response may be utilized in the present invention. In embodiments, any chimeric protein which induces an adaptive immune response may be utilized in the present invention. In an illustrative embodiment, a chimeric protein which induce an innate immune response is a chimeric protein comprising the extracellular domain of CSF1R at the N-terminus and the extracellular domain of CD40L at the C-terminus. In another embodiment, a chimeric protein which induces an innate immune response is a chimeric protein comprising the extracellular domain of SIRP $\alpha$  at the N-terminus and the extracellular domain of CD40L at the C-terminus. In an illustrative embodiment, a chimeric protein which induce an adaptive immune response is a chimeric protein comprising the extracellular domain of PD-1 at the N-terminus and the extracellular domain of OX40L at the C-terminus. In another embodiment, a chimeric protein which induces an adaptive immune response is a chimeric protein comprising the extracellular domain of VSIG8 at the N-terminus and the extracellular domain of OX40L at the C-terminus.

In embodiments, the present invention relates to the co-administration of a first chimeric protein, *e.g.*, which induces an innate immune response, and a second chimeric protein, *e.g.*, which induces an adaptive immune response. In such embodiments, the first chimeric protein may be administered before, concurrently with, or subsequent to administration of the second chimeric protein. For example, the chimeric proteins may be

administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart. In an illustrative embodiment, the first chimeric protein and the second chimeric protein are administered 1 week apart, or administered on alternate weeks (*i.e.*, administration of the first chimeric *i.e.*, protein is followed 1 week later with administration of the second chimeric protein and so forth).

Any chimeric protein disclosed herein can be a first chimeric protein, as described herein; any chimeric protein disclosed herein can be a second chimeric protein, as described herein.

In embodiments, a chimeric protein comprising an extracellular domain of TIM3 and an extracellular domain of OX40L is co-administered with a chimeric protein comprising an extracellular domain of CD172a(SIRP $\alpha$ ) and an extracellular domain of CD40L. In embodiments the chimeric protein comprising an extracellular domain of TIM3 and an extracellular domain of OX40L is administered before the chimeric protein comprising an extracellular domain of CD172a(SIRP $\alpha$ ) and an extracellular domain of CD40L. In embodiments the chimeric protein comprising an extracellular domain of TIM3 and an extracellular domain of OX40L is administered after the chimeric protein comprising an extracellular domain of CD172a(SIRP $\alpha$ ) and an extracellular domain of CD40L.

In embodiments, a chimeric protein comprising an extracellular domain of TIM3 and an extracellular domain of OX40L is co-administered with a chimeric protein comprising an extracellular domain of CSF1R and an extracellular domain of CD40L. In embodiments the chimeric protein comprising an extracellular domain of TIM3 and an extracellular domain of OX40L is administered before the chimeric protein comprising an extracellular domain of CSF1R and an extracellular domain of CD40L. In embodiments the chimeric protein comprising an extracellular domain of TIM3 and an extracellular domain of OX40L is administered after the chimeric protein comprising an extracellular domain of CSF1R and an extracellular domain of CD40L. In embodiments, co-administration includes twice administering the same chimeric protein with the first administering and the second administering separated in time. For example, the first administering and the second administering may be 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart.

In embodiments, inclusive of, without limitation, cancer applications, the present invention pertains to chemotherapeutic agents as additional agents. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and CYTOXAN cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines

and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; acetogenins (e.g., bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (e.g., cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall (see, e.g., Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994))); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabacin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino- doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitioctanol, mepitiostane, testolactone; anti-adrenals such as minogluthethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguanzone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (e.g., T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumburg, 111.), and TAXOTERE doxetaxel (Rhone-Poulenc Rorer, Antony, France); chlorambucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE. vinorelbine; novantrone; teniposide; edatraxate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen

of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (TYKERB); inhibitors of PKC- $\alpha$ , Raf, H-Ras, EGFR (e.g., erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation. In addition, the methods of treatment can further include the use of photodynamic therapy.

In embodiments, inclusive of, without limitation, cancer applications, the present additional agent is one or more immune-modulating agents selected from an agent that blocks, reduces and/or inhibits PD-1 and PD-L1 or PD-L2 and/or the binding of PD-1 with PD-L1 or PD-L2 (by way of non-limiting example, one or more of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, Merck), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), atezolizumab (TECENTRIQ, GENENTECH), MPDL3280A (ROCHE)), an agent that increases and/or stimulates CD137 (4-1BB) and/or the binding of CD137 (4-1BB) with one or more of 4-1BB ligand (by way of non-limiting example, urelumab (BMS-663513 and anti-4-1BB antibody), and an agent that blocks, reduces and/or inhibits the activity of CTLA-4 and/or the binding of CTLA-4 with one or more of AP2M1, CD80, CD86, SHP-2, and PPP2R5A and/or the binding of OX40 with OX40L (by way of non-limiting example GBR 830 (GLENMARK), MEDI6469 (MEDIMMUNE)).

In embodiments, inclusive of, without limitation, infectious disease applications, the present invention pertains to anti-infectives as additional agents. In embodiments, the anti-infective is an anti-viral agent including, but not limited to, Abacavir, Acyclovir, Adefovir, Amprenavir, Atazanavir, Cidofovir, Darunavir, Delavirdine, Didanosine, Docosanol, Efavirenz, Elvitegravir, Emtricitabine, Enfuvirtide, Etravirine, Famciclovir, and Foscarnet. In embodiments, the anti-infective is an anti-bacterial agent including, but not limited to, cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); monobactam antibiotics (aztreonam); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem). In embodiments, the anti-infectives include anti-malarial agents (e.g., chloroquine, quinine, mefloquine, primaquine, doxycycline, artemether/lumefantrine, atovaquone/proguanil and sulfadoxine/pyrimethamine), metronidazole, tinidazole, ivermectin, pyrantel pamoate, and albendazole.

In embodiments, inclusive, without limitation, of autoimmune applications, the additional agent is an immunosuppressive agent. In embodiments, the immunosuppressive agent is an anti-inflammatory agent such as a steroidal anti-inflammatory agent or a non-steroidal anti-inflammatory agent (NSAID). Steroids, particularly the adrenal corticosteroids and their synthetic analogues, are well known in the art. Examples of corticosteroids useful in the present invention include, without limitation, hydroxyltriamcinolone, alpha-methyl dexamethasone, beta-methyl betamethasone, beclomethasone dipropionate, betamethasone benzoate, betamethasone dipropionate, betamethasone valerate, clobetasol valerate, desonide, desoxymethasone, dexamethasone, diflorasone



diacetate, diflucortolone valerate, fluadrenolone, flucorolone acetonide, flumethasone pivalate, fluosinolone acetonide, fluocinonide, flucortine butylester, fluocortolone, fluprednidene (fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, clocortolone, clescinolone, dichlorisone, difluprednate, flucoronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate. (NSAIDS) that may be used in the present invention, include but are not limited to, salicylic acid, acetyl salicylic acid, methyl salicylate, glycol salicylate, salicylides, benzyl-2,5-diacetoxybenzoic acid, ibuprofen, fulindac, naproxen, ketoprofen, etofenamate, phenylbutazone, and indomethacin. In embodiments, the immunosuppressive agent may be cytostatics such as alkylating agents, antimetabolites (e.g., azathioprine, methotrexate), cytotoxic antibiotics, antibodies (e.g., basiliximab, daclizumab, and muromonab), anti-immunophilins (e.g., cyclosporine, tacrolimus, sirolimus), interferons, opioids, TNF binding proteins, mycophenolates, and small biological agents (e.g., fingolimod, myriocin).

In embodiments, the chimeric proteins (and/or additional agents) described herein, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the composition such that covalent attachment does not prevent the activity of the composition. For example, but not by way of limitation, derivatives include composition that have been modified by, *inter alia*, glycosylation, lipidation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of turicamycin, *etc.* Additionally, the derivative can contain one or more non-classical amino acids. In still other embodiments, the chimeric proteins (and/or additional agents) described herein further comprise a cytotoxic agent, comprising, in illustrative embodiments, a toxin, a chemotherapeutic agent, a radioisotope, and an agent that causes apoptosis or cell death. Such agents may be conjugated to a composition described herein.

The chimeric proteins (and/or additional agents) described herein may thus be modified post-translationally to add effector moieties such as chemical linkers, detectable moieties such as for example fluorescent dyes, enzymes, substrates, bioluminescent materials, radioactive materials, and chemiluminescent moieties, or functional moieties such as for example streptavidin, avidin, biotin, a cytotoxin, a cytotoxic agent, and radioactive materials.

### **Formulations**

The chimeric proteins (and/or additional agents) described herein can possess a sufficiently basic functional group, which can react with an inorganic or organic acid, or a carboxyl group, which can react with an inorganic or organic base, to form a pharmaceutically acceptable salt. A pharmaceutically acceptable acid addition salt is formed from a pharmaceutically acceptable acid, as is well known in the art. Such salts include the pharmaceutically acceptable salts listed in, for example, *Journal of Pharmaceutical Science*, 66, 2-19 (1977) and *The Handbook of*

*Pharmaceutical Salts; Properties, Selection, and Use*. P. H. Stahl and C. G. Wermuth (eds.), Verlag, Zurich (Switzerland) 2002, which are hereby incorporated by reference in their entirety.

In embodiments, the compositions described herein are in the form of a pharmaceutically acceptable salt.

Further, any chimeric protein (and/or additional agents) described herein can be administered to a subject as a component of a composition that comprises a pharmaceutically acceptable carrier or vehicle. Such compositions can optionally comprise a suitable amount of a pharmaceutically acceptable excipient so as to provide the form for proper administration. Pharmaceutical excipients can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical excipients can be, for example, saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one embodiment, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, specifically for injectable solutions. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Any agent described herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents.

In embodiments, the compositions described herein are resuspended in a saline buffer (including, without limitation TBS, PBS, and the like).

In embodiments, the chimeric proteins may be conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In embodiments, the chimeric proteins may be fused or conjugated with one or more of PEG, XTEN (e.g., as rPEG), polysialic acid (POLYXEN), albumin (e.g., human serum albumin or HAS), elastin-like protein (ELP), PAS, HAP, GLK, CTP, transferrin, and the like. In embodiments, each of the individual chimeric proteins is fused to one or more of the agents described in BioDrugs (2015) 29:215–239, the entire contents of which are hereby incorporated by reference.

#### **Administration, Dosing, and Treatment Regimens**

The present invention includes the described chimeric protein (and/or additional agents) in various formulations. Any chimeric protein (and/or additional agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. DNA or RNA constructs encoding the protein sequences may also be used. In one embodiment, the composition is in the form of a capsule (see, e.g., U.S. Patent No. 5,698,155). Other examples of suitable pharmaceutical excipients are described in *Remington's Pharmaceutical Sciences* 1447-1676 (Alfonso R. Gennaro eds., 19th ed. 1995), incorporated herein by reference.

Where necessary, the formulations comprising the chimeric protein (and/or additional agents) can also include a solubilizing agent. Also, the agents can be delivered with a suitable vehicle or delivery device as known in the art. Combination therapies outlined herein can be co-delivered in a single delivery vehicle or delivery device. Compositions for administration can optionally include a local anesthetic such as, for example, lignocaine to lessen pain at the site of the injection.

The formulations comprising the chimeric protein (and/or additional agents) of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing therapeutic agents into association with a carrier, which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation (*e.g.*, wet or dry granulation, powder blends, *etc.*, followed by tableting using conventional methods known in the art)

In one embodiment, any chimeric protein (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for a mode of administration described herein.

Routes of administration include, for example: intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically, particularly to the ears, nose, eyes, or skin. In embodiments, the administering is effected orally or by parenteral injection. In most instances, administration results in the release of any agent described herein into the bloodstream.

Any chimeric protein (and/or additional agents) described herein can be administered orally. Such chimeric proteins (and/or additional agents) can also be administered by any other convenient route, for example, by intravenous infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and can be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, capsules, *etc.*, and can be used to administer.

In specific embodiments, it may be desirable to administer locally to the area in need of treatment. In one embodiment, for instance in the treatment of cancer, the chimeric protein (and/or additional agents) are administered in the tumor microenvironment (*e.g.*, cells, molecules, extracellular matrix and/or blood vessels that surround and/or feed a tumor cell, inclusive of, for example, tumor vasculature; tumor-infiltrating lymphocytes; fibroblast reticular cells; endothelial progenitor cells (EPC); cancer-associated fibroblasts; pericytes; other stromal cells; components of the extracellular matrix (ECM); dendritic cells; antigen presenting cells; T-cells; regulatory T cells; macrophages; neutrophils; and other immune cells located proximal to a tumor) or lymph node and/or targeted to the tumor microenvironment or lymph node. In embodiments, for instance in the treatment of cancer, the chimeric protein (and/or additional agents) are administered intratumorally.

In the various embodiments, the present chimeric protein allows for a dual effect that provides less side effects than are seen in conventional immunotherapy (e.g., treatments with one or more of OPDIVO, KEYTRUDA, YERVOY, and TECENTRIQ). For example, the present chimeric proteins reduce or prevent commonly observed immune-related adverse events that affect various tissues and organs including the skin, the gastrointestinal tract, the kidneys, peripheral and central nervous system, liver, lymph nodes, eyes, pancreas, and the endocrine system; such as hypophysitis, colitis, hepatitis, pneumonitis, rash, and rheumatic disease. Further, the present local administration, e.g., intratumorally, obviate adverse event seen with standard systemic administration, e.g., IV infusions, as are used with conventional immunotherapy (e.g., treatments with one or more of OPDIVO, KEYTRUDA, YERVOY, and TECENTRIQ).

Dosage forms suitable for parenteral administration (e.g., intravenous, intramuscular, intraperitoneal, subcutaneous and intra-articular injection and infusion) include, for example, solutions, suspensions, dispersions, emulsions, and the like. They may also be manufactured in the form of sterile solid compositions (e.g., lyophilized composition), which can be dissolved or suspended in sterile injectable medium immediately before use. They may contain, for example, suspending or dispersing agents known in the art.

The dosage of any chimeric protein (and/or additional agents) described herein as well as the dosing schedule can depend on various parameters, including, but not limited to, the disease being treated, the subject's general health, and the administering physician's discretion. Any chimeric protein described herein, can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concurrently with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of an additional agent, to a subject in need thereof. In embodiments any chimeric protein and additional agent described herein are administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2 days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart.

In embodiments, the present invention relates to the co-administration of a chimeric protein which induces an innate immune response and another chimeric protein which induces an adaptive immune response. In such embodiments, the chimeric protein which induces an innate immune response may be administered before, concurrently with, or subsequent to administration of the chimeric protein which induces an adaptive immune response. For example, the chimeric proteins may be administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, 1 day apart, 2

days apart, 3 days apart, 4 days apart, 5 days apart, 6 days apart, 1 week apart, 2 weeks apart, 3 weeks apart, or 4 weeks apart. In an illustrative embodiment, the chimeric protein which induces an innate immune response and the chimeric protein which induces an adaptive response are administered 1 week apart, or administered on alternate weeks (*i.e.*, administration of the chimeric protein inducing an innate immune response is followed 1 week later with administration of the chimeric protein which induces an adaptive immune response and so forth).

The dosage of any chimeric protein (and/or additional agents) described herein can depend on several factors including the severity of the condition, whether the condition is to be treated or prevented, and the age, weight, and health of the subject to be treated. Additionally, pharmacogenomic (the effect of genotype on the pharmacokinetic, pharmacodynamic or efficacy profile of a therapeutic) information about a particular subject may affect dosage used. Furthermore, the exact individual dosages can be adjusted somewhat depending on a variety of factors, including the specific combination of the agents being administered, the time of administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disease being treated, the severity of the disorder, and the anatomical location of the disorder. Some variations in the dosage can be expected.

For administration of any chimeric protein (and/or additional agents) described herein by parenteral injection, the dosage may be about 0.1 mg to about 250 mg per day, about 1 mg to about 20 mg per day, or about 3 mg to about 5 mg per day. Generally, when orally or parenterally administered, the dosage of any agent described herein may be about 0.1 mg to about 1500 mg per day, or about 0.5 mg to about 10 mg per day, or about 0.5 mg to about 5 mg per day, or about 200 to about 1,200 mg per day (*e.g.*, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,100 mg, about 1,200 mg per day).

In embodiments, administration of the chimeric protein (and/or additional agents) described herein is by parenteral injection at a dosage of about 0.1 mg to about 1500 mg per treatment, or about 0.5 mg to about 10 mg per treatment, or about 0.5 mg to about 5 mg per treatment, or about 200 to about 1,200 mg per treatment (*e.g.*, about 200 mg, about 300 mg, about 400 mg, about 500 mg, about 600 mg, about 700 mg, about 800 mg, about 900 mg, about 1,000 mg, about 1,100 mg, about 1,200 mg per treatment).

In embodiments, a suitable dosage of the chimeric protein (and/or additional agents) is in a range of about 0.01 mg/kg to about 100 mg/kg of body weight, or about 0.01 mg/kg to about 10 mg/kg of body weight of the subject, for example, about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5 mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, about 1.9 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg body weight, inclusive of all values and ranges therebetween.

In another embodiment, delivery can be in a vesicle, in particular a liposome (see Langer, 1990, *Science* 249:1527-1533; Treat *et al.*, in *Liposomes in therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989).

Any chimeric protein (and/or additional agents) described herein can be administered by controlled-release or sustained-release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,556, each of which is incorporated herein by reference in its entirety. Such dosage forms can be useful for providing controlled- or sustained-release of one or more active ingredients using, for example, hydropropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Controlled- or sustained-release of an active ingredient can be stimulated by various conditions, including but not limited to, changes in pH, changes in temperature, stimulation by an appropriate wavelength of light, concentration or availability of enzymes, concentration or availability of water, or other physiological conditions or compounds.

In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; see also Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, *Ann. Neurol.* 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 71:105).

In another embodiment, a controlled-release system can be placed in proximity of the target area to be treated, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, *Science* 249:1527-1533) may be used.

Administration of any chimeric protein (and/or additional agents) described herein can, independently, be one to four times daily or one to four times per month or one to six times per year or once every two, three, four or five years. Administration can be for the duration of one day or one month, two months, three months, six months, one year, two years, three years, and may even be for the life of the subject.

The dosage regimen utilizing any chimeric protein (and/or additional agents) described herein can be selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the subject; the severity of the condition to be treated; the route of administration; the renal or hepatic function of the subject; the pharmacogenomic makeup of the individual; and the specific compound of the invention employed. Any chimeric protein (and/or additional agents) described herein can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, any chimeric protein (and/or additional agents) described herein can be administered continuously rather than intermittently throughout the dosage regimen.

**Cells and Nucleic Acids**

In embodiments, the present invention provides an expression vector, comprising a nucleic acid encoding the chimeric protein described herein. In embodiments, the expression vector comprises DNA or RNA. In embodiments, the expression vector is a mammalian expression vector.

Both prokaryotic and eukaryotic vectors can be used for expression of the chimeric protein. Prokaryotic vectors include constructs based on *E. coli* sequences (see, e.g., Makrides, *Microbiol Rev* 1996, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* include lac, trp, lpp, phoA, recA, tac, T3, T7 and  $\lambda$ P<sub>L</sub>. Non-limiting examples of prokaryotic expression vectors may include the  $\lambda$ gt vector series such as  $\lambda$ gt11 (Huynh *et al.*, in "DNA Cloning Techniques, Vol. I: A Practical Approach," 1984, (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, *Methods Enzymol* 1990, 185:60-89). Prokaryotic host-vector systems cannot perform much of the post-translational processing of mammalian cells, however. Thus, eukaryotic host-vector systems may be particularly useful. A variety of regulatory regions can be used for expression of the chimeric proteins in mammalian host cells. For example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter can be used. Inducible promoters that may be useful in mammalian cells include, without limitation, promoters associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the  $\beta$ -interferon gene, and the hsp70 gene (see, Williams *et al.*, *Cancer Res* 1989, 49:2735-42; and Taylor *et al.*, *Mol Cell Biol* 1990, 10:165-75). Heat shock promoters or stress promoters also may be advantageous for driving expression of the chimeric proteins in recombinant host cells.

In embodiments, expression vectors of the invention comprise a nucleic acid encoding the chimeric proteins (and/or additional agents), or a complement thereof, operably linked to an expression control region, or complement thereof, that is functional in a mammalian cell. The expression control region is capable of driving expression of the operably linked blocking and/or stimulating agent encoding nucleic acid such that the blocking and/or stimulating agent is produced in a human cell transformed with the expression vector.

Expression control regions are regulatory polynucleotides (sometimes referred to herein as elements), such as promoters and enhancers, that influence expression of an operably linked nucleic acid. An expression control region of an expression vector of the invention is capable of expressing operably linked encoding nucleic acid in a human cell. In an embodiment, the cell is a tumor cell. In another embodiment, the cell is a non-tumor cell. In an embodiment, the expression control region confers regulatable expression to an operably linked nucleic acid. A signal (sometimes referred to as a stimulus) can increase or decrease expression of a nucleic acid operably linked to such an expression control region. Such expression control regions that increase expression in response to a signal are often referred to as inducible. Such expression control regions that decrease expression in response to a signal are often referred to as repressible. Typically, the amount of increase or decrease conferred by such elements is proportional to the amount of signal present; the greater the amount of signal, the greater the increase or decrease in expression.

In an embodiment, the present invention contemplates the use of inducible promoters capable of effecting high level of expression transiently in response to a cue. For example, when in the proximity of a tumor cell, a cell transformed with an expression vector for the chimeric protein (and/or additional agents) comprising such an expression control sequence is induced to transiently produce a high level of the agent by exposing the transformed cell to an appropriate cue. Illustrative inducible expression control regions include those comprising an inducible promoter that is stimulated with a cue such as a small molecule chemical compound. Particular examples can be found, for example, in U.S. Pat. Nos. 5,989,910, 5,935,934, 6,015,709, and 6,004,941, each of which is incorporated herein by reference in its entirety.

Expression control regions and locus control regions include full-length promoter sequences, such as native promoter and enhancer elements, as well as subsequences or polynucleotide variants which retain all or part of full-length or non-variant function. As used herein, the term "functional" and grammatical variants thereof, when used in reference to a nucleic acid sequence, subsequence or fragment, means that the sequence has one or more functions of native nucleic acid sequence (*e.g.*, non-variant or unmodified sequence).

As used herein, "operable linkage" refers to a physical juxtaposition of the components so described as to permit them to function in their intended manner. In the example of an expression control element in operable linkage with a nucleic acid, the relationship is such that the control element modulates expression of the nucleic acid. Typically, an expression control region that modulates transcription is juxtaposed near the 5' end of the transcribed nucleic acid (*i.e.*, "upstream"). Expression control regions can also be located at the 3' end of the transcribed sequence (*i.e.*, "downstream") or within the transcript (*e.g.*, in an intron). Expression control elements can be located at a distance away from the transcribed sequence (*e.g.*, 100 to 500, 500 to 1000, 2000 to 5000, or more nucleotides from the nucleic acid). A specific example of an expression control element is a promoter, which is usually located 5' of the transcribed sequence. Another example of an expression control element is an enhancer, which can be located 5' or 3' of the transcribed sequence, or within the transcribed sequence.

Expression systems functional in human cells are well known in the art, and include viral systems. Generally, a promoter functional in a human cell is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and typically a TATA box located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A promoter will also typically contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.



Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40. Introns may also be included in expression constructs.

There are a variety of techniques available for introducing nucleic acids into viable cells. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, polymer-based systems, DEAE-dextran, viral transduction, the calcium phosphate precipitation method, *etc.* For *in vivo* gene transfer, a number of techniques and reagents may also be used, including liposomes; natural polymer-based delivery vehicles, such as chitosan and gelatin; viral vectors are also suitable for *in vivo* transduction. In some situations, it is desirable to provide a targeting agent, such as an antibody or ligand specific for a tumor cell surface membrane protein. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.*, capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al.*, J. Biol. Chem. 262, 4429-4432 (1987); and Wagner *et al.*, Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990).

Where appropriate, gene delivery agents such as, *e.g.*, integration sequences can also be employed. Numerous integration sequences are known in the art (see, *e.g.*, Nunes-Duby *et al.*, Nucleic Acids Res. 26:391-406, 1998; Sadwoski, J. Bacteriol., 165:341-357, 1986; Bestor, Cell, 122(3):322-325, 2005; Plasterk *et al.*, TIG 15:326-332, 1999; Kootstra *et al.*, Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). These include recombinases and transposases. Examples include Cre (Sternberg and Hamilton, J. Mol. Biol., 150:467-486, 1981), lambda (Nash, Nature, 247, 543-545, 1974), Flp (Broach, *et al.*, Cell, 29:227-234, 1982), R (Matsuzaki, *et al.*, J. Bacteriology, 172:610-618, 1990), cpC31 (see, *e.g.*, Groth *et al.*, J. Mol. Biol. 335:667-678, 2004), sleeping beauty, transposases of the mariner family (Plasterk *et al.*, *supra*), and components for integrating viruses such as AAV, retroviruses, and antiviruses having components that provide for virus integration such as the LTR sequences of retroviruses or lentivirus and the ITR sequences of AAV (Kootstra *et al.*, Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). In addition, direct and targeted genetic integration strategies may be used to insert nucleic acid sequences encoding the chimeric fusion proteins including CRISPR/CAS9, zinc finger, TALEN, and meganuclease gene-editing technologies.

In one aspect, the invention provides expression vectors for the expression of the chimeric proteins (and/or additional agents) that are viral vectors. Many viral vectors useful for gene therapy are known (see, *e.g.*, Lundstrom, Trends Biotechnol., 21: 117, 122, 2003). Illustrative viral vectors include those selected from Antiviruses (LV), retroviruses (RV), adenoviruses (AV), adeno-associated viruses (AAV), and  $\alpha$  viruses, though other viral vectors may also be used. For *in vivo* uses, viral vectors that do not integrate into the host genome are suitable for use, such as  $\alpha$  viruses and adenoviruses. Illustrative types of  $\alpha$  viruses include Sindbis virus, Venezuelan equine

encephalitis (VEE) virus, and Semliki Forest virus (SFV). For *in vitro* uses, viral vectors that integrate into the host genome are suitable, such as retroviruses, AAV, and Antiviruses. In one embodiment, the invention provides methods of transducing a human cell *in vivo*, comprising contacting a solid tumor *in vivo* with a viral vector of the invention.

In embodiments, the present invention provides a host cell, comprising the expression vector comprising the chimeric protein described herein.

Expression vectors can be introduced into host cells for producing the present chimeric proteins. Cells may be cultured *in vitro* or genetically engineered, for example. Useful mammalian host cells include, without limitation, cells derived from humans, monkeys, and rodents (see, for example, Kriegler in "Gene Transfer and Expression: A Laboratory Manual," 1990, New York, Freeman & Co.). These include monkey kidney cell lines transformed by SV40 (e.g., COS-7, ATCC CRL 1651); human embryonic kidney lines (e.g., 293, 293-EBNA, or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J Gen Virol* 1977, 36:59); baby hamster kidney cells (e.g., BHK, ATCC CCL 10); Chinese hamster ovary-cells-DHFR (e.g., CHO, Urlaub and Chasin, *Proc Natl Acad Sci USA* 1980, 77:4216); DG44 CHO cells, CHO-K1 cells, mouse Sertoli cells (Mather, *Biol Reprod* 1980, 23:243-251); mouse fibroblast cells (e.g., NIH-3T3), monkey kidney cells (e.g., CV1 ATCC CCL 70); African green monkey kidney cells (e.g., VERO-76, ATCC CRL-1587); human cervical carcinoma cells (e.g., HELA, ATCC CCL 2); canine kidney cells (e.g., MDCK, ATCC CCL 34); buffalo rat liver cells (e.g., BRL 3A, ATCC CRL 1442); human lung cells (e.g., W138, ATCC CCL 75); human liver cells (e.g., Hep G2, HB 8065); and mouse mammary tumor cells (e.g., MMT 060562, ATCC CCL51). Illustrative cancer cell types for expressing the chimeric proteins described herein include mouse fibroblast cell line, NIH3T3, mouse Lewis lung carcinoma cell line, LLC, mouse mastocytoma cell line, P815, mouse lymphoma cell line, EL4 and its ovalbumin transfectant, E.G7, mouse melanoma cell line, B16F10, mouse fibrosarcoma cell line, MC57, and human small cell lung carcinoma cell lines, SCLC#2 and SCLC#7.

Host cells can be obtained from normal or affected subjects, including healthy humans, cancer patients, and patients with an infectious disease, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

Cells that can be used for production of the present chimeric proteins *in vitro*, *ex vivo*, and/or *in vivo* include, without limitation, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells (e.g., as obtained from bone marrow), umbilical cord blood, peripheral blood, fetal liver, *etc.* The choice of cell type depends on the type of tumor or infectious disease being treated or prevented, and can be determined by one of skill in the art.

Production and purification of Fc-containing macromolecules (such as monoclonal antibodies) has become a standardized process, with minor modifications between products. For example, many Fc containing macromolecules are produced by human embryonic kidney (HEK) cells (or variants thereof) or Chinese Hamster Ovary (CHO) cells (or variants thereof) or in some cases by bacterial or synthetic methods. Following production,

the Fc containing macromolecules that are secreted by HEK or CHO cells are purified through binding to Protein A columns and subsequently 'polished' using various methods. Generally speaking, purified Fc containing macromolecules are stored in liquid form for some period of time, frozen for extended periods of time or in some cases lyophilized. In embodiments, production of the chimeric proteins contemplated herein may have unique characteristics as compared to traditional Fc containing macromolecules. In certain examples, the chimeric proteins may be purified using specific chromatography resins, or using chromatography methods that do not depend upon Protein A capture. In embodiments, the chimeric proteins may be purified in an oligomeric state, or in multiple oligomeric states, and enriched for a specific oligomeric state using specific methods. Without being bound by theory, these methods could include treatment with specific buffers including specified salt concentrations, pH and additive compositions. In other examples, such methods could include treatments that favor one oligomeric state over another. The chimeric proteins obtained herein may be additionally 'polished' using methods that are specified in the art. In embodiments, the chimeric proteins are highly stable and able to tolerate a wide range of pH exposure (between pH 3-12), are able to tolerate a large number of freeze/thaw stresses (greater than 3 freeze/thaw cycles) and are able to tolerate extended incubation at high temperatures (longer than 2 weeks at 40 degrees C). In embodiments, the chimeric proteins are shown to remain intact, without evidence of degradation, deamidation, etc. under such stress conditions.

***Subjects and/or Animals***

In embodiments, the subject and/or animal is a mammal, e.g., a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, rabbit, sheep, or non-human primate, such as a monkey, chimpanzee, or baboon. In embodiments, the subject and/or animal is a non-mammal, such, for example, a zebrafish. In embodiments, the subject and/or animal may comprise fluorescently-tagged cells (with e.g., GFP). In embodiments, the subject and/or animal is a transgenic animal comprising a fluorescent cell.

In embodiments, the subject and/or animal is a human. In embodiments, the human is a pediatric human. In embodiments, the human is an adult human. In embodiments, the human is a geriatric human. In embodiments, the human may be referred to as a patient.

In certain embodiments, the human has an age in a range of from about 0 months to about 6 months old, from about 6 to about 12 months old, from about 6 to about 18 months old, from about 18 to about 36 months old, from about 1 to about 5 years old, from about 5 to about 10 years old, from about 10 to about 15 years old, from about 15 to about 20 years old, from about 20 to about 25 years old, from about 25 to about 30 years old, from about 30 to about 35 years old, from about 35 to about 40 years old, from about 40 to about 45 years old, from about 45 to about 50 years old, from about 50 to about 55 years old, from about 55 to about 60 years old, from about 60 to about 65 years old, from about 65 to about 70 years old, from about 70 to about 75 years old, from about 75 to about 80 years old, from about 80 to about 85 years old, from about 85 to about 90 years old, from about 90 to about 95 years old or from about 95 to about 100 years old.

In embodiments, the subject is a non-human animal, and therefore the invention pertains to veterinary use. In a specific embodiment, the non-human animal is a household pet. In another specific embodiment, the non-human animal is a livestock animal.

### **Kits**

The invention provides kits that can simplify the administration of any agent described herein. An illustrative kit of the invention comprises any composition described herein in unit dosage form. In one embodiment, the unit dosage form is a container, such as a pre-filled syringe, which can be sterile, containing any agent described herein and a pharmaceutically acceptable carrier, diluent, excipient, or vehicle. The kit can further comprise a label or printed instructions instructing the use of any agent described herein. The kit may also include a lid speculum, topical anesthetic, and a cleaning agent for the administration location. The kit can also further comprise one or more additional agent described herein. In one embodiment, the kit comprises a container containing an effective amount of a composition of the invention and an effective amount of another composition, such those described herein.

Any aspect or embodiment described herein can be combined with any other aspect or embodiment as disclosed herein

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

## **EXAMPLES**

### *Example 1: In Silico Predicted Structure of Monomeric Human CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric Protein*

An *in silico* structure prediction of the monomeric human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein (SL-172154) having 792 amino acid residues was generated, with a p-value  $1.14 \times 10^{-21}$ . The molecular weight of the monomeric protein was predicted to be 88.1 kDa. A structure of the chimeric protein is provided in **FIG. 2**.

Specifically, the structure prediction revealed that 48 positions (6%) may be disordered. Secondary structure prediction of the entire sequence of the chimeric protein showed that the protein has the composition of 2%  $\alpha$ -helix (H), 50%  $\beta$ -sheet (E), and 47% coil (C). The GDT (global distance test) and uGDT (un-normalized GDT) for the absolute global quality were also calculated for the chimeric protein to give an overall uGDT(GDT) of 429(54). The three-state prediction for solvent accessibility of the protein residues were 33% exposed (E), 48% intermediate (M), and 17% buried (B).

A schematic diagram illustrating a mechanism of action of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein for stimulating active tumor destruction is shown in **FIG. 3**. The chimeric protein may then “dangle” from the surface of the tumor cell, and the CD40L portion of the chimeric protein may then bind to CD40 expressed on the surface of the T cell. This would result in replacement of an inhibitory hCD172a(SIRP $\alpha$ ) signal with a co-stimulatory CD40L signal to enhance the anti-tumor activity of T cells.

Many tumor types express high levels of membrane-bound CD47, which can bind to CD172a(SIRP $\alpha$ ) on the surface of a macrophage, thereby inducing a 'don't eat me' signal that inhibits macrophage engulfment or phagocytosis of the tumor cell. Without wishing to be bound by theory, it is believed that the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein (SL-172154) binds tumor that express CD47, blocking its interaction with macrophages, thereby allowing macrophage maturation and phagocytosis-mediated destruction of the tumor cells. This in turn results in enhanced tumor-antigen release and cross-presentation back to other macrophages. Interestingly, the cross-presentation of antigens occurs at the same time and in the same tumor microenvironmental context as co-stimulation between macrophage/APC bound CD40 and CD40L from the chimeric protein. The CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein therefore stimulates both active tumor destruction, and also immune recognition of tumor antigens, which are essential in programming a memory response capable of preventing relapse (**FIG. 3**).

*Example 2: Characterization of Human CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric protein*

A human CD172a(SIRP $\alpha$ )-Fc-CD40L\_chimeric protein was constructed. The chimeric protein was characterized by performing a Western blot analysis against each individual domain of the chimeric protein, *i.e.*, *via* anti-CD172a(SIRP $\alpha$ ), anti-Fc, and anti-CD40L antibodies.

The Western blots indicated the presence of a dominant dimer band in the non-reduced lanes (**FIG. 4**, lane 2 in each blot), which was reduced to a glycosylated monomeric band in the presence of the reducing agent,  $\beta$ -mercaptoethanol (**FIG. 4**, lane 3 in each blot). As shown in **FIG. 4**, lane 4 in each blot, the chimeric protein ran as a monomer at the predicted molecular weight of 88.1 kDa in the presence of both a reducing agent ( $\beta$ -mercaptoethanol) and an endoglycosidase (PNGase).

*Example 3: Characterization of the Binding Affinity of the Different Domains of the CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric Protein Using ELISA*

Functional ELISA (enzyme-linked immunosorbent assay) assays were developed to demonstrate the binding affinity of the different domains of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to their respective binding partners. The CD172a(SIRP $\alpha$ ) domain of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was detected by capturing to a plate-bound recombinant human CD47 protein and detected *via* an HRP conjugated anti-human IgG antibody. Recombinant hCD172a(SIRP $\alpha$ )-Fc protein was used to generate a standard curve. The Fc portion of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was detected by capturing to a plate-bound human IgG and detected *via* an HRP conjugated anti-human IgG antibody (hIg). Recombinant hIg protein was used to generate a standard curve. The CD40L domain of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was detected by capturing to a plate-bound recombinant human CD40 protein and detected *via* a CD40L-specific antibody. Recombinant hCD40L protein was used to generate a standard curve.

As shown in **FIG. 6**, the different domains of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein effectively interacted with their respective binding partners in a concentration-dependent manner and with high affinity. Moreover, as shown in the right panel of **FIG. 6**, the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein is able to contemporaneously bind to both CD40 and CD47. Nevertheless, it was observed that in ELISA assays, using the central Fc region to

detect chimeric proteins tended to underestimate the actual protein content in a sample. Therefore, low level of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was detected compared to standard in this assay. Based on this data an EC<sub>50</sub> value for CD47 of 1.39 nM, for mCD172a(SIRP $\alpha$ ) of 1.12 nM, and for contemporaneous CD47 and CD40 of 18.1 nM were calculated for the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein.

*Ex vivo* cell binding assays were also utilized to assess the ability of the different domains of the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to bind their respective binding partners. Here, a cell line was engineered to overexpress human CD47 (i.e., HeLa/hCD47) and a cell line was engineered to overexpress murine CD40 (i.e., CHOK1/mCD40).

Human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein or murine CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was incubated with the parental and over-expressing cell lines for 2 hours. Cells were collected, washed, and stained with antibodies for the detection of the chimeric protein binding by flow cytometry. As shown in **FIG. 7A** and **FIG. 7B**, and as expected, the chimeric proteins did not significantly bind the parental cell lines. However, the hCD172a(SIRP $\alpha$ )-Fc-CD40L bound the HeLa/hCD47 engineered cell line in a concentration-dependent manner; based on this data an EC<sub>50</sub> value of 21.51 nM was calculated (**FIG. 7A**). Similarly, the mCD172a(SIRP $\alpha$ )-Fc-CD40L bound the CHOK1/mCD40 engineered cell line in a concentration-dependent manner; based on this data an EC<sub>50</sub> value of 20.2 nM was calculated.

*Example 4: Characterization of the Binding Affinity of the CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric protein by Surface Plasmon Resonance (SPR)*

The binding affinity of the different domains of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was measured by surface plasmon resonance (SPR) using the BioRad ProteOn XPR 360 system. Specifically, the affinity of the chimeric protein for CD47, Fc $\gamma$ R1A, and FcRn was determined and compared to recombinant control proteins, and the results are shown in **FIG. 8A** to **FIG. 8C**, respectively. Kinetic data collected is summarized in the table shown in **FIG. 8D**.

As shown in **FIG. 8A**, the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein bound to CD47 and with high affinity. The 'on-rate' of CD172a(SIRP $\alpha$ )-Fc-CD40L to human CD47 was rapid, however the 'off-rate' was much lower, in fact ~40-fold slower than the 'off-rate' of recombinant CD47-Fc, indicating that CD172a(SIRP $\alpha$ )-Fc-CD40L bound quickly and stably, with long on-target residence time. The K<sub>D</sub> of CD172a(SIRP $\alpha$ )-Fc-CD40L binding to human CD47 was calculated to be 3.59 nM. The affinity measurements demonstrated high-affinity binding to the chimeric protein, except against Fc receptors with effector function (**FIG. 8B** and **FIG. 8C**). Importantly, the off-rates of the chimeric protein were much slower than those of benchmark control proteins; the chimeric protein dissociation from CD47 was 2.78 fold longer than CD172a(SIRP $\alpha$ )-Fc.

Additionally, the binding affinity of murine CD172a(SIRP $\alpha$ )-Fc-CD40L to mCD40 was assessed by SPR. It was determined that the chimeric protein bound to mCD40 tightly at a K<sub>d</sub> of 0.756 nM, as shown in the in **FIG. 8E**.

*Example 5: Functional Assays of the CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric protein*

Functional assays, ELISA-based blocking assay and macrophage engulfment assays, were performed to demonstrate functional activity of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein. ELISA-based blocking assay was performed to demonstrate that hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein binding to cells over-expressing human CD47 (HeLa/hCD47 and Jurkat/endogenous-CD47) can be disrupted by pre-incubating cells with a human CD47 blocking antibody. HeLa cells stably transfected with a human CD47-expressing plasmid, were incubated with increasing concentrations of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein, alone, or after HeLa/hCD47 cells were pre-incubated with a human CD47 blocking antibody (**FIG. 9A**, middle panel). HeLa/hCD47 bound the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein in a concentration-dependent manner (**FIG. 9A**, middle panel, top curve). The binding was blocked when HeLa/hCD47 cells were pre-treated with the CD47 blocking antibody (**FIG. 9A**, middle panel, bottom curve).

Jurkat cells, which expressed high levels of human CD47 endogenously, bound the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein in a concentration-dependent manner (**FIG. 9A**, right panel, top curve). Similarly, this binding was blocked when Jurkat cells were pre-treated with the same CD47 blocking antibody (**FIG. 9A**, right panel, bottom curve).

Together, these data indicated that the binding of the CD172a(SIRP $\alpha$ ) component of hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was highly specific to both over-expressed and endogenous CD47, since binding of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was impeded when CD47 access is blocked.

In a macrophage engulfment assay, primary derived human macrophages were incubated with CD47-expressing cells in the presence or absence of the hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein, in order to assess suppression of the 'don't eat me' signal produced when macrophage bound CD172a(SIRP $\alpha$ ) was freely able to interact with CD47. A schematic showing the basic principles of the macrophage engulfment assay is shown in **FIG. 9B**. Primary human monocytes were isolated and differentiated *in vitro* into macrophages. As the donor of CD47, the suspension cell line Jurkat was identified as expressing high levels of membrane bound CD47. Macrophages and Jurkat cells were incubated with different cell trace dyes, and co-cultured, in the presence or absence of the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein (**FIG. 9B**). In the absence of the chimeric protein, Jurkat-CD47 interacted with macrophage-CD172a(SIRP $\alpha$ ), blocking phagocytosis, resulting in baseline levels of cells which were double positive for both cell trace dyes (**FIG. 9C**, left bar). When the chimeric protein was added to the Jurkat/macrophage co-culture, increased levels of double positive cells were detected, which increased in a concentration-dependent manner (**FIG. 9C**, right three bars). The results of the macrophage engulfment assay indicated that the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was able to promote macrophage phagocytosis of CD47+ cells.

#### *Example 6: Characterization of the Murine CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric protein*

The murine CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein was characterized by performing a Western blot analysis against each individual domain of the chimeric protein, *i.e.*, via anti-CD172a(SIRP $\alpha$ ), anti-Fc, and anti-CD40L antibodies. As shown in **FIG. 10A**, all three domains of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein were

detected under non-reduced (Lane 2), reduced (Lane 3) and reduced+PNGase treatments (Lane 4). The reduced, glycosylated form of the chimeric protein migrated at the expected molecular weight of approximately 110 kDa. The reduced, deglycosylated form was not detected by any of the antibodies, which could be due to its dependence on the protein being glycosylated.

ELISA assays were performed to demonstrate the binding affinity of the different domains of the mCD172a(SIRPα)-Fc-CD40L chimeric protein to interact with their predicted binding partners (*i.e.*, CD47 or CD40). Specifically, the CD172a(SIRPα) domain of the mCD172a(SIRPα)-Fc-CD40L chimeric protein was detected by capturing to a plate-bound recombinant CD47 protein and detecting *via* an HRP-conjugated anti-Fc antibody (**FIG. 10B**, left panel, square symbols). Recombinant mCD172a(SIRPα)-mFc was used to generate a standard curve (**FIG. 10B**, left panel, circle symbols). The CD40L domain of the chimeric protein was detected by capturing to a plate-bound recombinant murine CD40 protein and detecting *via* a CD40L-specific antibody (**FIG. 10B**, right panel, square symbols). Recombinant mCD40L was used to generate a standard curve (**FIG. 10B**, right panel, circle symbols). As shown in **FIG. 10B**, the different domains of the mCD172a(SIRPα)-Fc-CD40L chimeric protein effectively interacted with their respective binding partners and with high affinity.

The *in vivo* anti-tumor activity of the mCD172a(SIRPα)-Fc-CD40L chimeric protein was analyzed using the CT26 mouse colorectal tumor model. In one set of experiments, Balb/c mice were inoculated with CT26 tumor cells on day 0 and/or rechallenged with a second inoculation of CT26 tumor cells at day 30. Following 4 days of tumor growth, when tumors reached a diameter of 4-5 mm, mice were treated with anti-CD47, anti-CD40, a combination of the two antibodies, or with mCD172a(SIRPα)-Fc-CD40L chimeric protein.

The tumor growth for each treatment group was assessed as shown in **FIG. 11A**. Specifically, the untreated mice developed tumors quickly. Treatment with the anti-CD47, anti-CD40, or the combination of those two antibodies appeared to slightly delay the development of tumors. In comparison, treating mice with the mCD172a(SIRPα)-Fc-CD40L chimeric protein significantly prevented and/or delayed the development of tumors. Importantly, the CD172a(SIRPα)-Fc-CD40L chimeric protein is effectively able to kill tumor cells and/or reduce tumor growth when rechallenged (which illustrates a cancer relapse). Thus, the CD172a(SIRPα)-Fc-CD40L chimeric protein appears to generate a memory response which may be capable of preventing relapse.

The overall survival percentage of mice through forty-five days after tumor inoculation was also assessed. All of the untreated mice died within twenty-one days after tumor inoculation. Most of the mice treated with a single antibody died around day 30. The mice receiving a combination of anti-CD40 and anti-CD47 antibodies demonstrated only about a 30% survival at day 50. Significantly, all the mice treated with the mCD172a(SIRPα)-Fc-CD40L chimeric protein survived past fifty days after tumor inoculation as shown in **FIG. 11B**.

As shown in **FIG. 11C**, monotherapy with either anti-CD40 or anti-CD47 led to moderate extensions in the tumor growth rates for most mice, with one animal completely rejected the tumor (anti-CD40 group, out of twelve total mice treated). When the two antibodies were administered in combination, there was a synergistic effect, with two out six mice in the long-term follow-up group having complete tumor rejection. For mice treated with



mCD172a(SIRP $\alpha$ )-Fc-CD40L, there was significantly superior activity in both the 150 and 300  $\mu$ g dose levels as compared to the antibody combinations, with a slightly improved rejection rate in the 150 versus the 300  $\mu$ g group. In comparison to the antibody combination treatment, there was an 80% complete rejection rate observed with treatment using the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein.

Immune phenotyping was also performed by analyzing splenocytes, lymph node cells, and tumor infiltrating lymphocytes on day thirteen post tumor inoculation. As shown in **FIG. 12A**, **FIG. 12C**, and **FIG. 12D**, mice treated with the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein exhibited higher percentages of total CD4<sup>+</sup> T cells in the spleen, peripheral lymph nodes and tumor as compared to the control or the combination antibody treatment group (anti-CD40 and anti-CD47). Within the spleen, this increase mostly comprised an increase in CD4<sup>+</sup>CD25<sup>-</sup> T cells, which would be consistent with the notion that activation of non-regulatory T cells was involved (**FIG. 12B**). There was also an increase in CD8<sup>+</sup> T cells in mice treated with mCD172a(SIRP $\alpha$ )-Fc-CD40L in the peripheral lymph nodes, but this increase was not observed in the spleen or tumor (**FIG. 12A**, **FIG. 12C**, and **FIG. 12D**).

The ability of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to stimulate the recognition of tumor antigens by CD8<sup>+</sup> T cells was also analyzed. Specifically, **FIG. 12E** shows tetramer staining analysis for determining the fraction of CD8<sup>+</sup> T cells that recognized the AH1 tumor antigen natively expressed by CT26 tumors. Within the spleen and tumor infiltrated lymphocytes (TIL), a higher proportion of CD8<sup>+</sup> T cells was found to recognize the AH1 tumor antigen in mice treated with the chimeric protein as compared to the untreated mice.

One of the indicators of CD40 activation was the proportion of cells which upregulated IL-15 receptor alpha. Amongst the treatment groups, there was a significant increase in the mice treated with the 150  $\mu$ g dose of the mCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein, but not in the anti-CD40/CD47 treatment group (**FIG. 12F**).

Together, these data demonstrate that mCD172a(SIRP $\alpha$ )-Fc-CD40L was effective at eliminating established tumors and generating a memory immune response capable of preventing tumor growth upon re-challenge. Moreover, mCD172a(SIRP $\alpha$ )-Fc-CD40L outperformed the benchmark antibody combinations (anti-CD47 + anti-CD40) when administered at the optimal dose. Notably, the 150  $\mu$ g dose of mCD172a(SIRP $\alpha$ )-Fc-CD40L appeared to be more efficacious and with a better defined immune signature than the 300  $\mu$ g dose.

The above data clearly demonstrate, *inter alia*, functional activity of mCD172a(SIRP $\alpha$ )-Fc-CD40L *in vivo*, at least, in treating cancer.

#### *Example 7: Characterization of the Human CD172a(SIRP $\alpha$ )-Fc-CD40L Chimeric protein in vivo*

The inability of the human CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein to bind to red blood cells and, thereby, causing hemolysis was then tested. Here, hCD172a(SIRP $\alpha$ )-Fc-CD40L was contacted with cynomolgus macaque red blood cells (RBCs; **FIG. 13** left top panel). No significant change in RBC counts was detected in the two monkeys; thus, the chimeric protein did not cause significant lysis of cynomolgus macaque RBCs *in vivo*. The hCD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein likewise did not appear to significantly affect indicators hemolysis (See **FIG. 13**, remaining panels). No evidence of RBC lysis or platelet depletion was observed following treatment

with hCD172a(SIRP $\alpha$ )-Fc-CD40L. Gross safety assessments were made multiple times daily, and no additional safety signals were observed. These data indicate that hCD172a(SIRP $\alpha$ )-Fc-CD40L does not cause hemolysis of RBCs as an unwanted side effect.

*Example 8: Characterization of Combination Treatments with a Plurality of Chimeric Proteins*

In this example, anti-tumor potency and/or synergistic effects were determined for treatments comprising combinations of chimeric proteins. Specifically, murine models of colorectal cancer (MC38 or CT26) or of melanoma (B16.F10) were used to assess the effects of these treatments on tumor growth and overall survival.

Balb/c mice (n= 6, 8, or 9) described in **FIG. 14** were inoculated in the hind flank with  $2.5 \times 10^5$  MC38-ova tumor cells. On days 5 and 8, mice were treated with the OX86 (anti-OX40) antibody; the RMP1-14 or 29F.1.A12 (anti-PD-1) antibody; the RMT3-32 (anti-TIM3) antibody; the anti-TIM3 and the anti-OX40 antibodies; the anti-TIM3, the anti-OX40, and the anti-PD-1 (RMP1-14) antibodies; the anti-OX40, the anti-TIM3, and the 29F.1.A12/anti-PD-1 antibodies; or the TIM3-Fc-OX40L chimeric protein. When provided, 100  $\mu$ g of each antibody was administered *via* intraperitoneal injection. When provided, 300  $\mu$ g of the TIM3-Fc-OX40L chimeric protein was administered *via* intraperitoneal injection on day 5 and again on day 7. Tumor area was calculated on the 19th day after inoculation by taking perpendicular tumor diameter measurements using electronic calipers. Neither the mice treated with a single antibody nor the mice treated with both the anti-TIM3 and the anti-OX40 antibodies had a statistically-significant reduction in tumor size relative to control treatments. However, when compared to control treatments, a statistically-significant ( $p < 0.05$ ) reduction in tumor size was observed in mice treated with either combination of three antibodies and in mice treated with two sequential doses of the TIM3-Fc-OX40L chimeric protein.

Balb/c mice (n= 8 or 9) described in **FIG. 15A** and **FIG. 15B** were inoculated in the hind flank with CT26 tumor cells. Mice in a first treatment group, on days 5 and 7, were treated with 150  $\mu$ g of the TIM3-Fc-OX40L chimeric protein. Mice in a second treatment group were treated on day 5 and 7 with 150  $\mu$ g of the TIM3-Fc-OX40L chimeric protein and then treated on day 7 and 9 with 150  $\mu$ g of the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein. Mice in a third treatment group were treated on day 5 and 7 with 150  $\mu$ g of the TIM3-Fc-OX40L chimeric protein and then treated on day 7 and 9 with 150  $\mu$ g of the CSF1R-Fc-CD40L chimeric protein. Mice in a fourth treatment group were treated on day 5 and 7 with 150  $\mu$ g of the CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein and then treated on day 7 and 9 with 150  $\mu$ g of TIM3-Fc-OX40L chimeric protein. Mice in a fifth treatment group were treated on day 5 and 7 with 150  $\mu$ g of the CSF1R-Fc-CD40L chimeric protein and then treated on day 7 and 9 with 150  $\mu$ g of the TIM3-Fc-OX40L chimeric protein. Tumor areas were calculated periodically. Of the mice in the first, second, third, and fifth treatment groups, only one mouse in each treatment group rejected the tumor inoculation and two mice in the fourth group rejected the tumor inoculation.

As shown in **FIG. 15A**, mice in each of the treatment groups showed reductions in tumor size (relative to control treatments) over the course of the test period, with the greatest reduction observed for mice of the fourth treatment group, which were first treated with CD172a(SIRP $\alpha$ )-Fc-CD40L chimeric protein and then treated with the TIM3-Fc-OX40L chimeric protein. The next greatest tumor reduction was for the same CD172a(SIRP $\alpha$ )-Fc-CD40L

pairing TIM3-Fc-OX40L yet in a reversed order. Thus, for this pairing, treating with the TIM3-Fc-OX40L chimeric protein second provided the greater reduction in tumor size. Surprisingly, an opposite pattern was observed for the other pairing of chimeric proteins. Indeed, a greater reduction in tumor size resulted when the TIM3-Fc-OX40L chimeric protein is treated before the CSF1R-Fc-CD40L when compared to a treatment with the CSF1R-Fc-CD40L chimeric protein first. Thus, for this pairing, treating with the TIM3-Fc-OX40L chimeric protein first provides the greater reduction in tumor size. Finally, for the treatment groups in which TIM3-Fc-OX40L was treated first, a greater reduction in tumor size was observed when a different chimeric protein was provided second relative to the mice who receive TIM3-Fc-OX40L as both a first and a second treatment. Thus, for certain chimeric proteins, there may be an advantage from administering different first and second chimeric proteins.

Similarly, as shown in **FIG. 15B**, mice in each of the treatment group had improved survival relative to the control treated mice; with survival at about 30 days of between about 50% and 75%. Again, for the treatment groups in which TIM3-Fc-OX40L was treated first, improved survival was observed when a different chimeric protein was provided second relative to the mice who receive TIM3-Fc-OX40L as both a first and a second treatment. Again, for certain chimeric proteins, there may be an advantage from administering different first and second chimeric proteins.

These data demonstrate that treatments including sequential treatments two chimeric proteins (either identical or different chimeric proteins) provides enhanced anti-tumor efficacy and improved survival. Moreover, for certain chimeric proteins, efficacy and survival may be affected by the order that two different chimeric proteins are administered. Furthermore, for certain chimeric proteins, efficacy and survival may be affected when the second chimeric protein administered differs from the first-administered chimeric protein. These data support the understanding that a combined regimen involving the administration of one or more chimeric proteins which induce an innate immune response before, concurrently with, or subsequent to administration of one or more chimeric proteins which induce an adaptive immune response may provide synergistic effects (e.g., synergistic anti-tumor effects).

*Example 9: Characterization of the Contribution of an Fc Domain in a Linker to Functionality of Chimeric Proteins*

In this example, the contribution of an Fc domain in a linker to functionality of chimeric proteins of the present invention was assayed. Here, a PD-1-Fc-OX40L was used as a model for Fc-containing chimeric proteins. Thus, the data presented below is relevant to chimeric proteins of the present invention.

In its native state, PD-1 exists as monomer whereas OX40Ls tend to dimerize due to electrostatic interactions between the OX40L domains; Fc domains associate with each other *via* disulfide bonds, e.g., *via* their cysteine residue(s). Together, several inter-molecular interactions may contribute to the quaternary structure of PD-1-Fc-OX40L. There are, at least, four potential configurations of PD-1-Fc-OX40L, with the chimeric protein existing as a monomer, a dimer, a trimer, or a hexamer. See, **FIG. 16**.

The existence of monomeric and dimeric configurations of the chimeric protein was tested by exposing chimeric proteins to reducing and non-reducing conditions and then running the proteins on SDS-PAGE. Under non-

reducing conditions (Reduced: "-"), the chimeric protein migrated in SDS-PAGE at about 200 kDa. Here, Western blots were probed with antibodies directed against PD-1, Fc, or OX40L in, respectively, the left, middle, and right blots shown in **FIG. 17**. Since, the predicted monomeric molecular weight of the chimeric protein is 57.6 kDa, the 200 kDa species was expected to be, at least a dimer. However, under reduced conditions (Reduced: "+"), which reduces disulfide bonds (*e.g.*, between Fc domains), the chimeric protein migrated in SDS-PAGE at about 100 kDa. Since the 100 kDa species was heavier than expected, it was predicted that the extra mass was due to glycosylation. Finally, chimeric proteins were treated with Peptide-N-Glycosidase F (PNGaseF "+") and run on SDS-PAGE under reduced conditions. Under these conditions, the chimeric protein migrated at about 57.6 kDa. These data suggest that the chimeric protein is glycosylated and exists naturally, at least, as a dimer; with dimerization likely due to disulfide bonding between Fc domains *e.g.*, via their cysteine residue(s).

SDS-PAGE gel methods do not accurately predict the molecular weight for highly charged and/or large molecular weight proteins. Thus, chimeric proteins were next characterized using Size Exclusion Chromatography (SEC). Unlike SDS-PAGE, in which the negatively-charged SDS reduces charge-based interactions between peptides, SEC does not use detergents or reducing agents. When the PD-1-Fc-OX40L chimeric protein was run on SEC, none of the peaks were around 200 kDa. This suggests, that natively, the chimeric protein does not exist as a dimer. Instead, a peak having a size greater than 670 kDa was detected. See, **FIG. 18**. This and the prior data suggests that the PD-1-Fc-OX40L chimeric protein exists as a hexamer in its native state.

As shown above, when run on SDS-PAGE under non-reducing conditions or under reducing conditions, SDS in the sample and/or running buffer converts the hexameric PD-1-Fc-OX40L chimeric protein into a predominant dimer or monomer, respectively, in the absence and presence of a reducing agent. See, **FIG. 19** (left gel). When run on native PAGE, which lacks SDS, and in the absence of a reducing agent, the chimeric protein exists as a hexamer. However, when run on native PAGE and in the presence of a reducing agent (which reduces disulfide bonds) the chimeric protein migrated heavier than expected; as shown in **FIG. 19** (right gel, lane 2), with the chimeric protein failed to substantially migrate out of the loading well. This data suggests that the chimeric protein oligomerized into a higher-order protein. Thus, in chimeric proteins, disulfide bonding appears to be important for controlling higher-order oligomerization.

To further confirm this, chimeric proteins lacking an Fc domain were constructed, *e.g.*, "PD-1-No Fc-OX40L". Such chimeric proteins will not have the disulfide bonding which occurs between Fc domains in the chimeric proteins described previously. As shown in **FIG. 20**, when chimeric proteins lacking Fc domains are run on native PAGE, none of the protein substantially migrated out of its loading well; again, suggesting that the "No Fc" chimeric proteins have formed a concatemer-like complex comprising numerous proteins. Thus, omission of the Fc domain in a chimeric protein leads to formation of protein aggregates. These data indicate that disulfide bonding, *e.g.*, between Fc domains on different chimeric proteins, stabilizes the chimeric proteins and ensures that they each exist as a hexamer and not as a higher-order protein/concatemer. In other words, the Fc domain surprisingly puts order to chimeric protein complexes. Lanes 1 to 4 respectively include 2.5 µg, of PD-1-No Fc-OX40L, 5 µg of PD-1-No Fc-OX40L, 2.5 µg of PD-1-No Fc-OX40L, and 5 µg of PD-1-No Fc-OX40L

Shown in **FIG. 21** is a model summarizing the above data and showing how a hexamer and concatemers form from chimeric proteins of the present invention. The illustrative chimeric protein (PD-1-Fc-OX40L) naturally forms into a hexamer (due to electrostatic interactions between the OX40L domains and dimerization by Fc domains). However, in the absence of the controlling effects of disulfide bonding between Fc domains, under reduced conditions for the PD-1-Fc-OX40L protein and due to the absence of Fc domains in the PD-1-No Fc-OX40L, these latter chimeric proteins form concatemers.

Additionally, chimeric proteins were constructed in which the Fc domain (as described herein) was replaced with Ficolin (which lacks cysteine residues necessary for disulfide bonding between chimeric proteins). As with the No Fc chimeric proteins and chimeric proteins comprising an Fc and run on native PAGE and in the presence of a reducing agent (both of which formed aggregates that do not migrate into a gel) chimeric proteins comprising Ficolin appear to also form higher-order lattices which did not migrate into a gel. These data reinforce the conclusion that disulfide binding is important for proper folding and function of chimeric proteins of the present invention.

Finally, chimeric proteins were prepared using coiled Fc domains (CCDFc). Very little purified protein was delivered under functional evaluation.

Accordingly, including an Fc domain in a linker of a chimeric protein (which is capable of forming disulfide bonds between chimeric proteins), helps avoid formation of insoluble and, likely, non-functional protein concatemers and/or aggregates.

*Example 10: Characterization of Different Joining Linker Sequences for the Chimeric proteins*

Different unique joining linker sequences (17 linkers) were identified with varying characteristics (length, solubility, charge and flexibility). Constructs were then synthesized incorporating each of those 17 joining linker sequences into the 'linker 2' position, where the configuration of chimeric protein:

ECD 1 – Joining Linker 1 – Fc – Joining Linker 2 – ECD 2

The production levels for those 17 constructs were tested in CHO cells. The following table provides a summary for the different joining linker sequences, characteristics of those joining linkers, the production level (by A280), and the binding values ( $EC_{50}$ ) based on FACS analysis to PD-L1 or OX40. Some variations in production levels and activity between certain joining linker sequences were determined.

**TABLE 2: Summary for optional joining linker sequences**

Protein Name	Protein conc. A280	CHO-PD-L1 $EC_{50}$ (nM)	HeLa-OX40 $EC_{50}$ (nM)	Joining Linker 2 Sequence	Characteristics
PD-1_IgG4_OX40L (1)	0.17	27	6	IEGRMD (SEQ ID NO: 51)	Linker
PD-1_IgG4_OX40L (2)	0.12	23	67	SKYGPPCPPCP (SEQ ID NO: 49)	IgG4 Hinge Region

Protein Name	Protein conc. A280	CHO-PD-L1 EC50 (nM)	HeLa-OX40 EC50 (nM)	Joining Linker 2 Sequence	Characteristics
PD-1_IgG4_OX40L (3)	0.15	25	140	GGGSGGGS (SEQ ID NO: 54)	Flexible
PD-1_IgG4_OX40L (4)	0.11	36	125	GGGSGGGGSG GG (SEQ ID NO: 55)	Flexible
PD-1_IgG4_OX40L (5)	0.22	25	41	EGKSSGSGSES KST (SEQ ID NO: 56)	Flexible + soluble
PD-1_IgG4_OX40L (6)	0.12	26	171	GGSG (SEQ ID NO: 57)	Flexible
PD-1_IgG4_OX40L (7)	0.11	27	195	GGSGGGSGGG SG (SEQ ID NO: 58)	Flexible
PD-1_IgG4_OX40L (8)	0.21	20	48	EAAAKEAAAKE AAAK (SEQ ID NO: 59)	Rigid Alpha Helix
PD-1_IgG4_OX40L (9)	0.23	45	87	EAAAREAAARE AAAREAAAR (SEQ ID NO: 60)	Rigid Alpha Helix
PD-1_IgG4_OX40L (10)	0.13	52	62	GGGGSGGGGS GGGGSAS (SEQ ID NO: 61)	Flexible
PD-1_IgG4_OX40L (11)	0.07	25	100	GGGVPRDCG (SEQ ID NO: 52)	Flexible
PD-1_IgG4_OX40L (12)	0.11	33	70	GGGGAGGGG (SEQ ID NO: 62)	Flexible
PD-1_IgG4_OX40L (13)	0.12	38	60	GS (SEQ ID NO: 63)	Highly flexible
PD-1_IgG4_OX40L (14)	0.18	25	70	GSGSGS (SEQ ID NO: 64)	Highly flexible
PD-1_IgG4_OX40L (15)	0.19	24	67	GSGSGSGSGS (SEQ ID NO: 65)	Highly flexible
PD-1_IgG4_OX40L (16)	0.11	34	77	GGGGSAS (SEQ ID NO: 66)	Flexible
PD-1_IgG4_OX40L (17)	0.19	32	44	APAPAPAPAPA PAPAPAPAP (SEQ ID NO: 67)	Rigid

Characterization of PD-1-IgG4-OX40L chimeric proteins with different joining linker sequences (17 linkers) by Western blot analysis is shown in **FIG. 22A** to **FIG. 22Q**. Specifically, each individual domain of the fusion construct was probed using an anti-PD-1, anti-Fc, or anti-OX40L antibody. Results showed similar performance across each chimeric protein suggesting that all of the candidate joining linker sequences were functional.

Additionally, each purified protein with different linker sequences was also characterized by binding to PD-L1 or OX40 in ELISA assays (**FIG. 23**), as well as cell-based flow cytometry assays (**FIG. 24A** to **FIG. 24P**).

*Example 11: Characterization of Murine PD-1-Fc-OX40L Chimeric Proteins*

Tumor cells may express PD-L1 on their cell surface, which can bind to PD-1 expressed by a T cell (**FIG. 25A** and **FIG. 25B**). This interaction suppresses activation of T cells. A chimeric protein comprising the extracellular domain of PD-1, adjoined to the extracellular domain of OX40L (*i.e.*, PD-1-Fc-OX40L) may bind to PD-L1 on the surface of a tumor cell, preventing binding to PD-1 on the surface of a T cell (**FIG. 25C**). The chimeric protein may then “dangle” from the surface of the tumor cell, and the OX40L portion of the chimeric protein may then bind to OX40 expressed on the surface of the T cell. This would result in replacement of an inhibitory PD-L1 signal with a co-stimulatory OX40L signal to enhance the anti-tumor activity of T cells.

The binding affinity of the different domains of the murine PD-1-Fc-OX40L chimeric protein was measured by surface plasmon resonance (SPR) using the BioRad ProteOn XPR 360 system. Specifically, the affinity of the chimeric proteins for PD-L1, PD-L2, OX40, and FcRn were determined and compared to recombinant control proteins, and the results are shown in the Table below:

		$K_a$	$K_d$	$K_D$
Sample		(on-rate; 1/10s)	(off-rate; 1/s)	(Binding; nM)
Binding to: PD-L1	PD1-Fc	3.24 E+4	1.08 E-3	33.3 nM
	PD1-Fc-OX40L	4.97 E+4	4.87 E-4	9.8 nM
PD-L2	PD1-Fc	4.79 E+4	1.71 E-3	23.1 nM
	PD1-Fc-OX40L	5.29 E+4	5.61 E-4	10.6 nM
OX40	OX40L-Fc	6.74 E+5	7.12 E-4	1.06 nM
	PD1-Fc-OX40L	3.19 E+6	3.07 E-4	9.62 nM
FcRn	IgG2A	3.00 E+6	2.42 E-2	8.08 nM
	PD1-Fc-OX40L	4.72 E+4	2.62 E-3	55.6 nM

mPD-1-Fc-OX40L bound to chip-bound mPD-L1-His (9.8 nM), PD-L2-His (10.6 nM), OX40-His (9.62 nM), and FcRn-His (55.6 nM) using SPR. Binding of control proteins (PD-1-Fc, OX40L-Fc, and IgG2A) were also shown. No binding of mPD-1-Fc-OX40L was detected to FcγR1.

Additional analysis was carried out to determine whether the mPD-1-Fc-OX40L chimeric protein could bind its targets on the surface of living cells. To assess mPD-1-Fc-OX40L binding to murine PD-L1, PD-L2, and OX40, the Chinese hamster ovary cell line, CHOK1, was transfected to stably express murine PD-L1, PD-L2, and OX40 (**FIG. 26A**). mPD-1-Fc-OX40L chimeric protein was incubated with each parental and over-expressing cell line for 2 hours. Cells were collected, washed, and stained with antibodies for the detection of the chimeric protein binding by flow cytometry. All engineered cell lines (CHOK1/hPD-L1, CHOK1/hPD-L2, and CHOK1/hOX40) bound mPD-1-Fc-OX40L in a concentration-dependent manner at low nM as shown in **FIG. 26B**. mPD-1-Fc-OX40L did not bind to parental CHOK1 cells since they did not express detectable levels of human PD-L1, OX40, or PD-L2. However, nearly the entire population of CHO-K1-PD-L1, CHOK1-PD-L2, and Jurkat/hOX40 cells shifted significantly, indicating that the different components of the chimeric protein were capable of binding to its respective receptor/ligands on living cells (**FIG. 26A** and **FIG. 26B**).

The functional activity of mPD-1-Fc-OX40L chimeric protein was assessed using the superantigen cytokine release assay. In this assay, increasing concentrations of staphylococcus enterotoxin B (SEB) were used to activate human peripheral blood leukocytes in the presence of various test agents. The quantity of TNF $\alpha$  or IL-2 secreted into the culture supernatant was monitored as a functional readout in the ability of test agents to either block suppressive signaling events or co-stimulate immune activating signals. As shown in **FIG. 26C**, the mPD-1-Fc-OX40L chimeric protein induced secretion of IL2 at higher levels (top curve) in comparison of other test agents *i.e.*, PD-1-Fc, OX40L-Fc, and PD-1-Fc/OX40L-Fc. However, as shown in **FIG. 26D**, the mPD-1-Fc-OX40L chimeric protein and the PD-1-Fc/OX40L-Fc induced the highest level of secreted TNF $\alpha$  (top two curves) in comparison to other test agents: PD-1-Fc and OX40L-Fc. Media and IgG controls were used. Together, these results suggest that mPD-1-Fc-OX40L chimeric protein functionally activates primary leukocytes to release TNF $\alpha$  and IL2 *in vitro*.

**FIG. 27A** to **FIG. 27F** show results from *in vivo* tumor studies demonstrating that the mPD-1-Fc-OX40L chimeric protein has significant anti-tumor activity in a CT26 tumor rechallenge model. Mice inoculated with CT26 tumors were alternately treated with anti-PD-1, anti-PD-L1, anti-OX40, a combination of anti-PD-L1 and OX40 antibodies, a combination of anti-PD-1 and OX40 antibodies, with control antibodies, or with one of three doses of the mPD-1-Fc-OX40L chimeric protein (*i.e.*, 100  $\mu$ g, 150  $\mu$ g and 300  $\mu$ g) and on two occasions (see bottom panel). Specifically, Tumor inoculation occurred on day 0, first treatment on day 5, and second treatment on day 7; tumor re-challenge (implantation of a second tumor on the opposite flank without re-treatment with drug) occurred on day 30 in any mice that rejected the primary tumor. Mice were re-challenged with CT26 tumor cells. **FIG. 27A** shows the evolution of tumor size over sixty-five days after tumor inoculation for each group. Importantly, the PD-1-Fc-OX40L chimeric protein is effectively able to kill tumor cells and/or reduce tumor growth when rechallenged (which illustrates a cancer relapse). Thus, the PD-1-Fc-OX40L chimeric protein appears to generate a memory response which may be capable of preventing relapse.

**FIG. 27B** and **FIG. 27C** shows the overall survival percentage, and statistics, of mice and tumor rejection through forty days after tumor inoculation. **FIG. 27D** shows changes in CD4 $^{+}$  T-cells, CD4 $^{+}$ CD25 $^{-}$  effector T cells or CD4 $^{+}$ CD25 $^{+}$  regulatory T cells in the tumor of mice treated with the chimeric protein and other benchmark antibodies. **FIG. 27E** shows changes in CD4 $^{+}$  T-cells, CD4 $^{+}$ CD25 $^{-}$  effector T cells or CD4 $^{+}$ CD25 $^{+}$  regulatory T cells in the spleen of mice treated with the chimeric protein and other benchmark antibodies. **FIG. 27F** summarizes treatment outcomes for each group. For **FIG. 27D** and **FIG. 27E**, cohorts of treated mice were euthanized thirteen days after initial tumor inoculation. Tumors and spleens were isolated, dissociated, and analyzed for proportions of effector and non-effector/Treg populations by flow cytometry.

Overall, administration of mPD-1-Fc-OX40L significantly reduced tumor size in the CT26 colorectal cancer model. Particularly, use of mPD-1-Fc-OX40L resulted in greater tumor regression than the OX40 agonist and PD-L1 blocking antibodies (**FIG. 27G**). mPD-1-Fc-OX40L outperformed anti-OX40, anti-PD-1, or anti-PD-L1 antibodies and antibody combinations at low dose (100  $\mu$ g, **FIG. 27G**) and at a higher dose (300  $\mu$ g, **FIG. 27H**). Cytokine signature suggests that 100  $\mu$ g was a sub-optimal dose. At a higher dose (300  $\mu$ g chimeric protein vs. 2700  $\mu$ g



mAb), mPD-1-Fc-OX40L significantly outperformed anti-PD-1/L1 + anti-OX40 mAb combinations. In **FIG. 27G** and **FIG. 27H**, data identified as “ARC Fusion Protein” refers to the mPD-1-Fc-OX40L chimeric protein.

The above data clearly demonstrate, *inter alia*, functional activity of mPD-1-Fc-OX40L *in vivo*, at least, in treating cancer.

*Example 12: Characterization of Human PD-1-Fc-OX40L Chimeric Proteins*

ELISA (enzyme-linked immunosorbent assay) assays were developed to demonstrate the binding affinity of the different domains of the human PD-1-Fc-OX40L chimeric protein (also referred to as SL-279252) to their respective binding partners. **FIG. 28A** shows the binding and detection of human PD-1-Fc-OX40L chimeric protein to human IgG, the binding partner for Fc (square symbols). Human Ig (hIg) was used as a standard (circle symbols). It was observed that in ELISA assays generally, using the central Fc region to detect chimeric proteins tended to underestimate the actual protein content in a sample. Therefore, low level of the hPD-1-Fc-OX40L chimeric protein was detected compared to standard in this assay. **FIG. 28B** shows the binding and detection of human PD-1-Fc-OX40L chimeric protein to the receptor OX40, *i.e.*, the binding partner for OX40L (square symbols). Recombinant OX40L-Fc was used to generate a standard curve (circle symbols). **FIG. 28C** shows dual-binding ELISA assay demonstrating the ability of PD-1-Fc-OX40L to bind and engage both targets (PD-L1 and OX40-His) simultaneously. Increasing concentrations of hPD-1-Fc-OX40L chimeric protein were incubated with a fixed amount of plate-bound recombinant human PD-L1 protein. Thereafter, recombinant OX40-His protein or a control His-tagged protein (HVEM-His) was incubated with the complex and binding was detected *via* an HRP-conjugated anti-His antibody. The results clearly show that hPD-1-Fc-OX40L binds to PD-L1 and OX40 simultaneously and with high specificity.

Additional analyses were carried out to determine whether hPD-1-Fc-OX40L fusion protein could bind its targets on the surface of living cells *in vitro*. To assess hPD-1-Fc-OX40L's binding to the human OX40 receptor, the human AML T cell line Jurkat was engineered to overexpress OX40, creating Jurkat/hOX40 cells (verified by flow cytometry; **FIG. 29**, right panel). To assess binding to PD-L1, the Chinese hamster ovary cell line, CHOK1, which does not express human PD-L1, was transfected to stably express human PD-L1 (**FIG. 29A**, left panel). To assess binding to human PD-L2, CHOK1 cells were transfected to stably express human PD-L2 (**FIG. 29A**, middle panel). Human PD-1-Fc-OX40L chimeric protein was incubated with each parental cell line and each of the over-expressing cell lines for two hours. Cells were collected, washed, and stained with antibodies for the detection of chimeric protein binding by flow cytometry. For histograms to the left and in the middle, parental CHOK1 (cell population to the right) and CHOK1/hPD-L1 (cell population to the left) cells were assessed by flow cytometry using an hPD-L1 antibody or an hPD-L2 antibody, respectively. In the right histogram, parental Jurkat cells (cell population to the right) and Jurkat/hOX40 (cell population to the left) were assessed by flow cytometry using a hOX40 antibody. All engineered cell lines (CHOK1/hPD-L1, CHOK1/hPD-L2, and Jurkat/hOX40) bound hPD-1-Fc-OX40L in a concentration-dependent manner at low nM.

As shown in **FIG. 29B** to **FIG. 29D**, hPD-1-Fc-OX40L did not bind to parental CHO-K1 cells since they did not express detectable levels of human PD-L1 or PD-L2. Similarly, hPD-1-Fc-OX40L did not bind to parental Jurkat cells since they did not express detectable levels of OX40. However, nearly the entire population of CHO-K1-PD-L1, CHOK1-PD-L2, and Jurkat/hOX40 cells shifted significantly, indicating that the different components of the chimeric protein were each capable of binding its respective receptor/ligands on living cells. *in vitro* cell binding affinities of SL-279252-CHOK1/hPD-L1 at 26.11 nM, SL-279252-CHOK1/hPD-L2 at 7.60 nM, and SL-279252-Jurkat/hOX40 at 6.28 nM.

Next, surface plasmon resonance (SPR) analysis was performed to determine the affinity by which SL-279252 bound to hPD-L1, hPD-L2, and hOX40. Specifically, polyhistidine-tagged versions of recombinant human PD-L1, PD-L2, or OX40 was bound to ProteOn HTG tris-NTA chips (BIORAD). SL-279252 was then flowed over the bound ligands over a time course and a relative index of 'on-rate' ( $K_a$ ) and 'off-rate' ( $K_d$ ) was generated to calculate binding affinity ( $K_D$ ) of SL-279252 to each partner. Recombinant human PD-1-Fc and OX40L-Fc were used as positive controls for binding. These controls have a relatively fast 'on-rate' and an equally fast 'off-rate', resulting in low nanomolar binding affinities. The results of SPR binding affinity demonstrated high-affinity binding for each portion of the fusion protein (except against Fc receptors with effector function). Importantly, the off-rates of hPD-1-Fc-OX40L were much slower than those of benchmark control proteins: hPD-1-Fc-OX40L dissociation from PD-L1 was 18 fold longer than PD-1-Fc, from PD-L2 was 13.4 fold longer than PD-1-Fc, and from OX40 was 36.32 fold longer OX40L-Fc. Together, these results indicated that the hPD-1-Fc-OX40L fusion protein had a long residence time when bound to PD-L1 or PD-L2.

The above data clearly demonstrates that the different domains of the human PD-1-Fc-OX40L fusion protein (SL-279252) bind their native binding partners (e.g., receptor or ligand; PD-L1, PD-L2, and OX40) on the surface of a mammalian cell membrane.

To confirm that all three domains of the human PD-1-Fc-OX40L (SL-279252) are intact and recognizable by a protein detection assay, Western blot analysis was performed on purified fusion protein which were probed with human anti-PD-1, anti-Fc, and anti-OX40L (**FIG. 30**). SL-279252 was detected by all three antibodies and when the protein was run under reducing conditions, migrated at approximately 75 kDa. Approximately 50% of the non-reduced protein ran as a dimer, which was a potential advantage, given the *in vivo* oligomerization associated with OX40L signaling and function. The predicted molecular weight for SL-279252 was 60.3 kDa. The reduced fraction of SL-279252 was detected at a higher molecular weight, which, without wishing to be bound by theory, may be due to glycosylation. This was verified by treating SL-279252 with a protein deglycosylase, PNGase F. Following deglycosylation, the reduced fraction of SL-279252 migrated exactly at the predicted molecular weight of 60.3 kDa. This provided evidence that SL-279252 was co/post-translationally modified through glycosylation, which plays essential roles in the proper folding and stability of proteins, and cell-to-cell adhesion (Dalziel M, Dwek RA. Science 2014; Maverakis E, Lebrilla CB. J Autoimmun. 2015).

Since the human PD-1-Fc-OX40L chimeric protein retained glycosylase modifications, further analysis was performed to determine whether its glycosylation status impacted its function. hPD-1-Fc-OX40L was treated with the deglycosylase PNGase F, and then its binding to hOX40 was assessed in routine functional ELISA (**FIG. 31A**) and cell binding assays with Jurkat cells expressing hOX40 (**FIG. 31B**). Jurkat cell lines that do not express hOX40 were used as control. Results indicated that glycosylation did not play a significant role in hPD-1-Fc-OX40L binding to its interacting partners.

Next, an ELISA-based blocking/competition assay was performed to demonstrate that hPD-1-Fc-OX40L could out-compete human PD-1-biotin for binding to plate-bound recombinant human PD-L1. In this assay, recombinant human PD-L1 was coated on high-binding ELISA plates. Horseradish peroxidase (HRP) signal was produced using detection with recombinant human PD-1-Biotin, followed by an avidin-HRP avidin. As shown in **FIG. 32A**, in case of a negative control (a chimeric protein that does contain PD-L1 binding domain) the signal for PD-1-biotin was not disrupted (**FIG. 32A**, top curve/Square symbols). Notably, hPD-1-Fc-OX40L blocked PD-1-Biotin binding to PD-L1 (thereby decreasing HRP signal), in a concentration-dependent manner (**FIG. 32A**, bottom curve/circle symbols). The results of this assay demonstrated that hPD-1-Fc-OX40L strongly competes with PD-1-biotin for binding to recombinant human PD-L1, with a calculated  $IC_{50}$  of 6.68 nM.

A cytokine release, tumor co-culture assay was performed to demonstrate that the hPD-1-Fc-OX40L chimeric protein was capable of inducing the expression of IL-2 in T cells. As shown in **FIG. 32B**, primary human CD3+ T cells, in the presence or absence of hPD-1-Fc-OX40L, were incubated with PD-L1<sub>low</sub> or PD-L1<sub>high</sub> human tumor cells; thus, allowing assessment of the effector function and proliferation of T cells using IL2 secretion and flow cytometry-based immune assessment. Specifically, human peripheral blood leukocytes were isolated by density gradient centrifugation, followed by negative enrichment for CD3+ cells, and subsequent activation with CD3/CD28 beads. The activated cells were then co-cultured with either a PD-L1<sub>low</sub> prostate cancer cell (human PC3) or a PD-L1<sub>high</sub> lung adenocarcinoma cell (human HCC827) in the presence or absence of hPD-1-Fc-OX40L (500 ng and 5  $\mu$ g concentrations). The quantity of IL-2 produced and secreted into the cell culture supernatant was then measured by ELISA (**FIG. 32C**). The hPD-1-Fc-OX40L induced higher levels of secreted IL2 in PC3 cells (**FIG. 32C**, left bundle) than in HCC827 cells (**FIG. 32C**, right bundle). As human T cells produced significantly more IL-2 when co-cultured with the PC3 cell line than with the HCC827 cell line, this suggested that the quantity of PD-L1 inhibited IL-2 production (**FIG. 32C**). When hPD-1-Fc-OX40L was added to the co-cultures, however, increased IL-2 production was observed in both co-culture systems. In addition to measuring the amount of IL-2 secreted, the activated T cells from the co-culture assay were collected and analyzed by intracellular flow cytometry. These data indicated that hPD-1-Fc-OX40L increased Ki67, IFN $\gamma$ , and TNF $\alpha$  staining in both CD4+ and CD8+ T cells (**FIG. 32D**).

Another functional assay to characterize the functional activity of hPD-1-Fc-OX40L chimeric protein is the superantigen cytokine release assay. In this assay, increasing concentrations of staphylococcus enterotoxin B (SEB) were used to activate human peripheral blood leukocytes in the presence of various test agents; a flow chart of the steps is shown in **FIG. 32E**. The quantity of TNF $\alpha$  (**FIG. 32F**) or IL-2 (**FIG. 32G**) secreted into the culture

supernatant was monitored as a functional readout of the ability of test agents to either block suppressive signaling events or co-stimulate immune activating signals. As shown in **FIG. 32F** and **FIG. 32G**, the hPD-1-Fc-OX40L chimeric protein induced secretion of TNF $\alpha$  and the secretion of IL2 at higher levels (top curves) in comparison to other test agents PD-1-Fc, OX40L-Fc, and PD-1-Fc/OX40L-Fc. Media and IgG controls were used. Together, these results suggest that hPD-1-Fc-OX40L chimeric protein functionally activated primary human leukocytes cells *in vitro*.

Finally, ELISA assays were performed to demonstrate the binding affinity and cross-reactivity of the human PD-1-Fc-OX40L chimeric protein to OX40 from rhesus macaque and PD-L1 and PD-L2 from cynomolgus macaque. As shown in **FIG. 33A**, human PD-1-Fc-OX40L chimeric protein specifically bound to plate-bound recombinant cmPD-L1 and the binding was detected by an HRP conjugated anti-hFc antibody. Human PD-1-Fc-OX40L chimeric protein was used from two different sources (square and triangle symbols). Human CD120b-Fc-TGFB chimeric protein (inverted triangle symbols) was used as a control while recombinant cmPD-1-hFc was used to generate a standard curve (circle symbols). These results indicate that hPD-1-Fc-OX40L chimeric protein cross-reacted with cmPD-L1.

hPD-1-Fc-OX40L chimeric protein specifically bound to plate-bound recombinant cmPD-L2 and the binding was detected *via* an HRP-conjugated anti-Goat IgG binding to Goat anti-hOX40L antibody. Human PD-1-Fc-OX40L chimeric protein was used from two different sources (**FIG. 33B**, square and circle symbols). Human CD120b-Fc-TGFB chimeric protein (**FIG. 33B**, triangle symbols) was used as a control. As shown in **FIG. 33C**, the binding of human PD-1-Fc-OX40L chimeric protein from two different sources (square and circle symbols) and hCD120b-Fc-TGFB chimeric protein (triangle symbols) to a plate-bound rmOX40 was demonstrated. Binding was detected *via* an HRP-conjugated anti-Goat IgG binding to Goat anti-hOX40L antibody. Accordingly, the above data demonstrates that hPD-1-Fc-OX40L chimeric protein cross-reacted with cmPD-L1, cmPD-L2, and rmOX40.

#### *Example 12: Characterization of the Murine TIM3-Fc-OX40L Chimeric Protein*

An *in silico* structure prediction of a monomeric TIM3-Fc-OX40L chimeric protein (SL-366252) having 548 amino acid residues was generated, with a p-value  $5.1 \times 10^{-17}$ . The molecular weight of the monomeric protein was predicted to be 61.6 kDa. A structure of the chimeric protein is provided in **FIG. 34**.

Specifically, the structure prediction revealed that six positions (1%) may be disordered. Secondary structure prediction of the entire sequence of the chimeric protein showed that the protein has the composition of 3%  $\alpha$ -helix (H), 43%  $\beta$ -sheet (E), and 51% coil (C). The GDT (global distance test) and uGDT (un-normalized GDT) for the absolute global quality were also calculated for the chimeric protein to give an overall uGDT(GDT) of 481(87). The three-state prediction for solvent accessibility of the protein residues were 35% exposed (E), 49% intermediate (M), and 14% buried (B).

A murine TIM3-Fc-OX40L chimeric protein was constructed. The chimeric protein was characterized by performing a Western blot analysis against each individual domain of the chimeric protein, *i.e.*, *via* anti-TIM3, anti-Fc, and anti-OX40L antibodies. The Western blots indicated the presence of a dominant trimeric band in the non-reduced lanes

(**FIG. 35**, lane 2 in each blot), which was reduced to a glycosylated monomeric band in the presence of the reducing agent,  $\beta$ -mercaptoethanol (**FIG. 35**, lane 3 in each blot). As shown in **FIG. 35**, lane 4 in each blot, the chimeric protein ran as a monomer at the predicted molecular weight of 61.6 kDa in the presence of both a reducing agent ( $\beta$ -mercaptoethanol) and an endoglycosidase (PNGase).

Cell binding assays were performed to demonstrate the binding affinity of the different domains of the mTIM3-Fc-OX40L chimeric protein towards their respective binding partners on the surface of a mammalian cell membrane. For the cell binding assays, immortalized cell lines were engineered to stably express murine receptor OX40 (CHOK1-mOX40). Increasing concentrations of mTIM3-Fc-OX40L were incubated with each parental (control) and over-expressing cell lines for 2 hours. Cells were collected, washed, and stained with antibodies for the detection of chimeric protein binding by flow cytometry. As shown in **FIG. 36**, mTIM3-Fc-OX40L bound to the engineered cell line (CHOK1-mOX40) in a concentration-dependent manner with low nM affinity. Specifically, the CHOK1 parental cell line (bottom curve) was not responsive to increasing concentrations of the mTIM3-Fc-OX40L chimeric protein as it did not overexpress mOX40. In comparison, the CHOK1-mOX40 cell line, which overexpressed mOX40, bound to mTIM3-Fc-OX40L in a concentration-dependent manner. The cell binding assay also indicated that mTIM3-Fc-OX40L bound to mOX40 with an affinity of 15.2 nM.

*In vivo* functional assays were performed to demonstrate the functional activity of the mTIM3-Fc-OX40L chimeric protein. Mice were inoculated with CT26 tumors on day 0. Once the tumors were palpable and at least 4-6 mm in diameter, mice were treated with two doses of 150  $\mu$ g of the mTIM3-Fc-OX40L chimeric protein. Immunophenotyping was performed on various tissues collected from the mice on day 13 after implantation.

Immune profiling was performed on tumor-bearing mice treated with the murine TIM3-Fc-OX40L chimeric protein. As shown in **FIG. 37A**, mice treated with the mTIM3-Fc-OX40L chimeric protein exhibited higher percentages of total CD4<sup>+</sup> T cells in the spleen, peripheral lymph nodes and tumor (right bundle in **FIG. 37A**) as compared to the control treatment groups (left bundle in **FIG. 37A**). Within the spleen and the tumor, this increase in CD4<sup>+</sup> T cell population was mostly due to an increase in CD4<sup>+</sup>CD25<sup>-</sup> effector T cells, consistent with the notion that activation of non-regulatory T cells was involved (**FIG. 37B**). The treated mice also exhibited a lower percentage of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, suggesting that regulatory T cells may be suppressed by the chimeric protein (**FIG. 37B**).

The ability of the chimeric protein to stimulate the recognition of tumor antigens by CD8<sup>+</sup> T cells was also analyzed. Specifically, **FIG. 37C** shows tetramer staining analysis for determining the fraction of CD8<sup>+</sup> T cells that recognized the AH1 tumor antigen natively expressed by CT26 tumors. Within the spleen, a higher proportion of CD8<sup>+</sup> T cells was found to recognize the AH1 tumor antigen in mice treated with the mTIM3-Fc-OX40L chimeric protein (right bundle in **FIG. 37C**) as compared to the untreated mice (left bundle in **FIG. 37C**). Notably, a much higher proportion of the AH1 tetramer positive CD8<sup>+</sup> T cells was observed within tumor infiltrated lymphocytes (TIL) for mice treated with the chimeric protein (right bundle in **FIG. 37C**) as compared to the untreated control mice (right bundle in **FIG. 37C**).

The *in vivo* anti-tumor activity of the mTIM3-Fc-OX40L chimeric protein was analyzed using the MC38 and CT26 mouse colorectal tumor models. In one set of experiments, Balb/c mice were inoculated with CT26 tumor cells on day 0 and/or rechallenged with a second inoculation of CT26 tumor cells at day thirty. Following four days of tumor growth, when tumors reached a diameter of 4-5 mm, mice were treated with either control antibodies or 150 µg of the mTIM3-Fc-OX40L chimeric protein. Treatments were repeated on day seven. An analysis of the evolution of tumor size over forty-five days after tumor inoculation was conducted.

As shown in **FIG. 38A**, the untreated mice developed significant tumors, whereas none of the mice treated with the mTIM3-Fc-OX40L chimeric protein developed tumors of detectable size. Importantly, the mTIM3-Fc-OX40L chimeric protein is effectively able to kill tumor cells and/or reduce tumor growth when rechallenged (which illustrates a cancer relapse). Thus, the mTIM3-Fc-OX40L chimeric protein appears to generate a memory response which may be capable of preventing relapse. The overall survival percentage of mice (**FIG. 38B**) through fifty days after tumor inoculation shows that all of the untreated mice died within twenty-one days after tumor inoculation, whereas mice treated the mTIM3-Fc-OX40L chimeric protein showed a 100% survival rate at fifty days after tumor inoculation. **FIG. 38C** summarizes the treatment outcomes for each group.

The above data clearly demonstrate, *inter alia*, functional activity of mTIM3-Fc-OX40L *in vivo*, at least, in treating cancer.

### EQUIVALENTS

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

### INCORPORATION BY REFERENCE

All patents and publications referenced herein are hereby incorporated by reference in their entireties.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

As used herein, all headings are simply for organization and are not intended to limit the disclosure in any manner. The content of any individual section may be equally applicable to all sections.

## CLAIMS

What is claimed is:

1. A method of treating cancer or an inflammatory disease, comprising administering to a subject in need thereof:
  - (i) a first chimeric protein of a general structure of N terminus – (a) – (b) – (c) – C terminus, wherein:
    - (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of a Type II transmembrane protein, and
    - the first chimeric protein modulates the innate immune system; and
  - (ii) a second chimeric protein of a general structure of N terminus – (a) – (b) – (c) – C terminus, wherein:
    - (a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and
    - the second chimeric protein modulates the adaptive immune system.
2. The method of claim 1, wherein the first chimeric protein is administered before the second chimeric protein.
3. The method of claim 1, wherein the first chimeric protein is administered after the second chimeric protein.
4. The method of any one of claims 1 to 3, wherein the first chimeric protein comprises at least one of: TIGIT, CSF1R, CD172a/SIRP $\alpha$ , VSIG8, TIM3, 41BBL, CD40L, SIGLEC7, SIGLEC9, and LIGHT.
5. The method of any one of claims 1 to 4, wherein the second chimeric protein comprises at least one of: PD-1, TIM3, VSIG8, CD172a/SIRP $\alpha$ , OX40L, GITRL, TL1A, and IL-2.
6. The method of any one of claims 1 to 5, wherein the first chimeric protein and the second chimeric protein are independently selected from TIM3-Fc-OX40L, CD172a-Fc-CD40L, and CSF1R-Fc-CD40L.
7. The method of claim 6, wherein TIM3-Fc-OX40L is administered before CD172a-Fc-CD40L.
8. The method of claim 6, wherein TIM3-Fc-OX40L is administered before CSF1R-Fc-CD40L.
9. The method of claim 6, wherein CD172a(SIRP1 $\alpha$ )-Fc-CD40L is administered before TIM3-Fc-OX40L.
10. The method of claim 6, wherein CD172a(SIRP1 $\alpha$ )-Fc-CD40L is administered before TIM3-Fc-OX40L.
11. A method of treating cancer or an inflammatory disease, comprising administering to a subject in need thereof: a second chimeric protein of a general structure of N terminus – (a) – (b) – (c) – C terminus, wherein:

(a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and

the second chimeric protein modulates the adaptive immune system,

wherein:

the subject is undergoing or has undergone treatment with a first chimeric protein of a general structure of N terminus – (a) – (b) – (c) – C terminus, wherein:

(a) is a first domain comprising an extracellular domain of a Type I transmembrane protein, (b) is a linker comprising at least one cysteine residue capable of forming a disulfide bond, and (c) is a second domain comprising an extracellular domain of Type II transmembrane protein, and

the first chimeric protein modulates the innate immune system.

12. The method of claim 11 wherein the first chimeric protein comprises at least one of: TIGIT, CSF1R, CD172a(SIRP1 $\alpha$ ), VSIG8, TIM3, 41BBL, CD40L, SIGLEC7, SIGLEC9, and LIGHT.

13. The method of claim 11 or claim 12, wherein the second chimeric protein comprises at least one of: PD-1, TIM3, VSIG8, CD172a(SIRP1 $\alpha$ ), OX40L, GITRL, TL1A, and IL-2.

14. The method of any one of claims 11 to 13, wherein the first chimeric protein and the second chimeric protein are independently selected from TIM3-Fc-OX40L, CD172a(SIRP1 $\alpha$ )-Fc-CD40L, and CSF1R-Fc-CD40L.

15. The method of claim 14, wherein TIM3-Fc-OX40L is administered before CD172a(SIRP1 $\alpha$ )-Fc-CD40L.

16. The method of claim 15, wherein TIM3-Fc-OX40L is administered before CSF1R-Fc-CD40L.

17. The method of claim 16, wherein CD172a(SIRP1 $\alpha$ )-Fc-CD40L is administered before TIM3-Fc-OX40L.

18. The method of claim 17, wherein CSF1R-Fc-CD40L is administered before TIM3-Fc-OX40L.

19. The method of any one of claims 1 to 18, wherein the first chimeric protein and/or the second chimeric protein causes activation of antigen presenting cells.

20. The method of any one of claims 1 to 19, wherein the first chimeric protein and/or the second chimeric protein enhances the ability of antigen presenting cells to present antigen.

21. The method of any one of claims 1 to 20, wherein the first chimeric protein and/or the second chimeric protein provides a sustained immunomodulatory effect.

22. The method of any one of claims 1 to 21, wherein the first chimeric protein and/or the second chimeric protein prevents a tumor cell from transmitting an immunosuppressive signal.

23. The method of any one of claims 1 to 22, wherein the second chimeric protein enhances tumor killing activity by T cells.



24. The method of any one of claims 1 to 23, wherein the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, or an antibody sequence.
25. The method of any one of claims 1 to 24, wherein the linker comprises hinge-CH2-CH3 Fc domain derived from IgG4.
26. The method of claim 25, wherein the hinge-CH2-CH3 Fc domain is derived from human IgG4.
27. The method of claim 25 or claim 26, wherein the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 45.
28. The method of claim 25 or claim 26, wherein the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 46.
29. The method of claim 25 or claim 26, wherein the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 47.
30. The method of any one of claims 25 to 29, wherein the linker further comprises at least one joining linker selected from SEQ ID NOs: 48 to 94.
31. The method of claim 30, wherein the linker comprises at least two joining linkers each joining linker independently selected from SEQ ID NOs: 48 to 94; wherein one joining linker is located N terminal to the hinge-CH2-CH3 Fc domain and another joining linker is located C terminal to the hinge-CH2-CH3 Fc domain.
32. The chimeric protein as described herein for use as a medicament.
33. The chimeric protein as described herein for use in the treatment of cancer.
34. The chimeric protein as described herein for use in the treatment of an inflammatory disease.
35. Use of the chimeric protein as described herein in the manufacture of a medicament.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00; C07K 14/705 (2018.01)

CPC - A61K 39/0011; C07K 14/70503; C07K 14/70596; C07K 2319/32; C07K 2319/74 (2018.02)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 424/192.1; 435/69.7; 514/19.3 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016/0340430 A1 (THE JOHNS HOPKINS UNIVERSITY) 24 November 2016 (24.11.2016) entire document	1, 4
Y		2, 3, 11-13
Y	MARCUS et al. "Recognition of tumors by the innate immune system and natural killer cells," <i>Advances in Immunology</i> , 01 January 2015 (01.01.2015), Vol. 122, Pgs. 01-128. entire document	2, 11-13
Y	DE VISSER et al. "The interplay between innate and adaptive immunity regulates cancer development," <i>Cancer Immunology, Immunotherapy</i> , 12 May 2005 (12.05.2005), Vol. 54, No. 11, Pgs. 1143-1152. entire document	3
A	WO 2016/166139 A1 (EBERHARD KARLS UNIVERSITÄT TÜBINGEN) 20 October 2016 (20.10.2016) entire document	1-4, 11-13
A	WO 2013/000234 A1 (HUABO BIOPHARM CO., LTD et al) 03 January 2013 (03.01.2013) entire document	1-4, 11-13
P, A	US 2017/0095531 A1 (HEAT BIOLOGICS, INC.) 06 April 2017 (06.04.2017) entire document	1-4, 11-13

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

\* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

23 April 2018

Date of mailing of the international search report

23 MAY 2018

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, VA 22313-1450

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Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

FIG. 1A

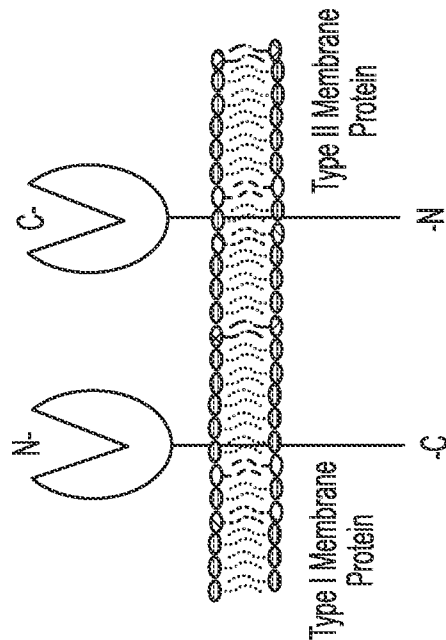


FIG. 1B

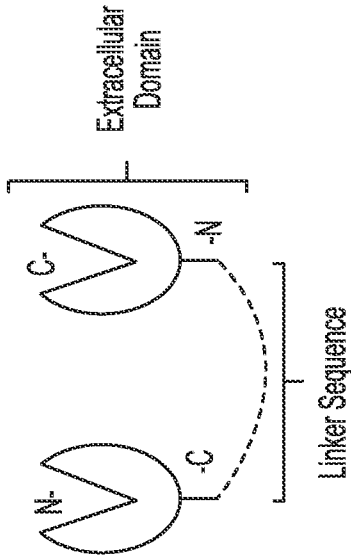


FIG. 1C

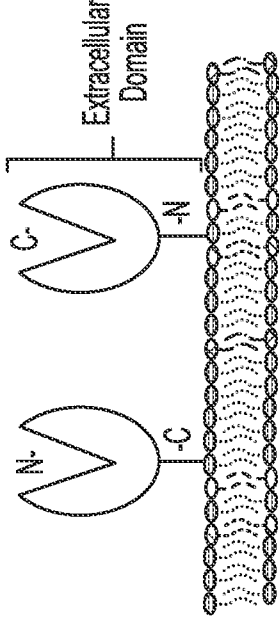
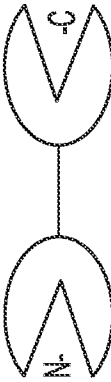


FIG. 1D



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FIG. 2

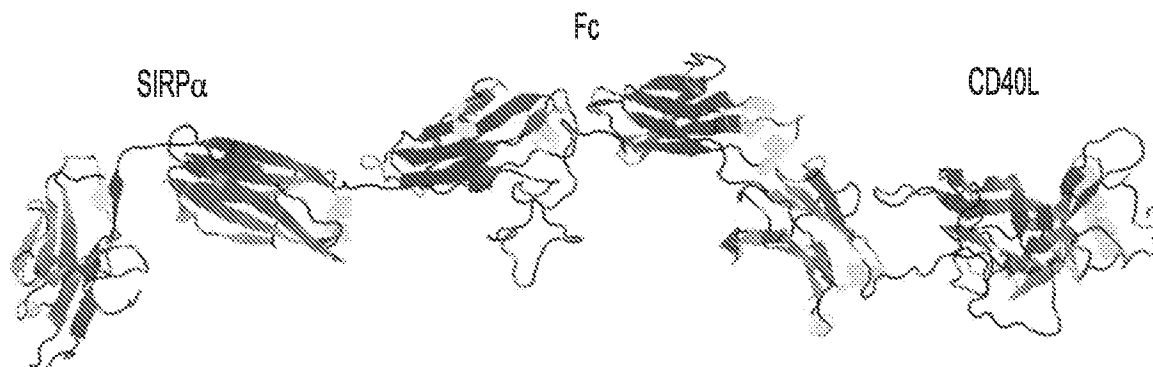
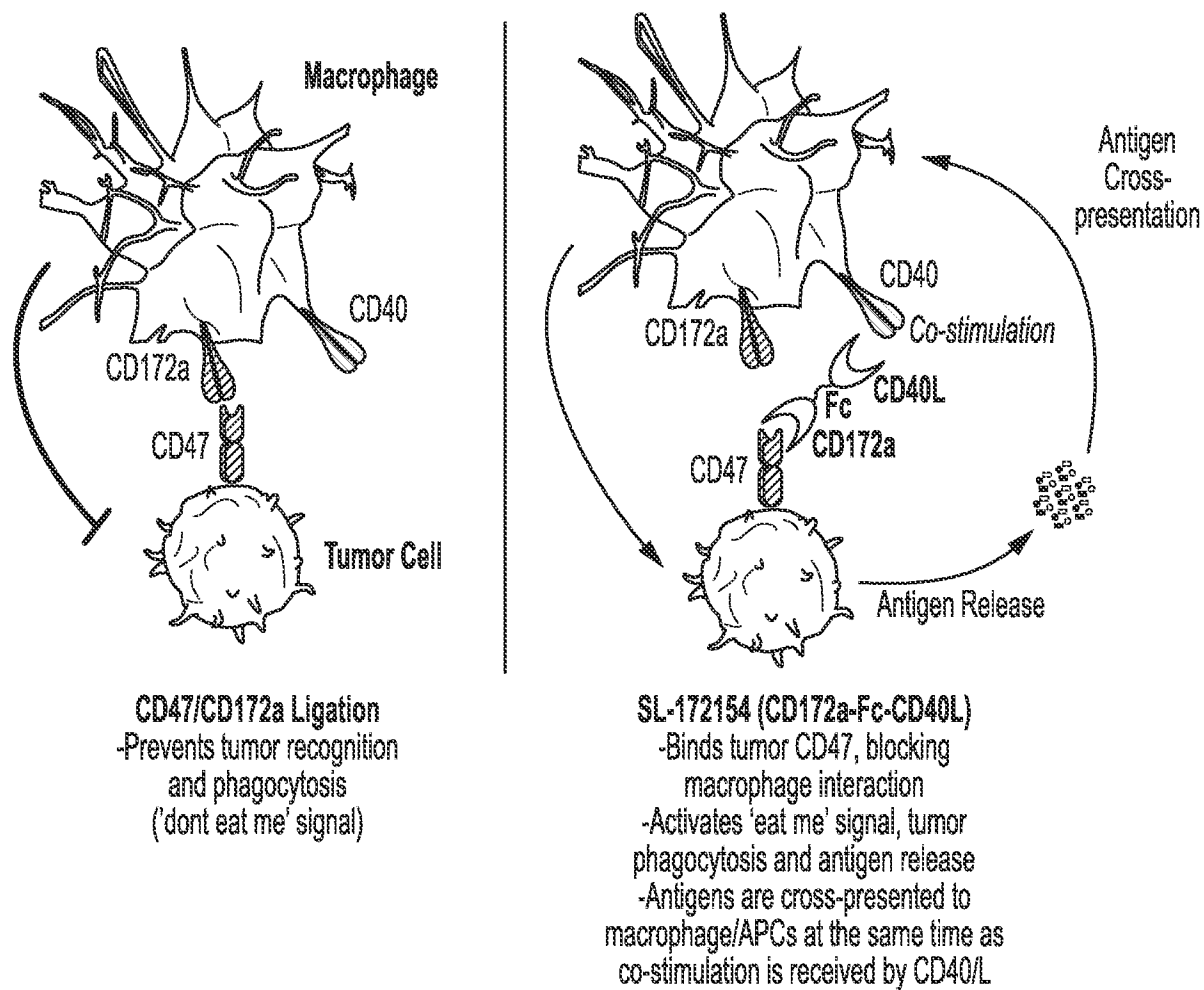
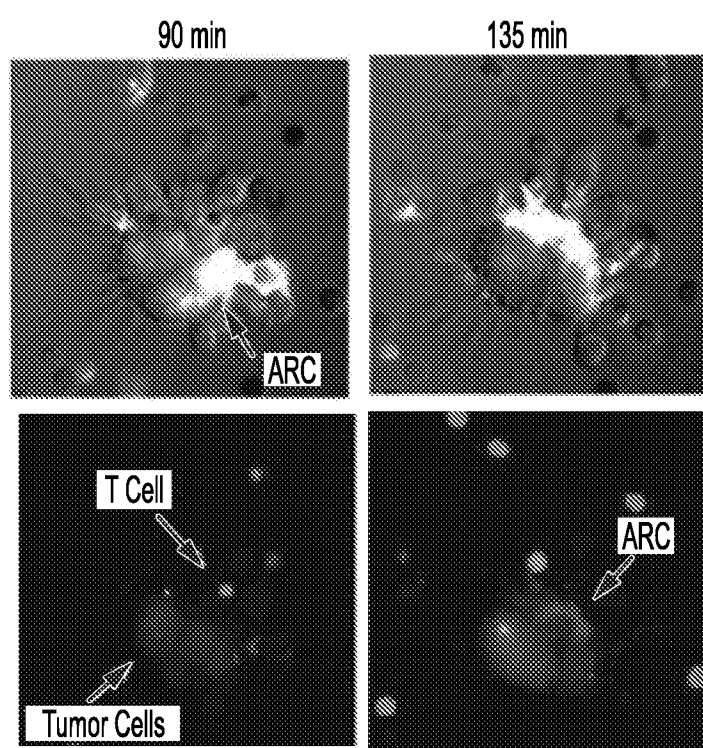


FIG. 3A



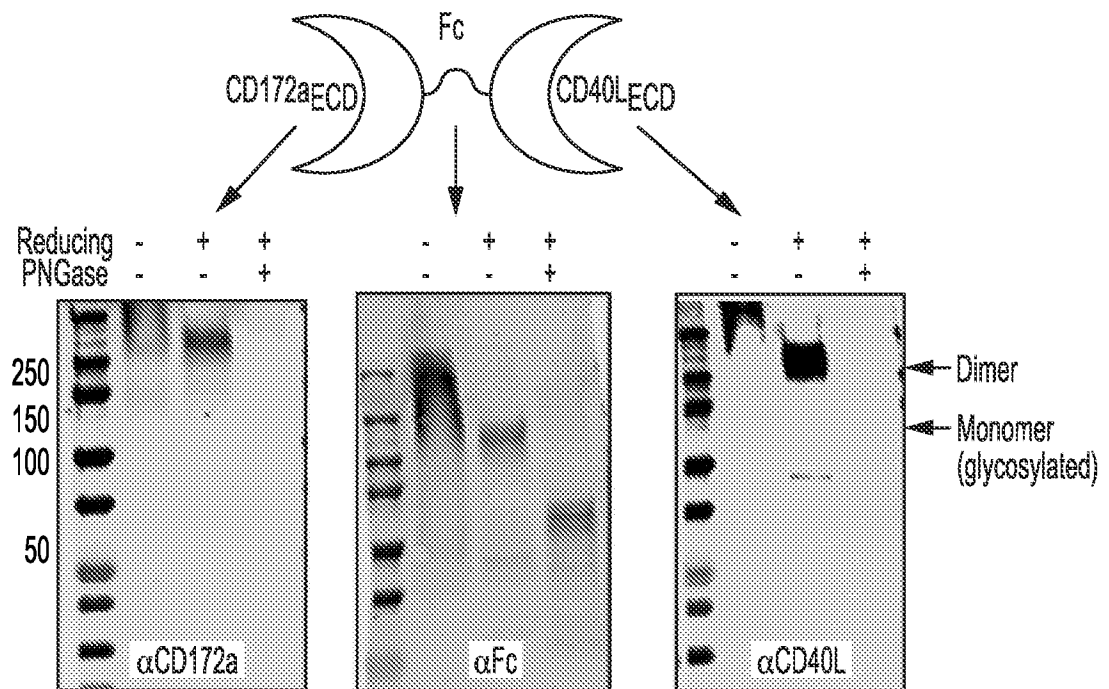
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FIG. 3B



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FIG. 4



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FIG. 5A

Gene Id	Protein Name	Accession	Primary Screen		Confirmation Screen		Comment
			Rep 1	Rep 2	Rep 1	Rep2	
PD1-Fc-OX40L	PDL1 (CD274)	NM_014143.3	strong	strong	strong	strong	isoform 1
	PDCD1LG2	BC113678	strong	strong	strong	strong	
	OX40		n/a	n/a	strong	strong	
	PDL1		n/a	n/a	strong	strong	
	TNFRSF4	NM_003327.3	n/a	n/a	medium	medium	canonical isoform
	LGALS1	BC001693	med/strong	med/strong	medium	medium	soluble; seen with other Fc fusion proteins

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FIG. 5B

Gene Id	Protein Name	Accession	Primary Screen		Confirmation Screen		Comment
			Rep 1	Rep 2	Rep 1	Rep2	
CD40	CD40 Molecule	BC012419	strong	strong	strong	strong	canonical isoform
CSF1	Colony Stimulating Factor 1	NM_000757.4	med/strong	med/strong	strong	strong	canonical isoform
CSF1	Colony Stimulating Factor 1	NM_172210.2	strong	strong	strong	strong	isoform 2
LGALS1	Galectin 1	BC001693	med/strong	med/strong	weak/med	weak/med	soluble; seen with other Fc fusion proteins
SLC51B	Solute Carrier Family 51 Beta Subunit	BC103822.1	v. weak	v. weak	weak	weak	

CSF1R-Fc-CD40L



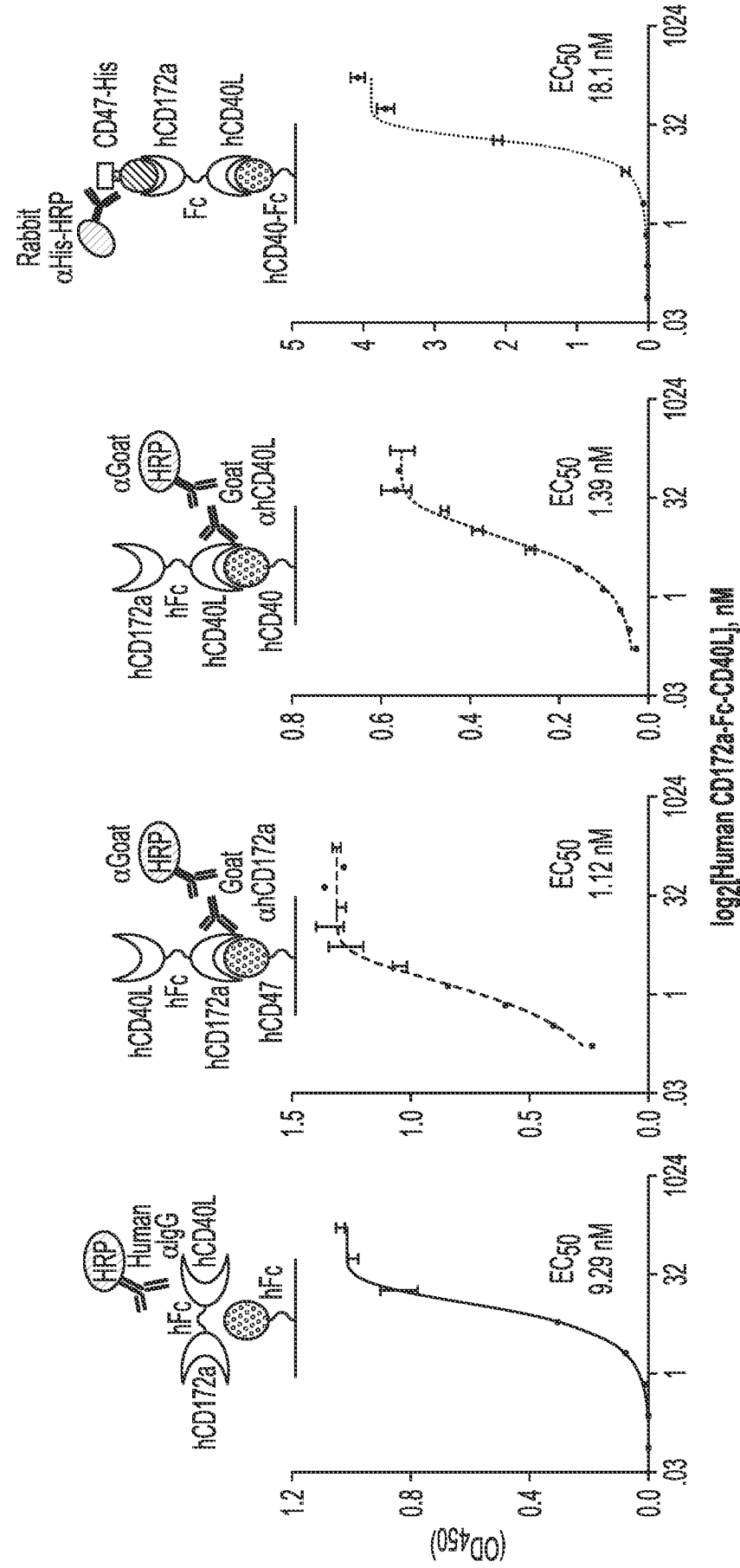
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FIG. 5C

Gene Id	Protein Name	Accession	Primary Screen		Confirmation Screen		Comment
			Rep 1	Rep 2	Rep 1	Rep2	
CD47	CD 47 Molecule	BC012884	medium	medium	strong	strong	isoform OA3-305
CD47	CD 47 Molecule	BC037306	medium	medium	strong	strong	isoform OA3-305
CD40	CD40 Molecule	BC012419	strong	strong	strong	strong	canonical isoform
CD47	CD 47 Molecule	NM_001777.3	med/strong	med/strong	med/strong	med/strong	canonical isoform
SIRPA	Signal Regulatory Protein Alpha	BC033092.1	inverse	inverse	inverse	inverse	binding partner of CD47
LGALS1	Galectin 1	BC001693	med/strong	med/strong	weak/med	weak/med	soluble; seen with other Fc fusion proteins

mCD172a(SIRPα)-  
Fc-CD40L

FIG. 6



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FIG. 7A

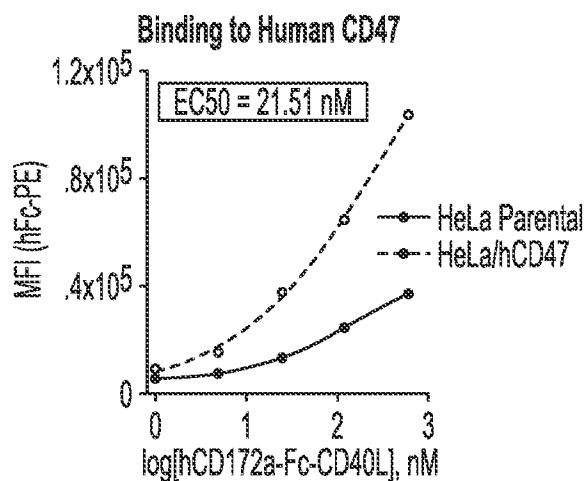


FIG. 7B

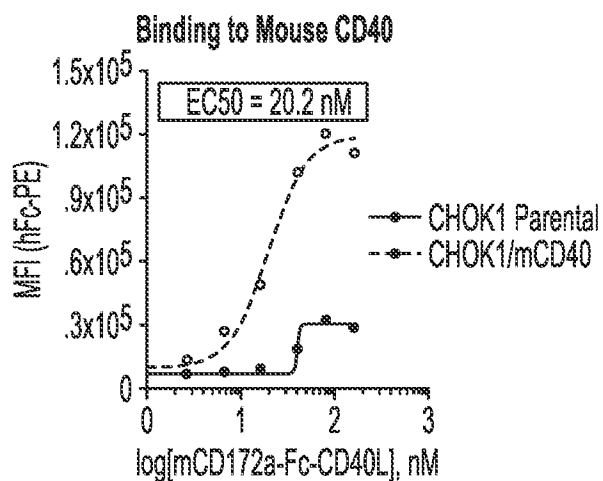


FIG. 8A

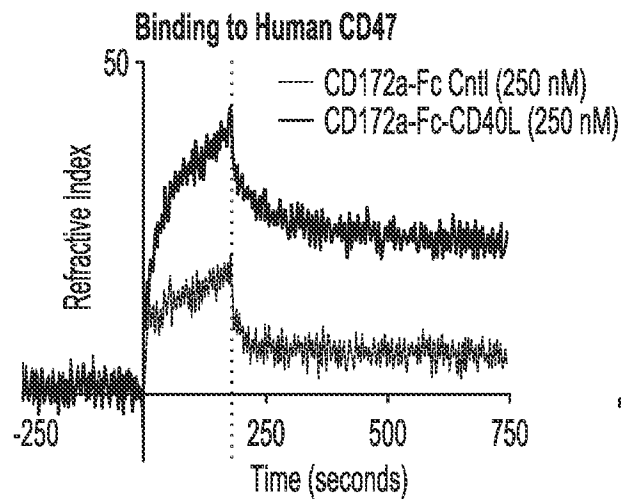
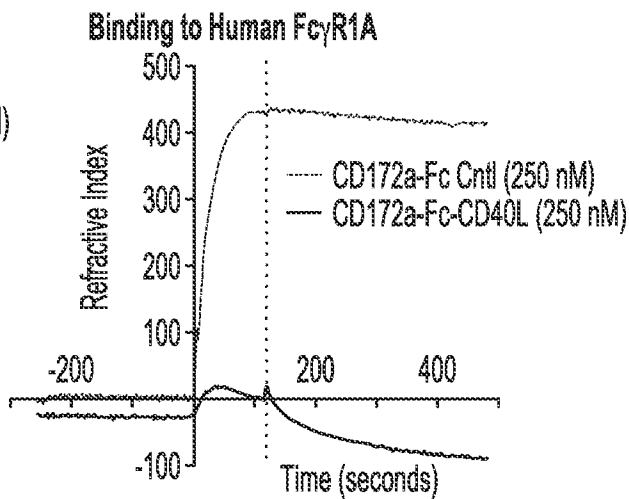


FIG. 8B



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FIG. 8C

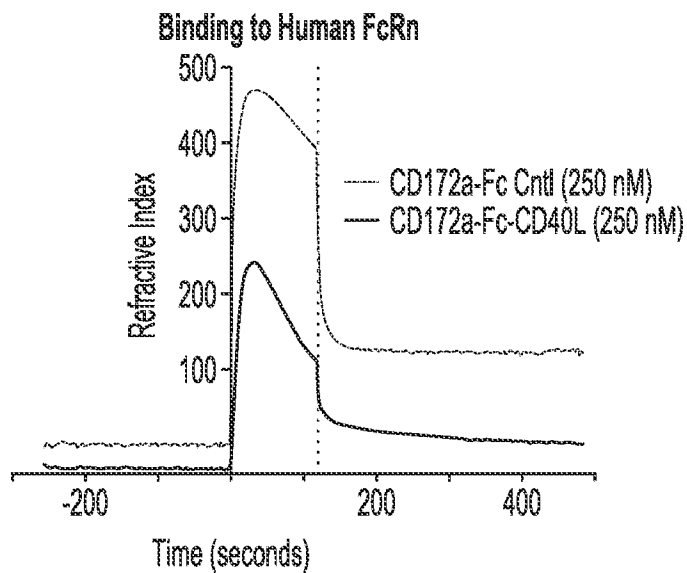


FIG. 8D

		K <sub>a</sub>	K <sub>d</sub>	K <sub>D</sub>
		(on-rate; 1/Ms)	(off-rate; 1/s)	(binding; M)
Human Binding to:	CD172a-Fc	7.56 E+5	2.39 E-3	3.16 nM
	CD172a-Fc-CD40L	2.42 E+5	8.61 E-4	3.59 nM
	IgG1	2.49 E+4	4.40 E-4	17.7 nM
	CD172a-Fc-CD40L	ND	ND	ND
	IgG1	9.45 E+5	4.60 E-3	4.87 nM
	CD172a-Fc-CD40L	9.56 E+3	7.56 E-3	790 nM

FIG. 8E

		Mouse CD172-Fc-CD40L		
		K <sub>a</sub>	K <sub>d</sub>	K <sub>D</sub>
		(on-rate; 1/Ms)	(off-rate; 1/s)	(binding; M)
Mouse Binding to:	mCD40L-Fc	3.04 E+4	9.81 E-5	3.23 nM
	CD172a-Fc-CD40L	7.28 E+4	5.51 E-5	.756 nM

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FIG. 9A

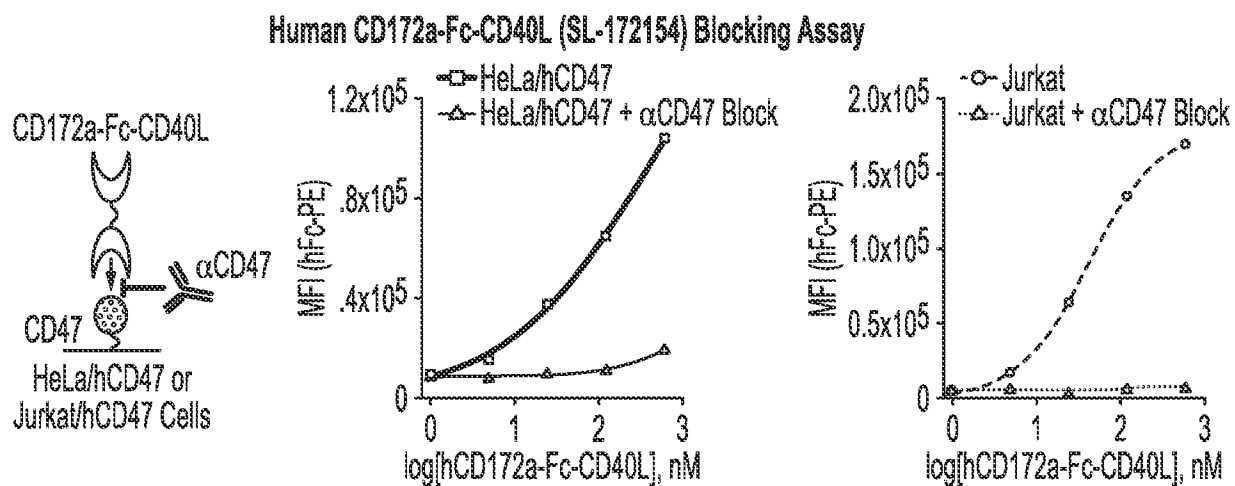
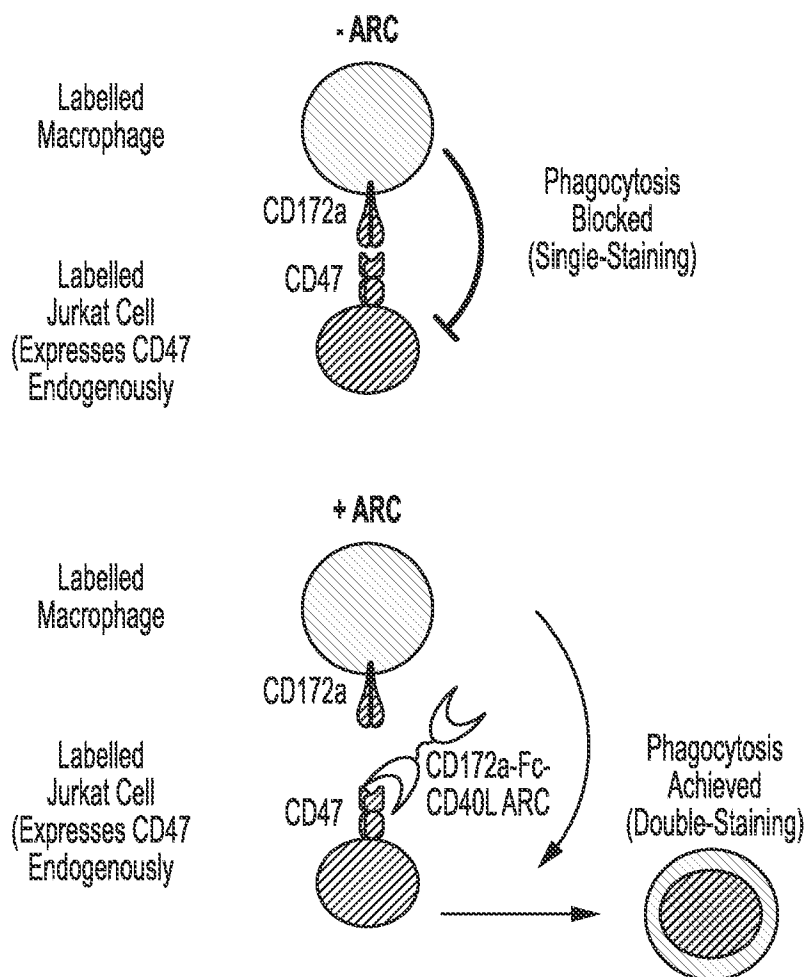
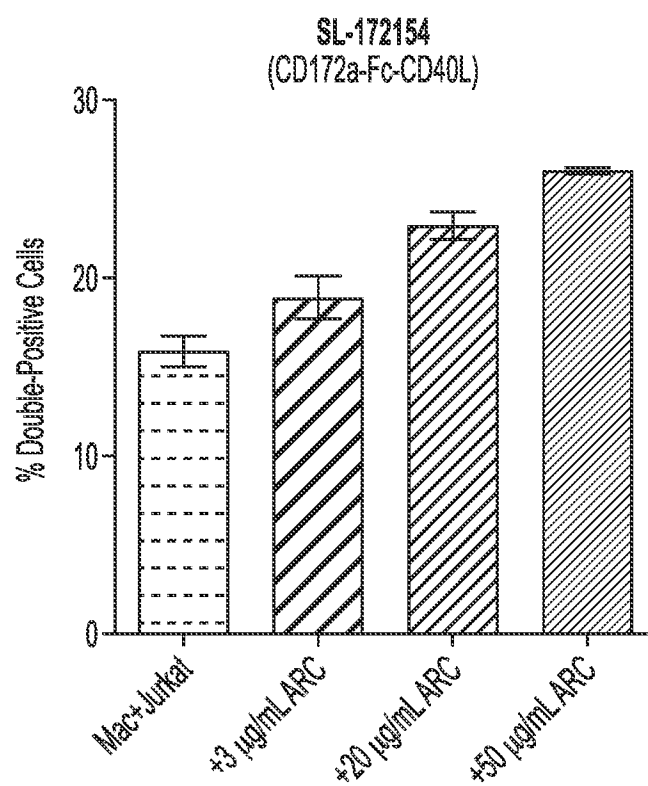


FIG. 9B



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FIG. 9C



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FIG. 10A

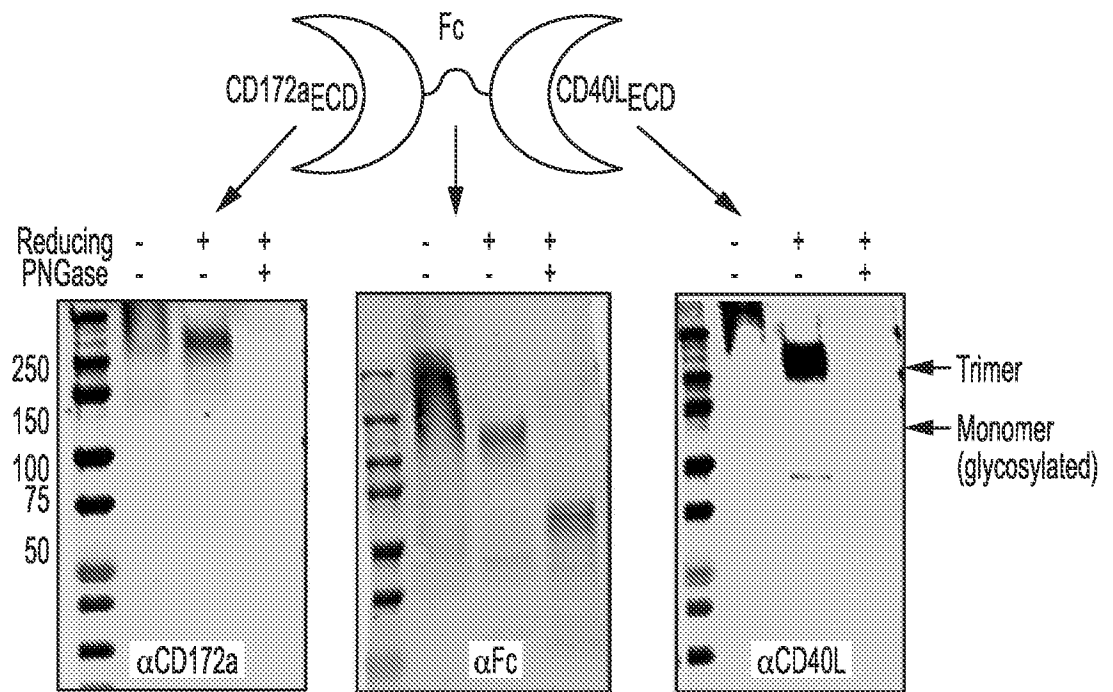
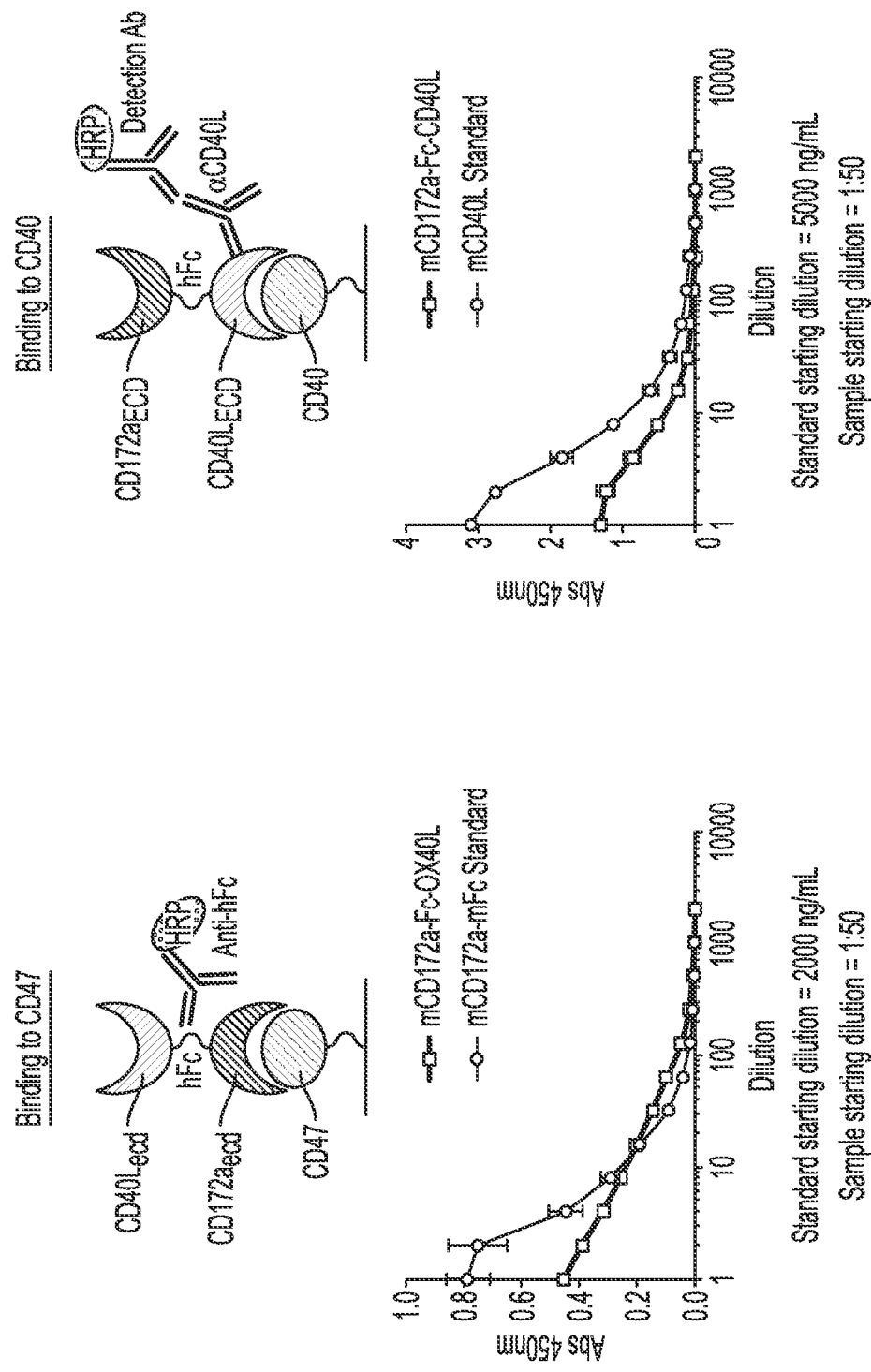


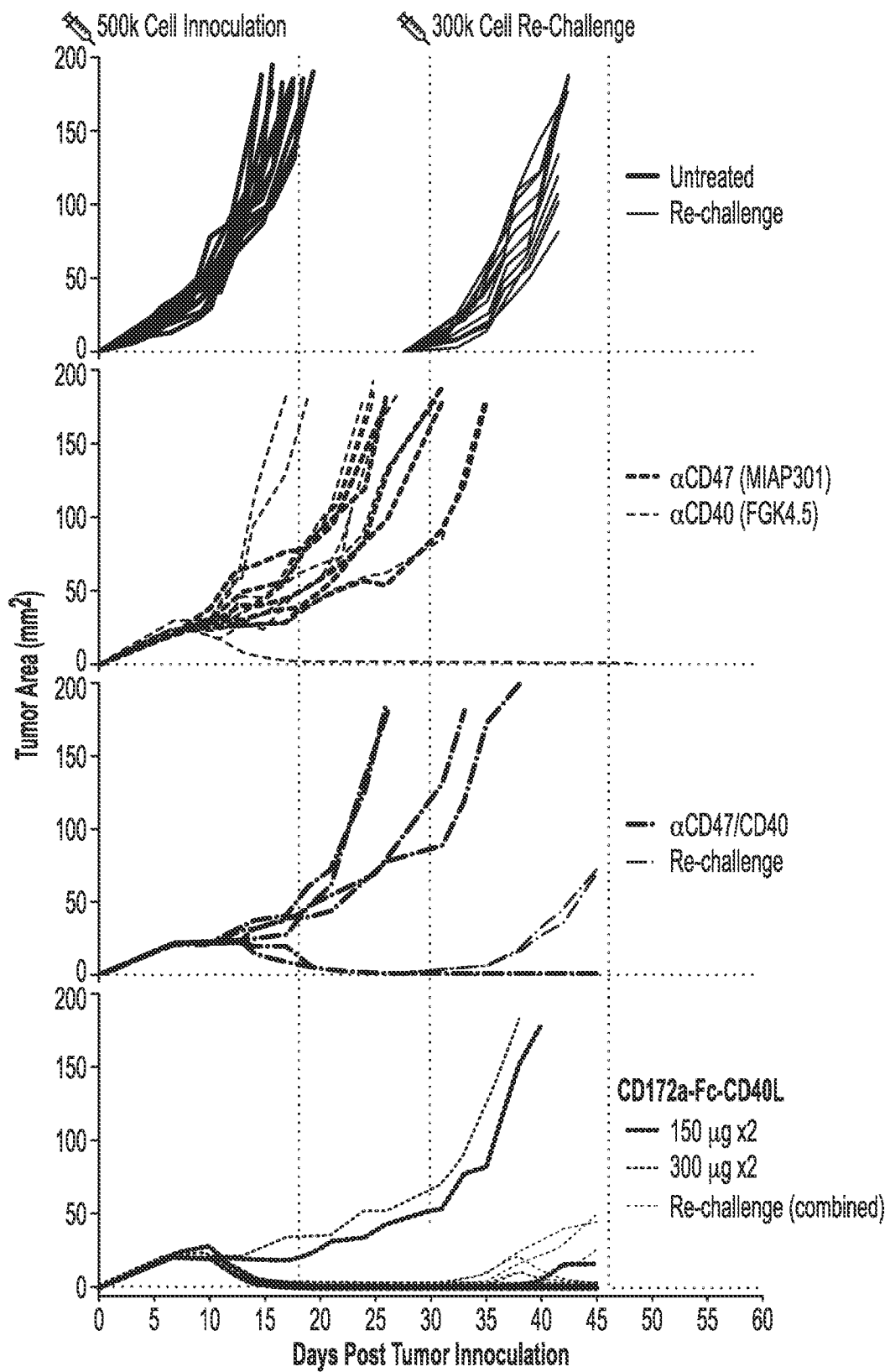
FIG. 10B





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FIG. 11A



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FIG. 11B

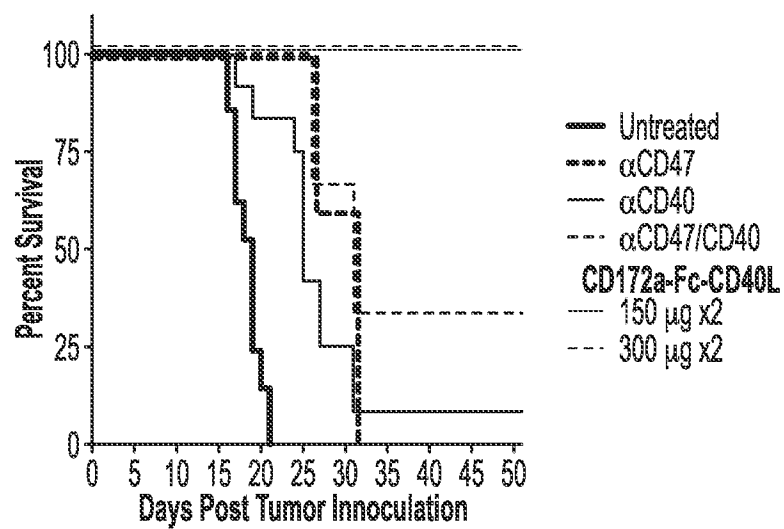


FIG. 11C

Group	Total, N	Short-Term, N (Immune Profiling)	Long-Term, N (Tumor Growth/Survival)	% Rejection (Primary Tumor)	% Rejection (Re-challenge)
Untreated	33	12	21	0.0	0.0
$\alpha$ CD47 (MIAP301)	5	/	5	0.0	N/A
$\alpha$ CD40 (FGK4.5)	12	/	12	8.3	N/A
$\alpha$ CD47 (CD40)	10	4	6	33.3	0.0
CD172a-Fc-CD40L (150 $\mu$ g x2)	11	6	5	80.0	75.0
CD172a-Fc-CD40L (350 $\mu$ g x2)	8	4	4	75.0	33.3

FIG. 12A

FIG. 12B

FIG. 12C

FIG. 12D

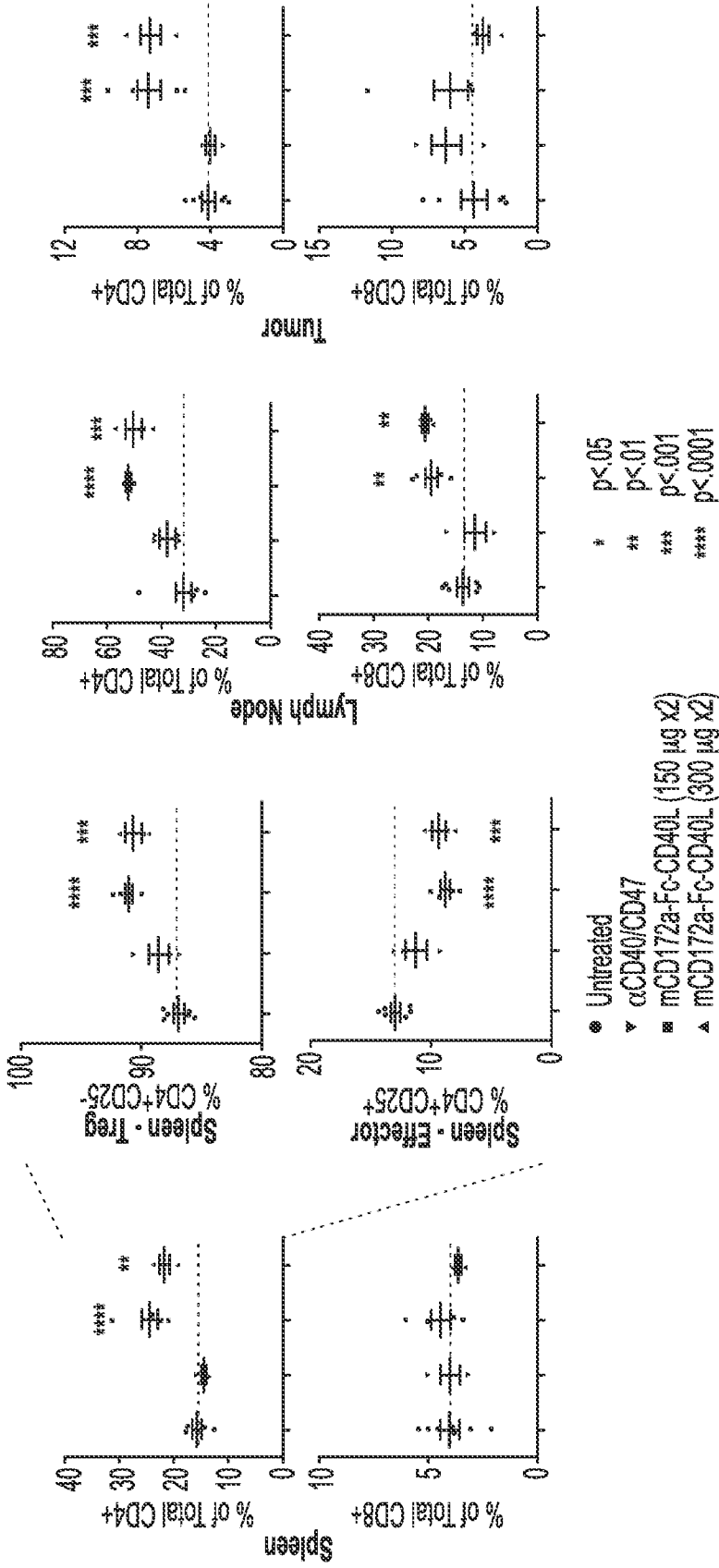


FIG. 12E

FIG. 12F

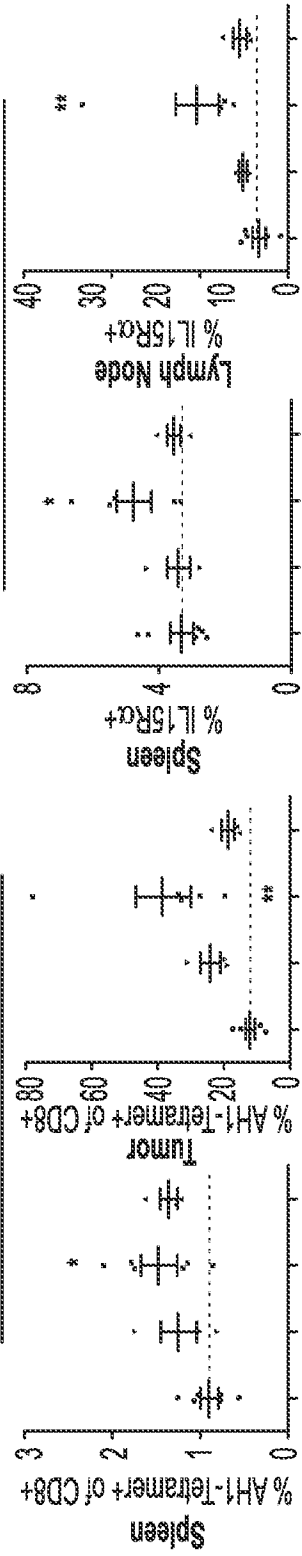
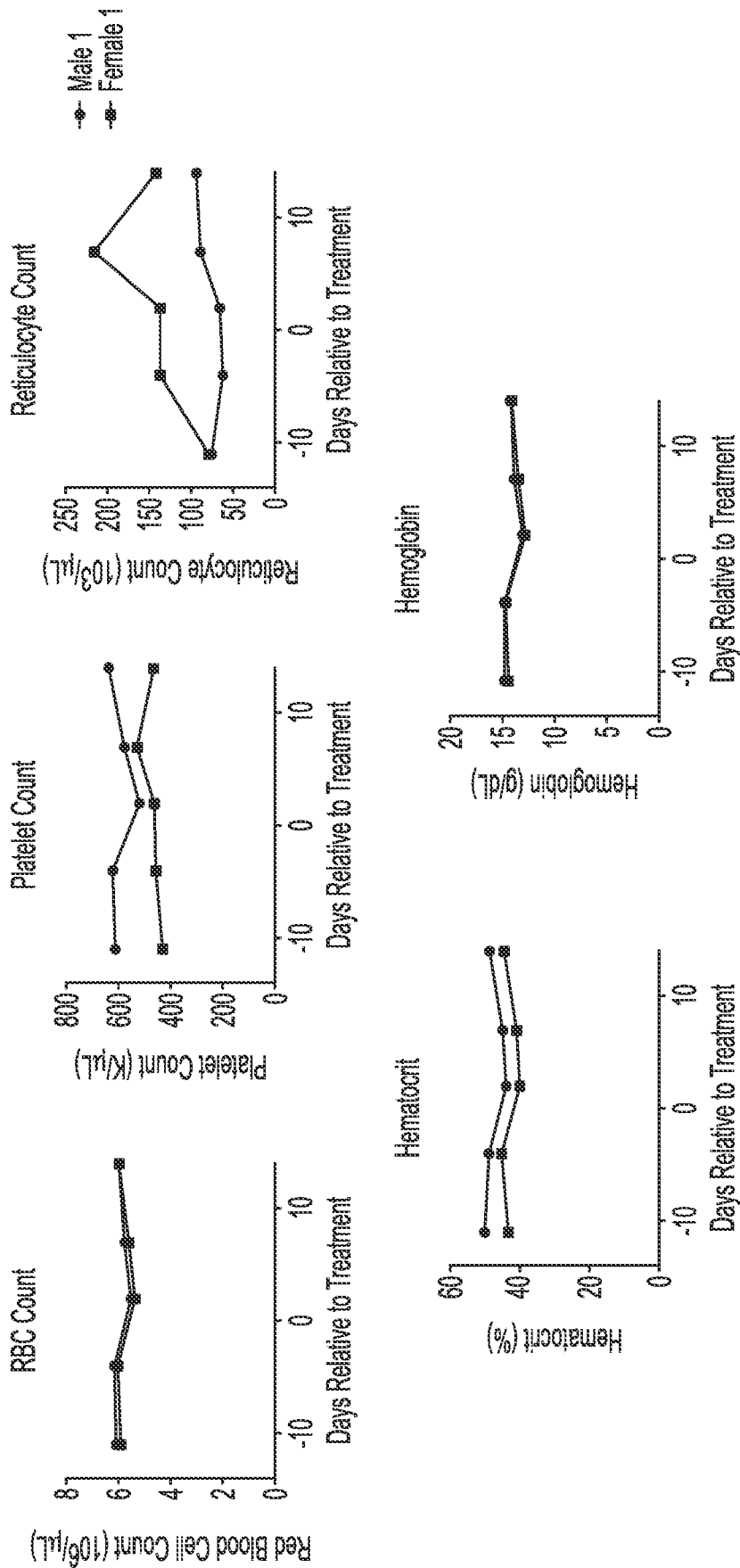
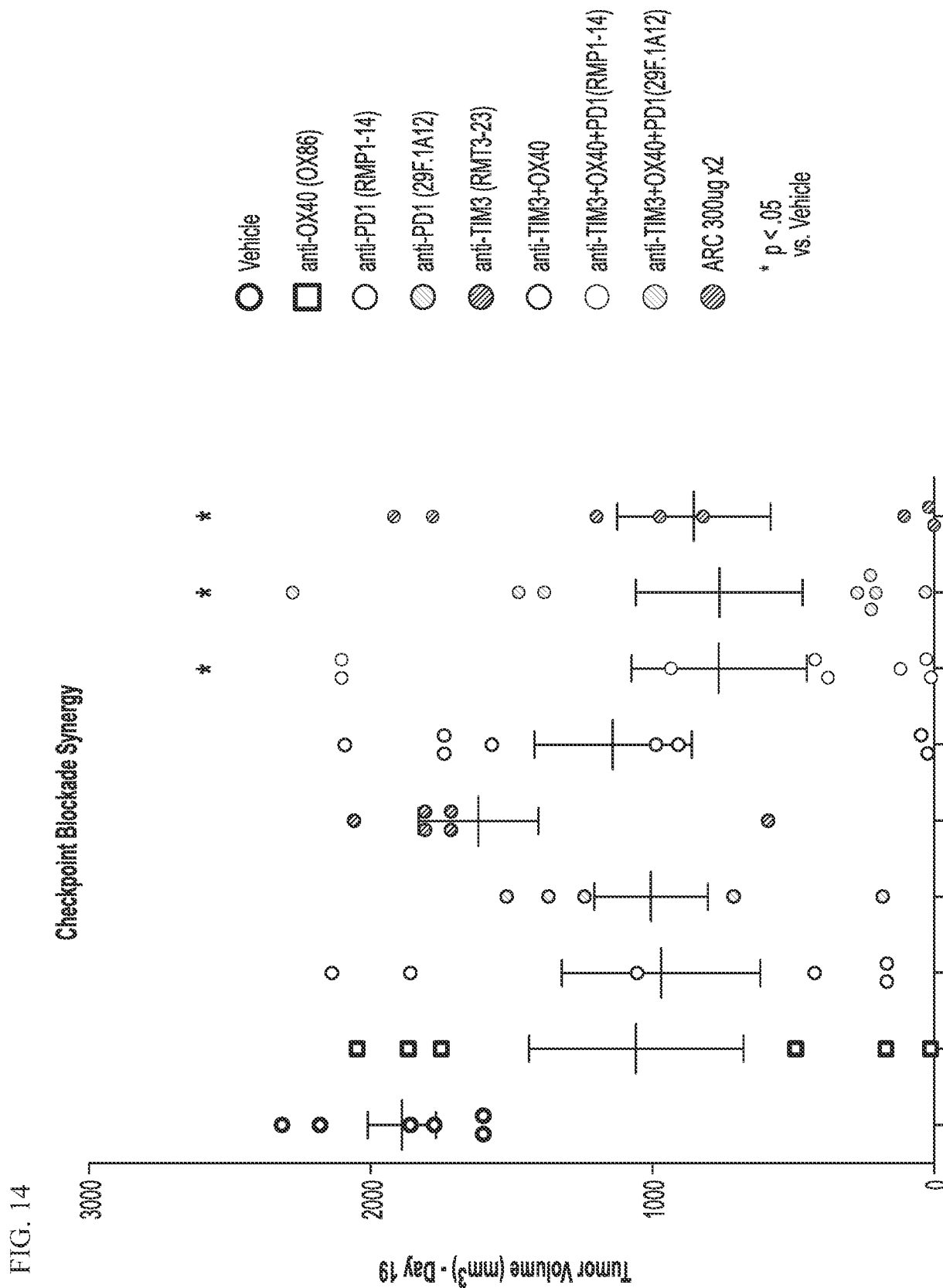


FIG. 13



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FIG. 15A

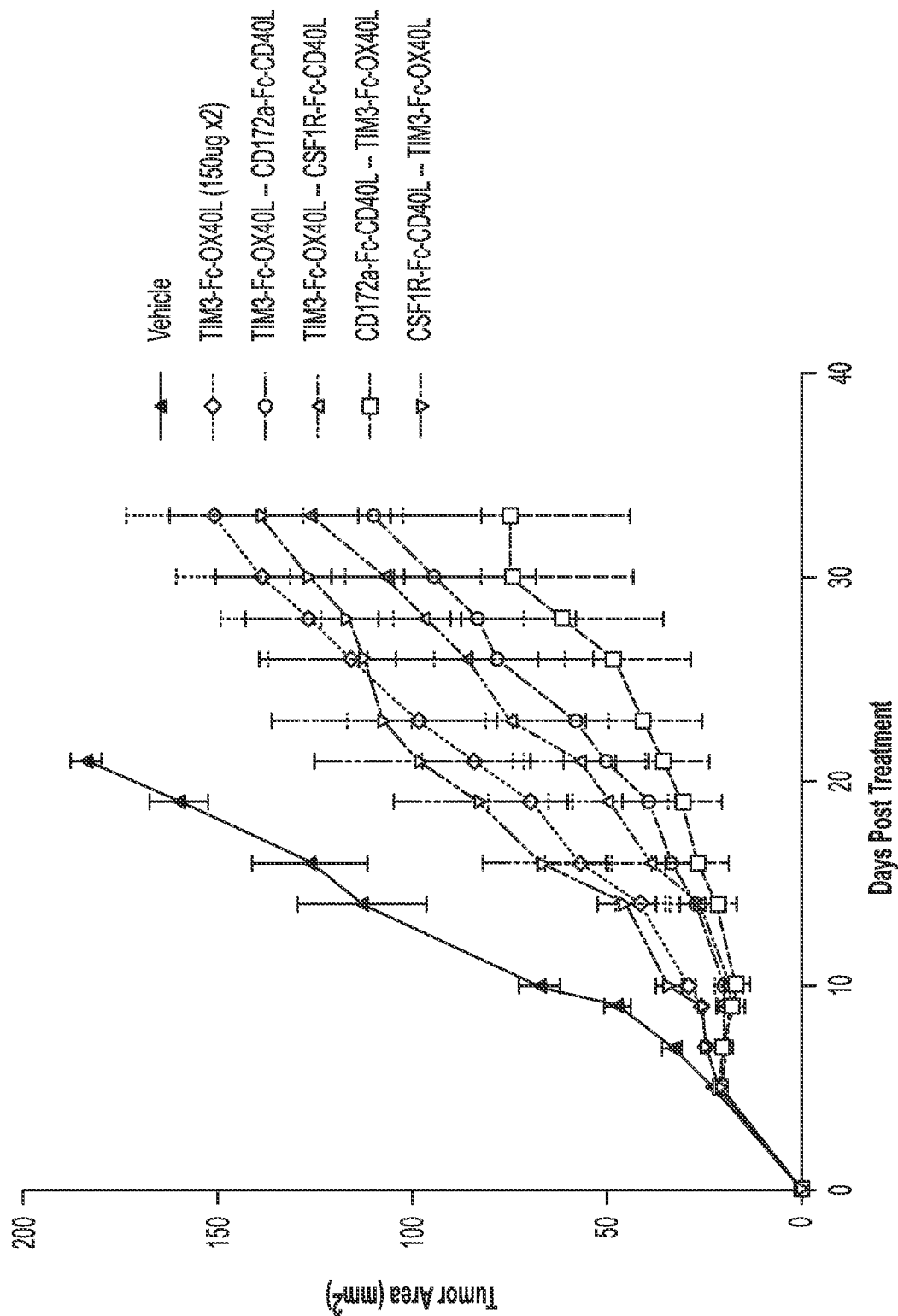
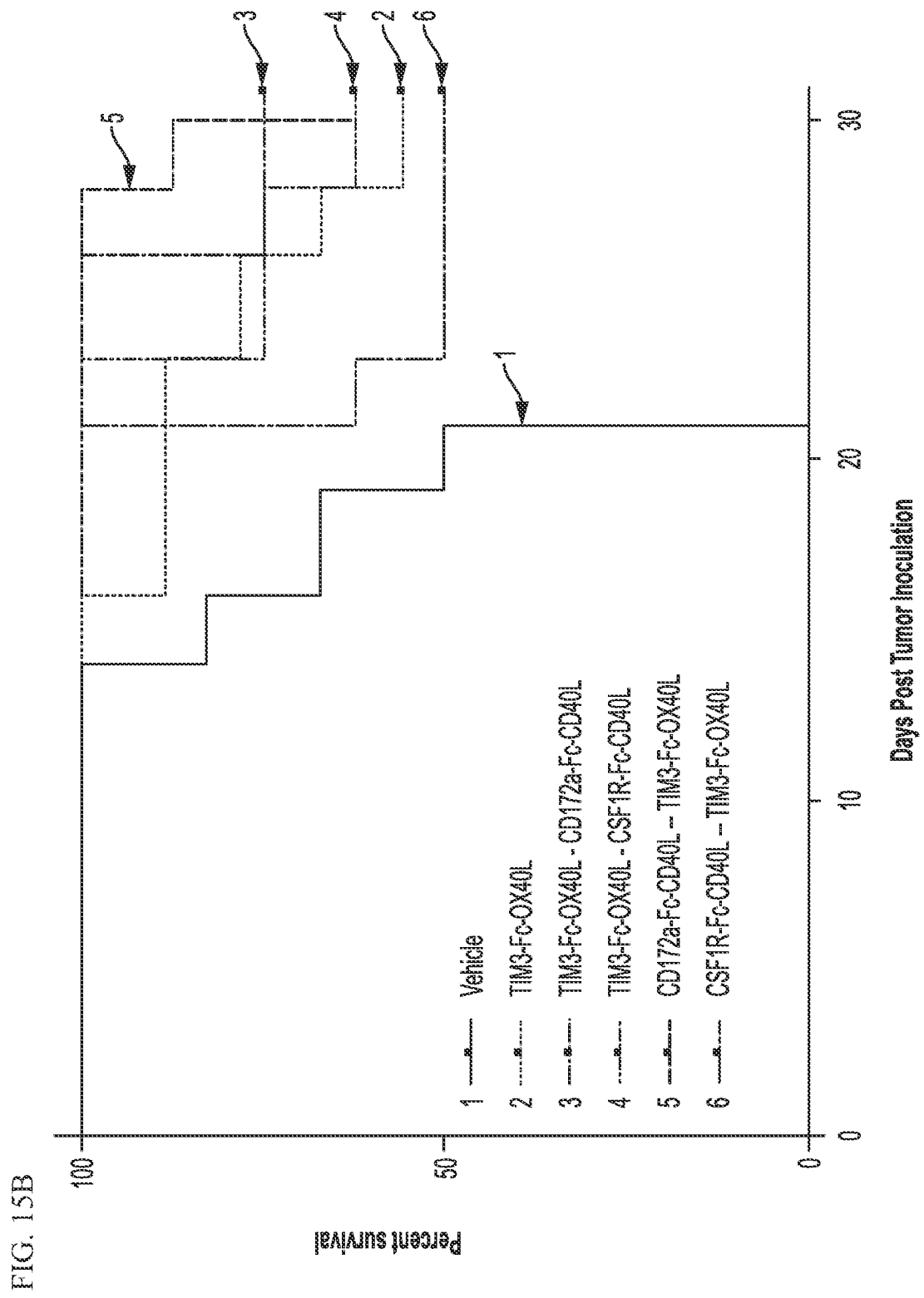
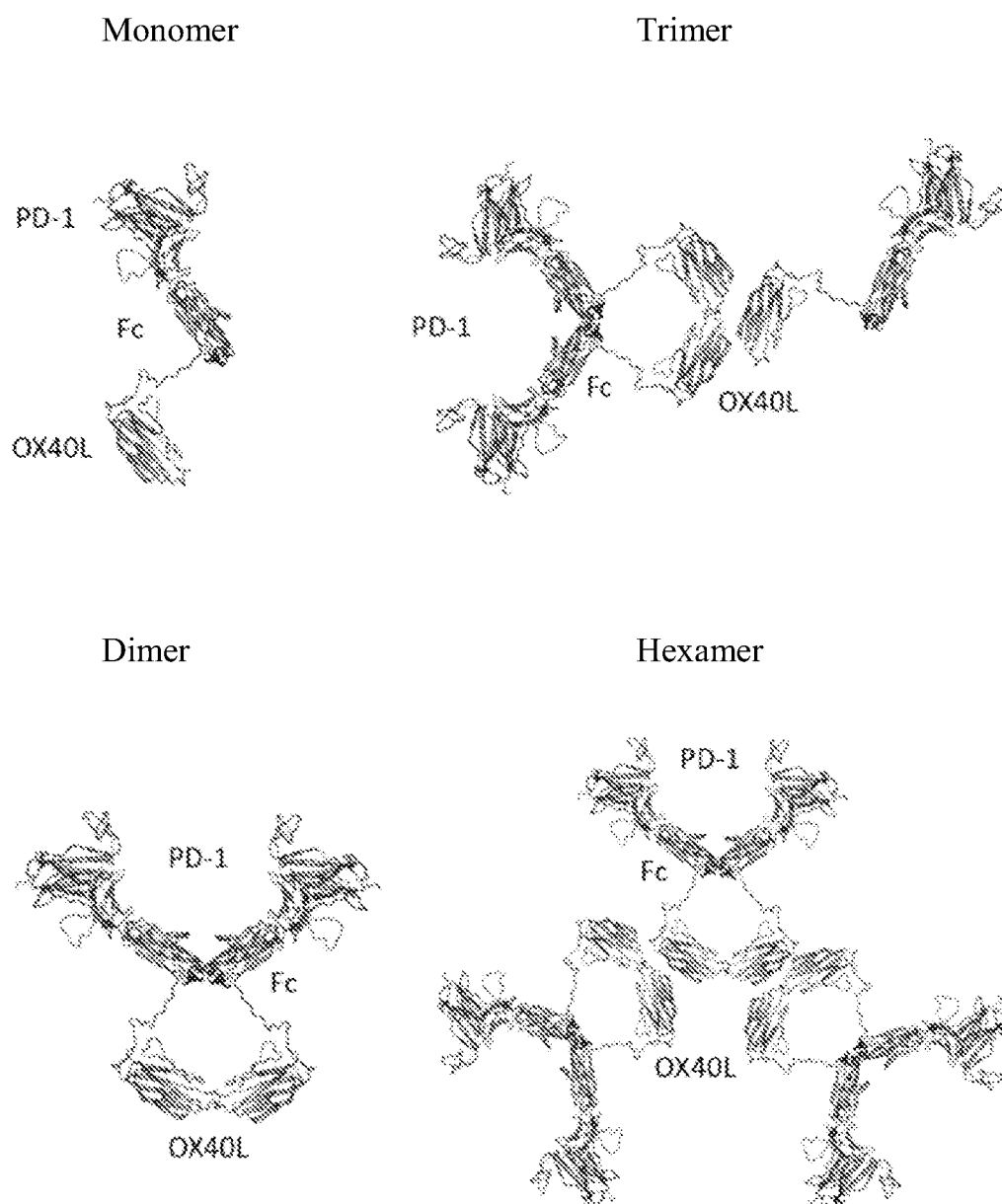


FIG. 15A



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FIG. 16





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FIG. 17

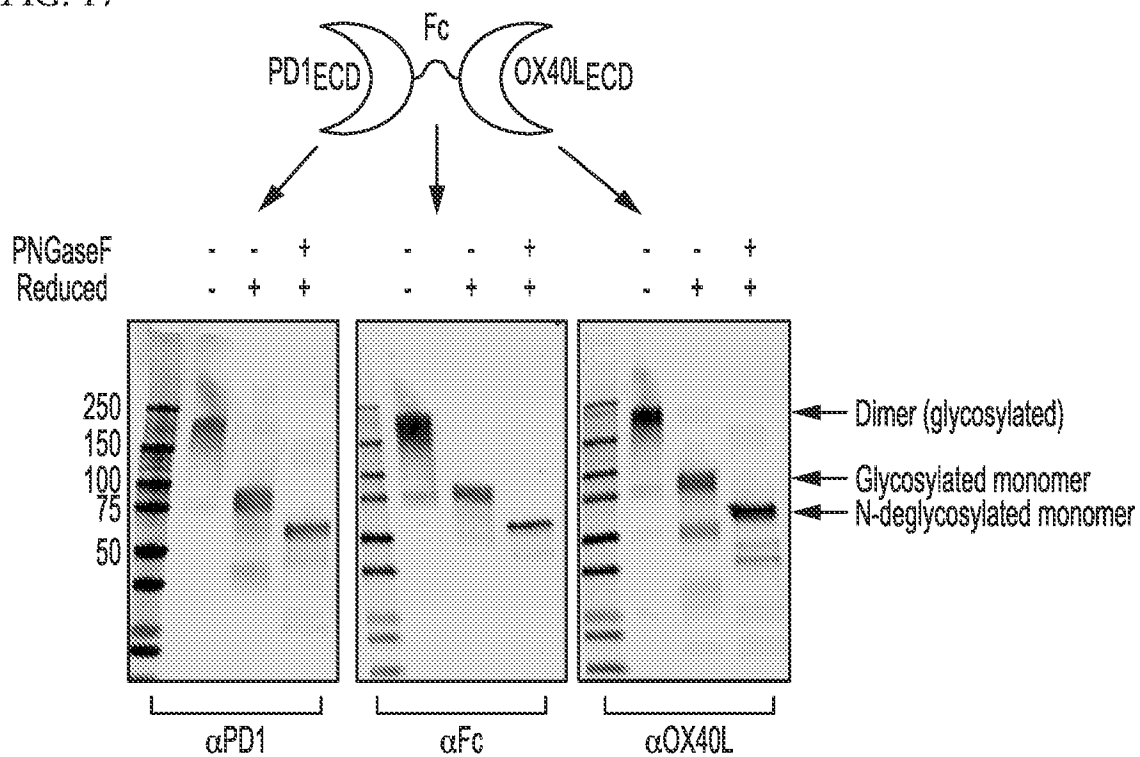
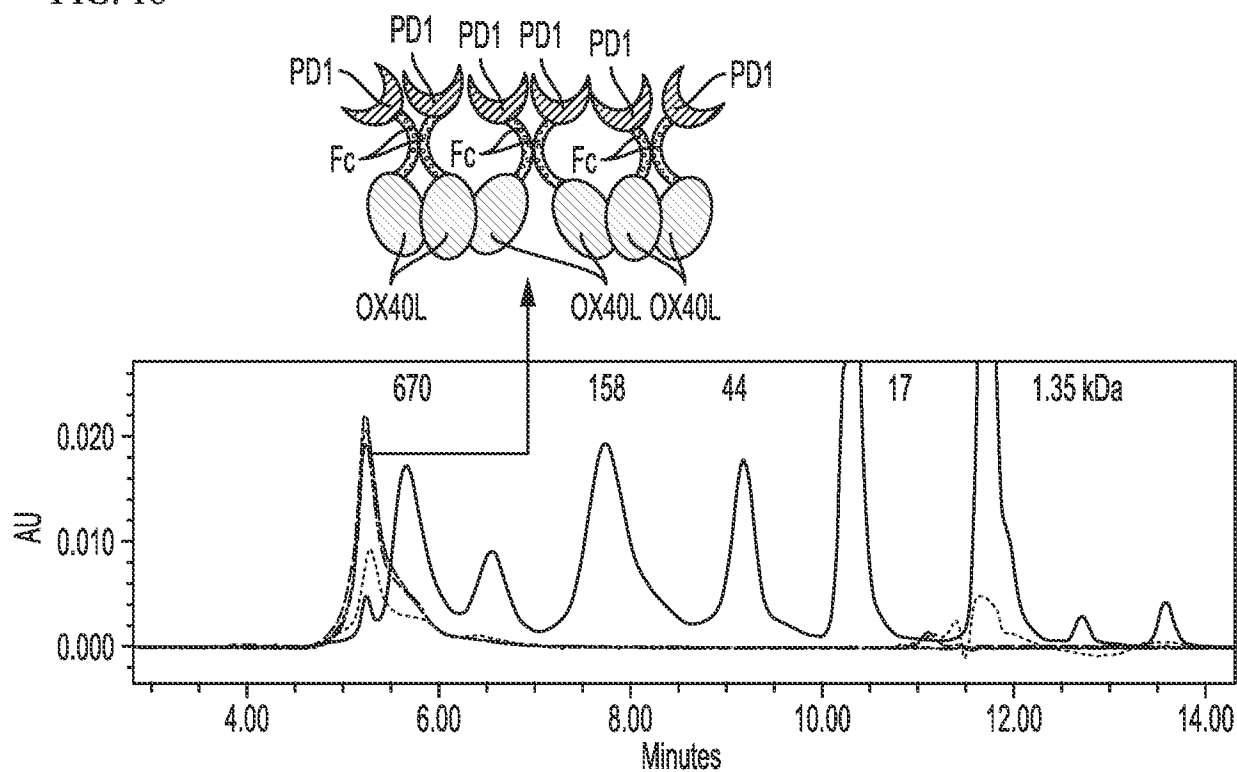


FIG. 18



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FIG. 19

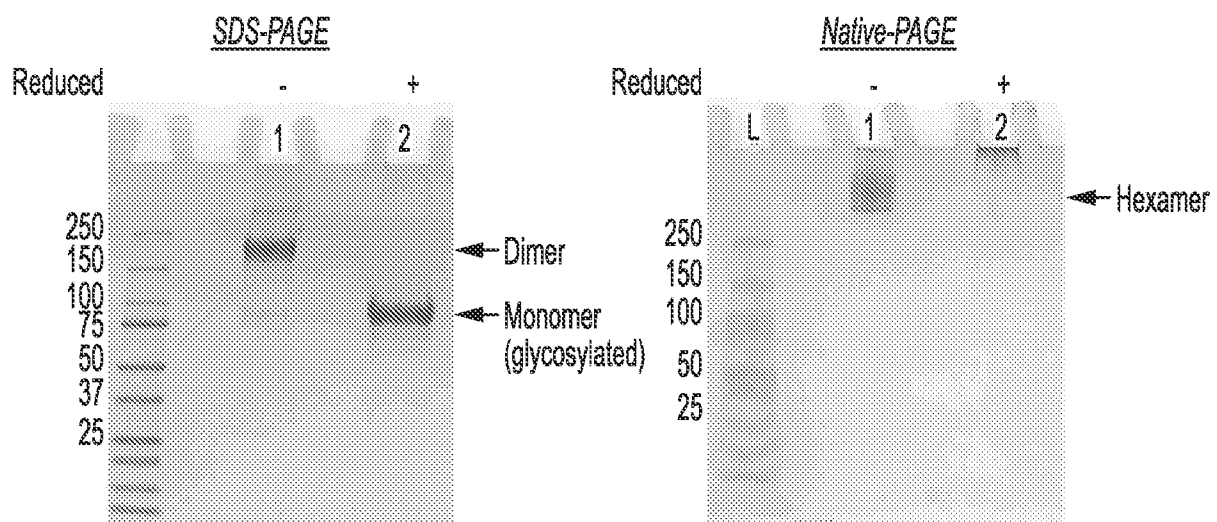
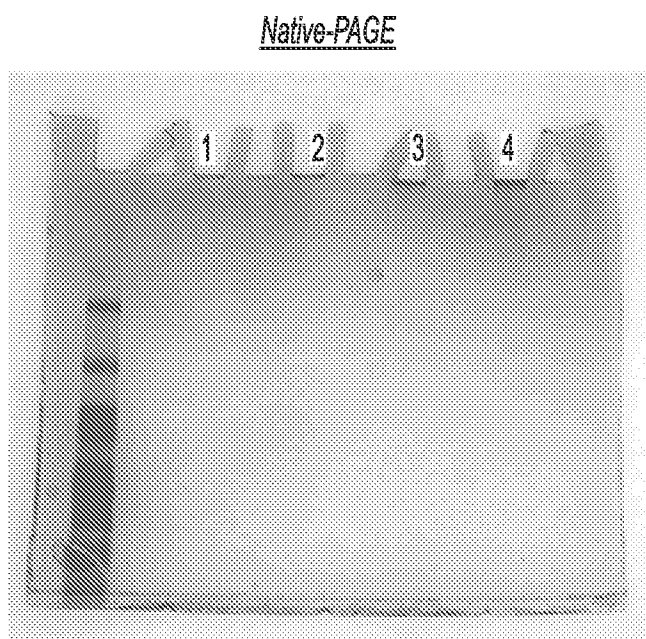


FIG. 20



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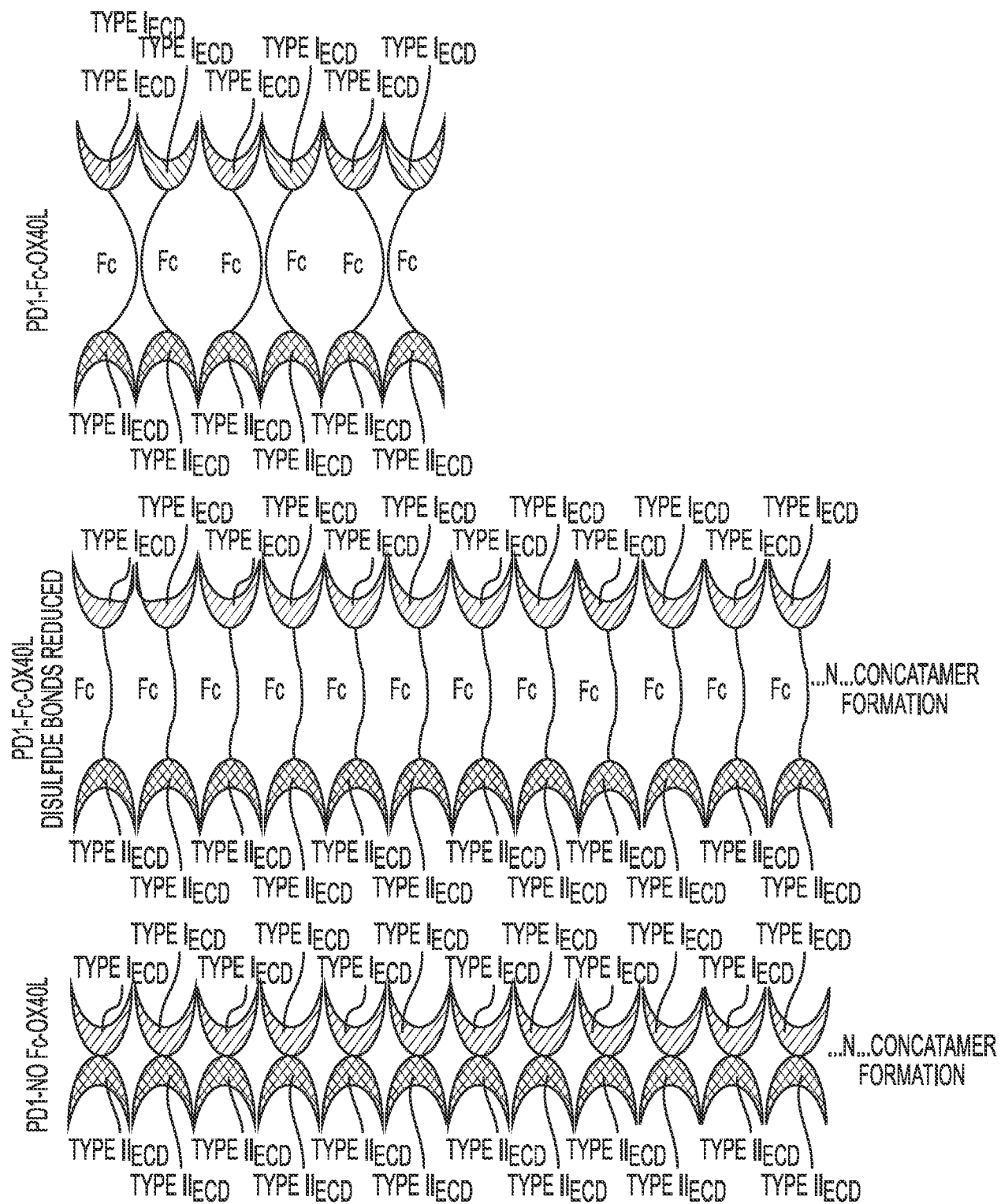


FIG. 21

FIG. 22A

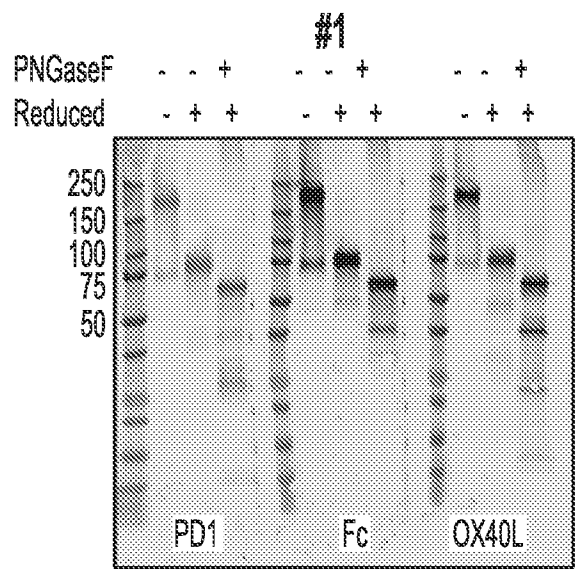


FIG. 22B

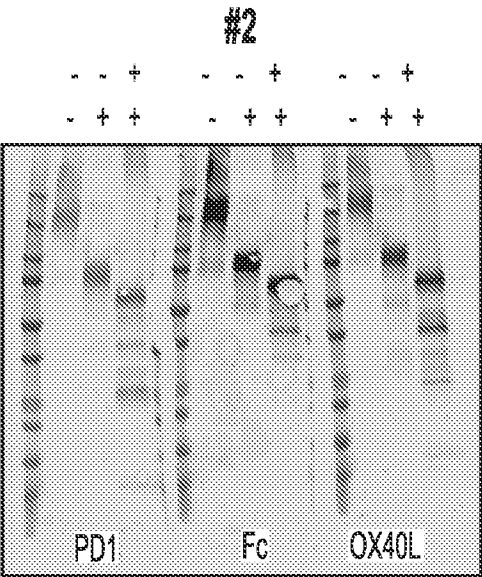


FIG. 22C

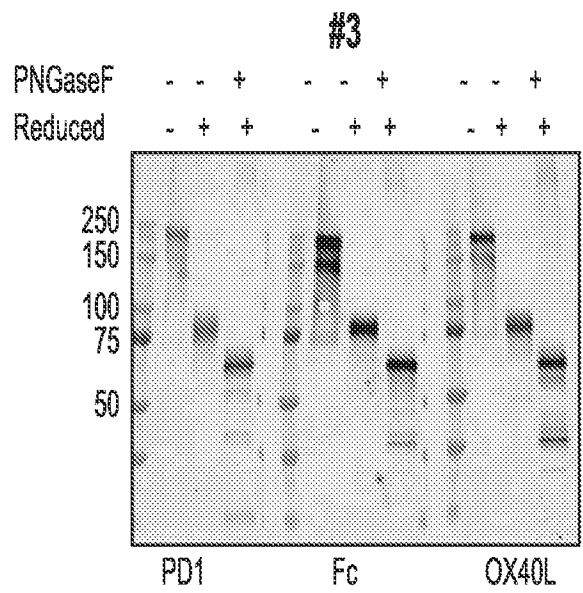


FIG. 22D

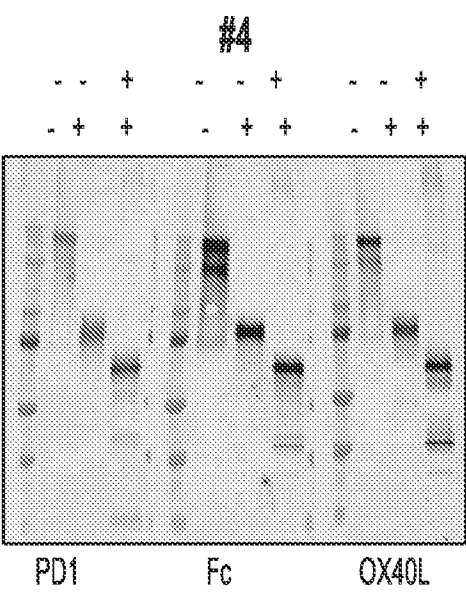


FIG. 22E

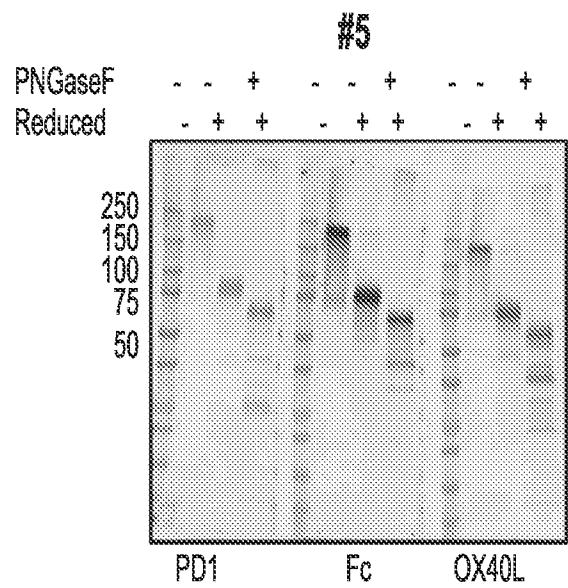


FIG. 22F

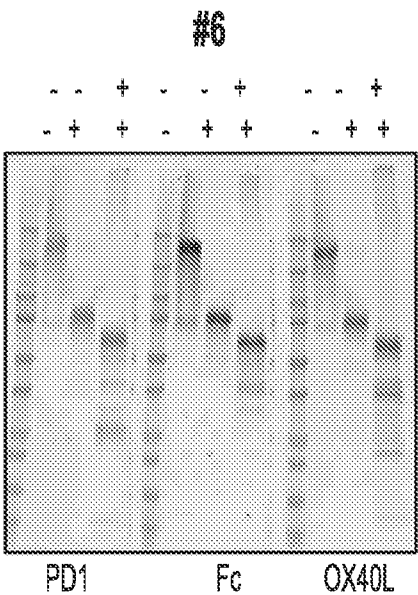


FIG. 22G

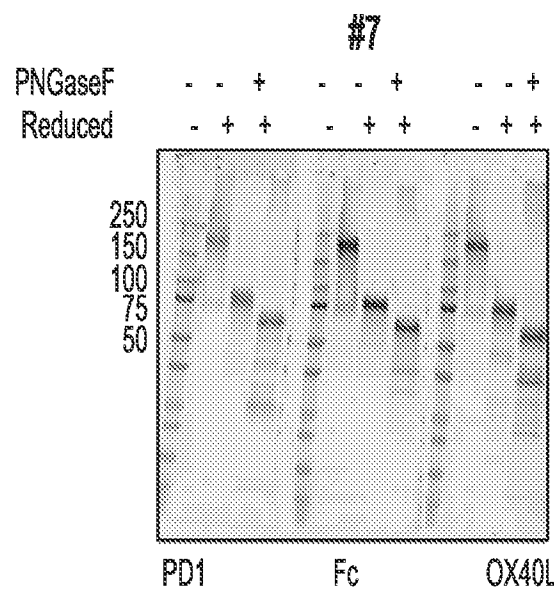


FIG. 22H

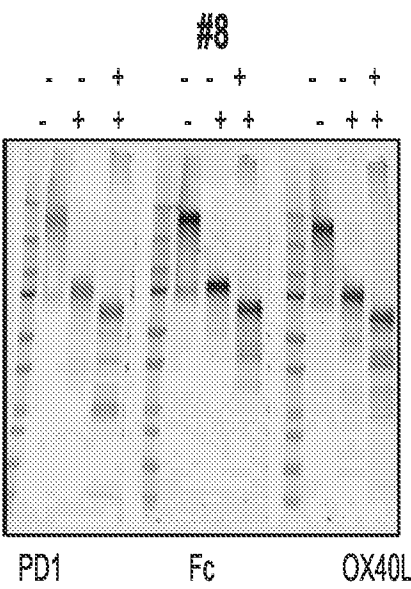


FIG. 22I

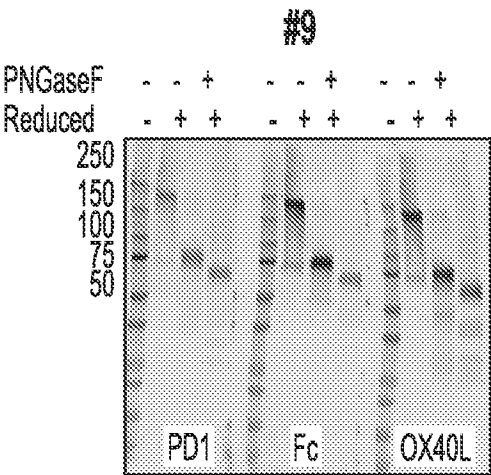


FIG. 22J

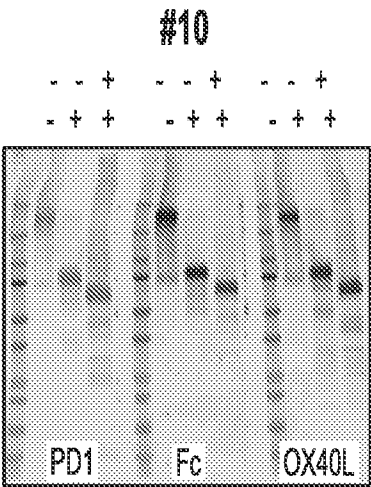


FIG. 22K

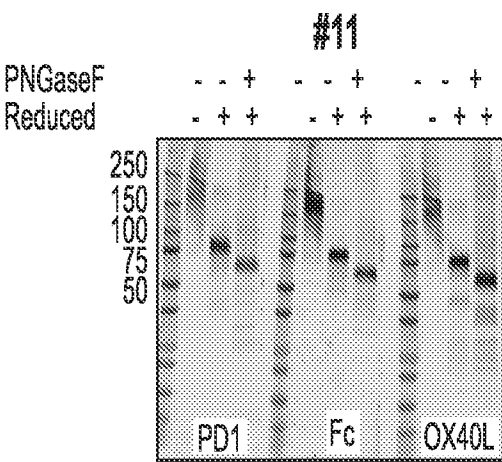
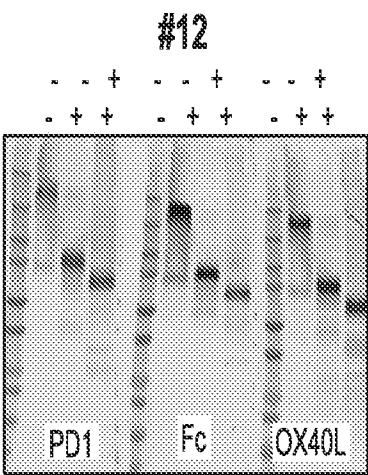


FIG. 22L



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FIG. 22M

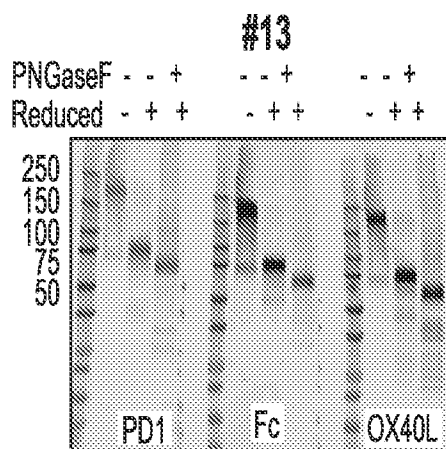


FIG. 22N

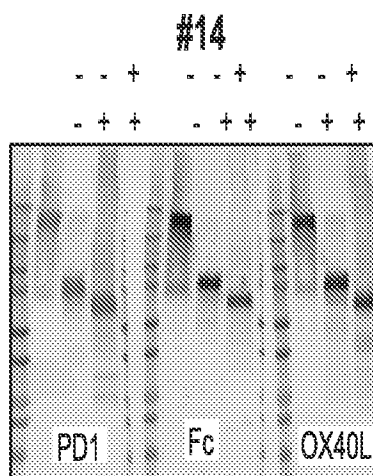


FIG. 22O

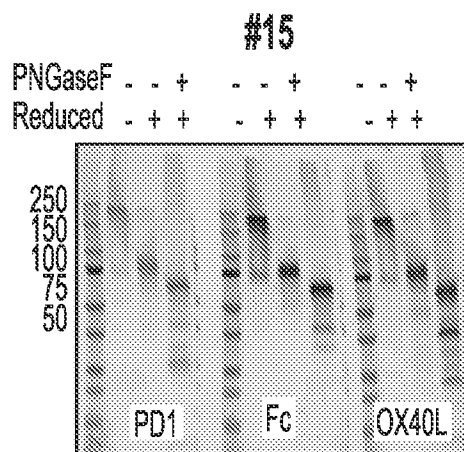


FIG. 22P

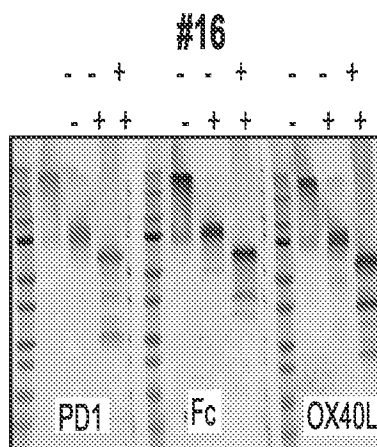


FIG. 22Q

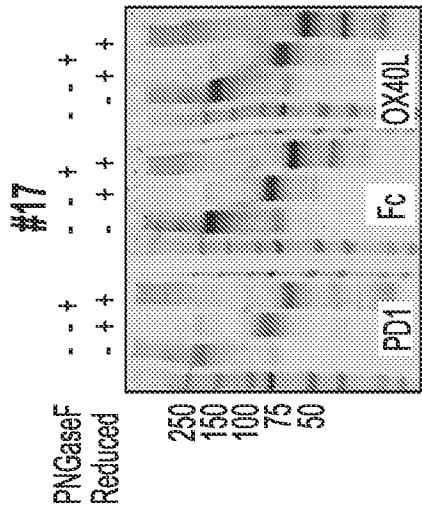
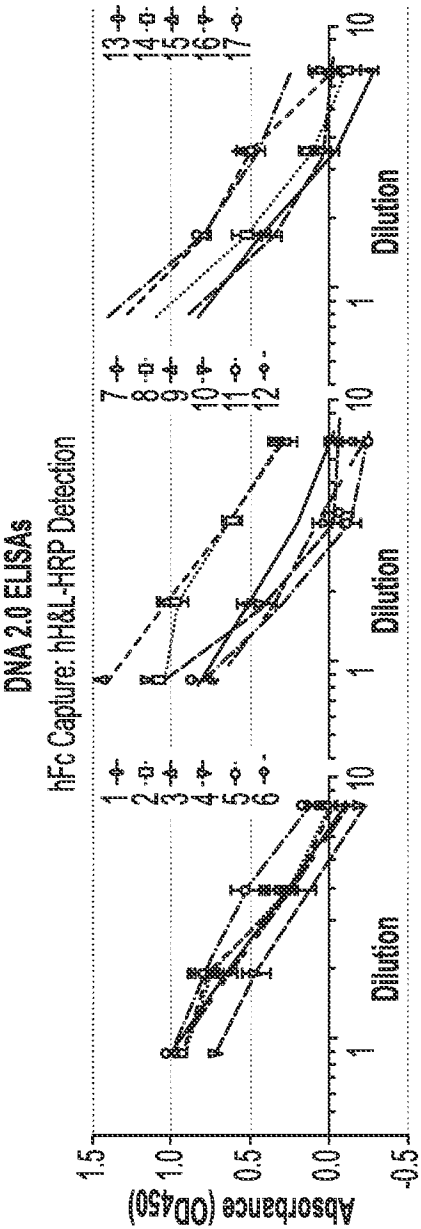


FIG. 23





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FIG. 24A

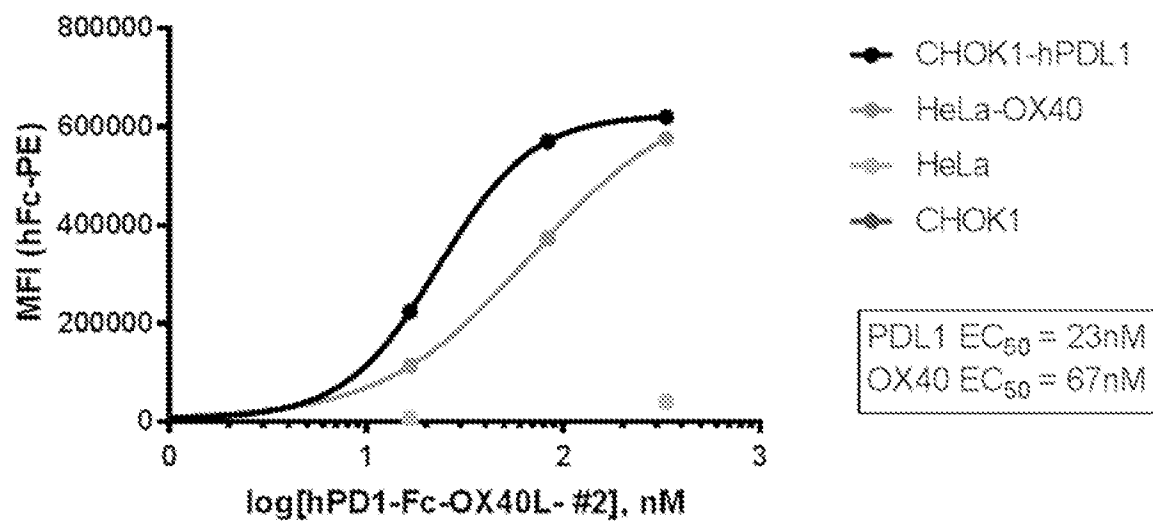
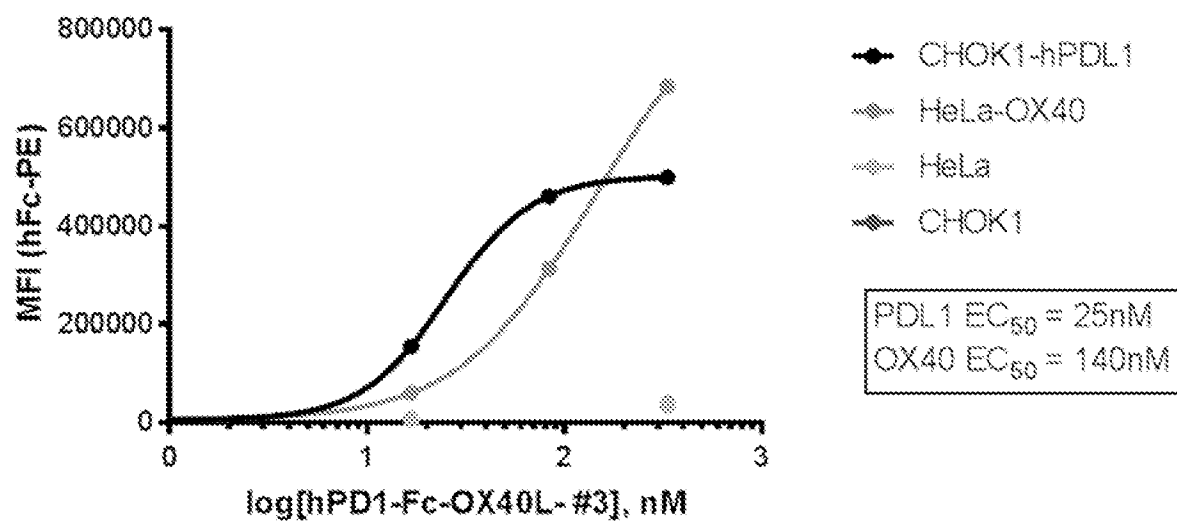


FIG. 24B



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FIG. 24C

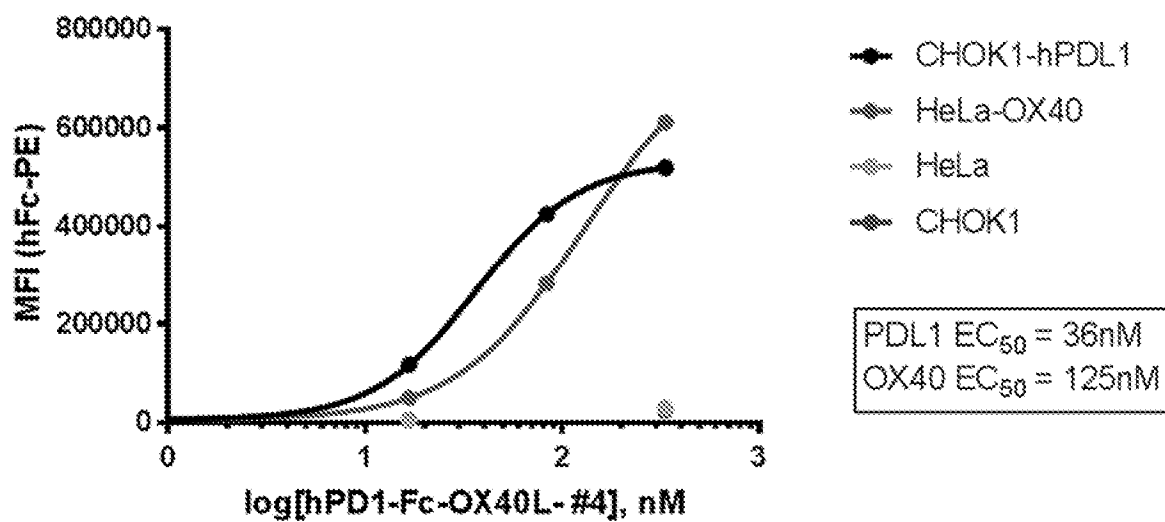
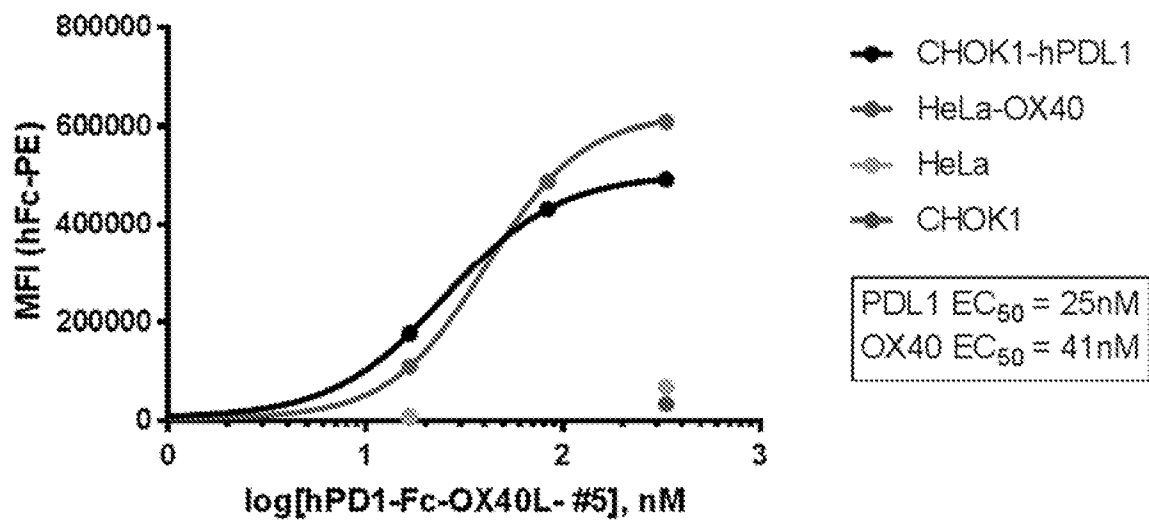


FIG. 24D



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FIG. 24E

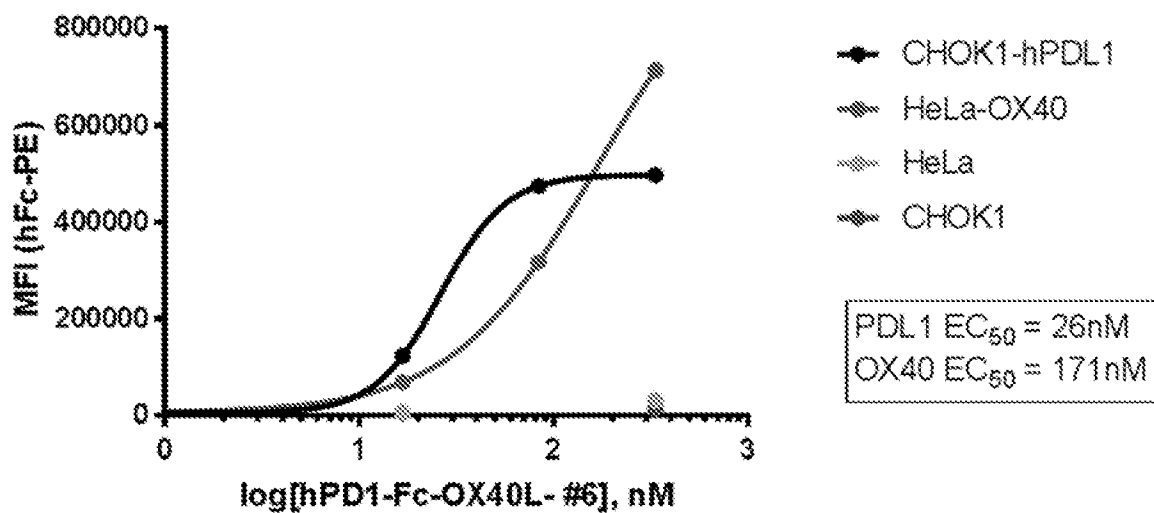
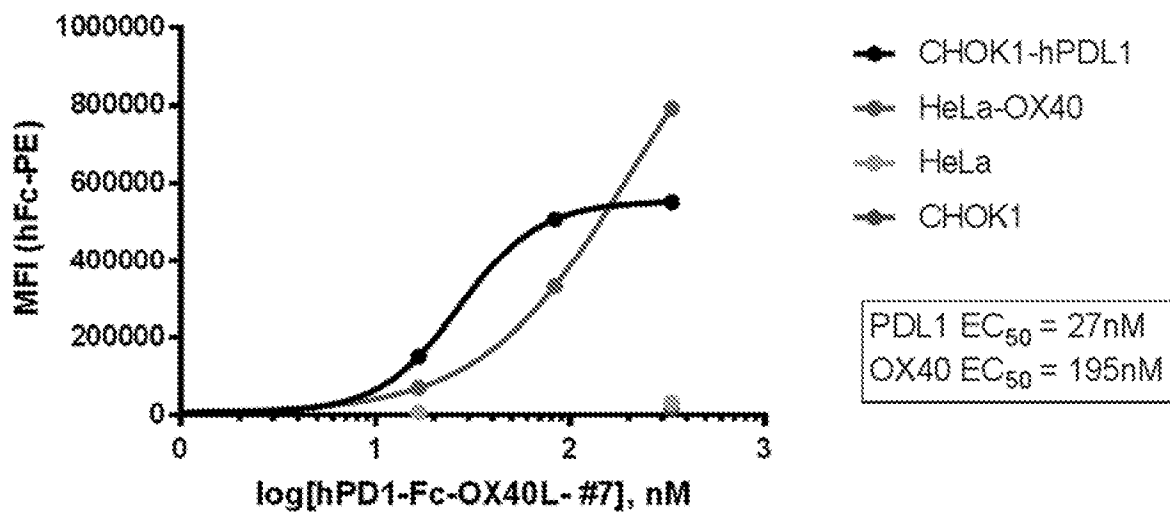


FIG. 24F



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FIG. 24G

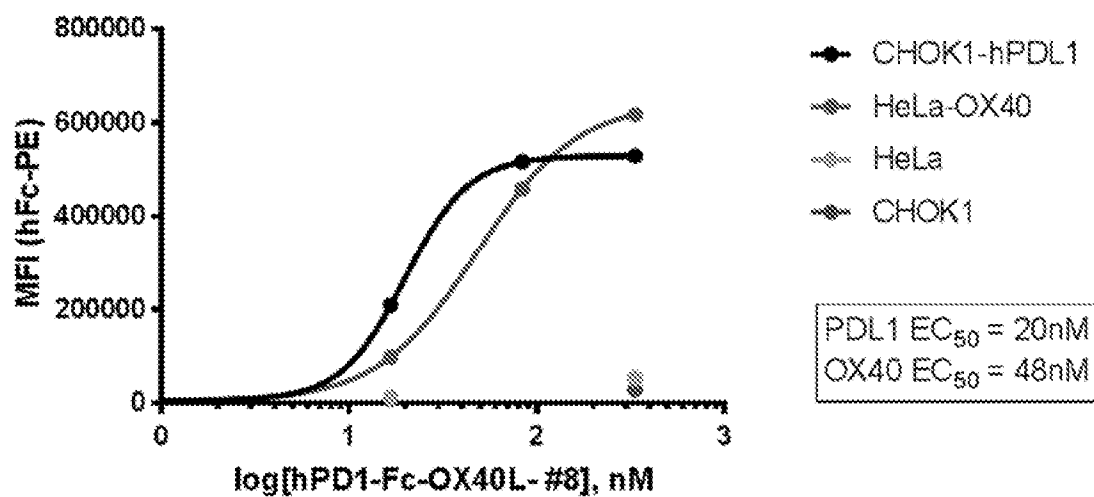
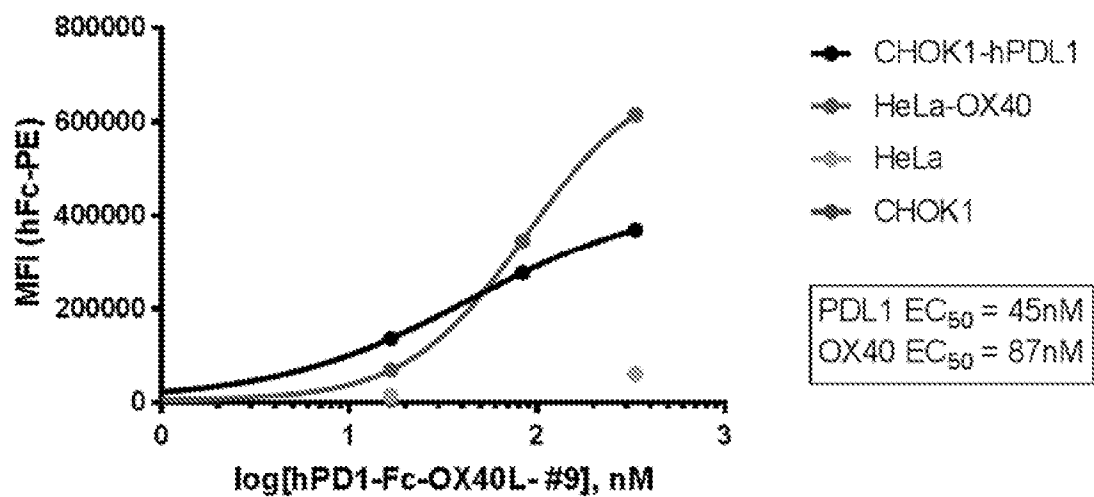


FIG. 24H



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FIG. 24I

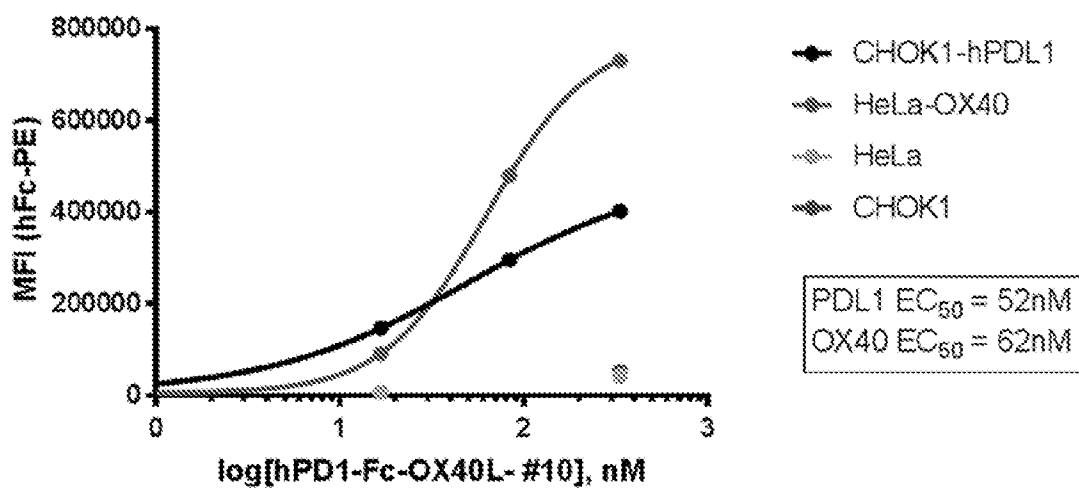
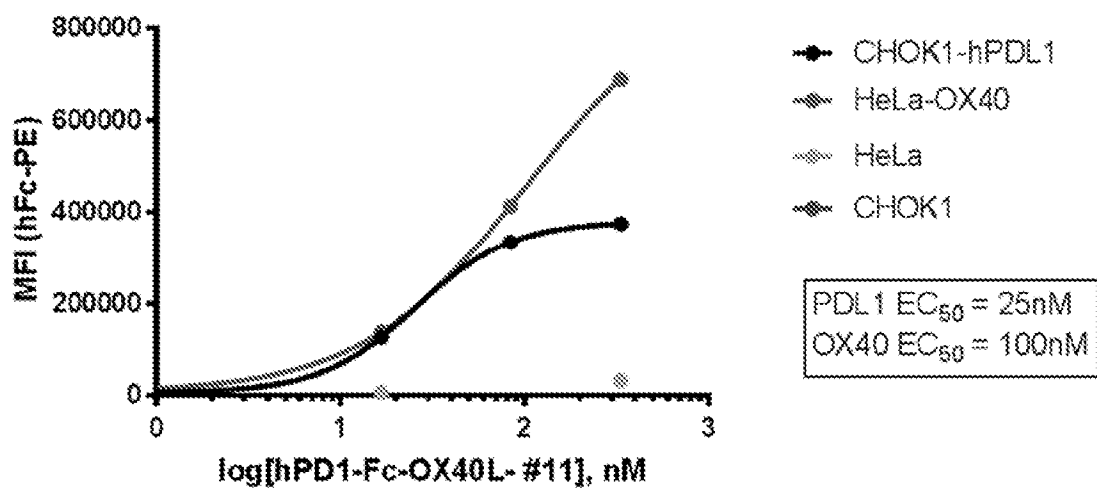


FIG. 24J



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FIG. 24K

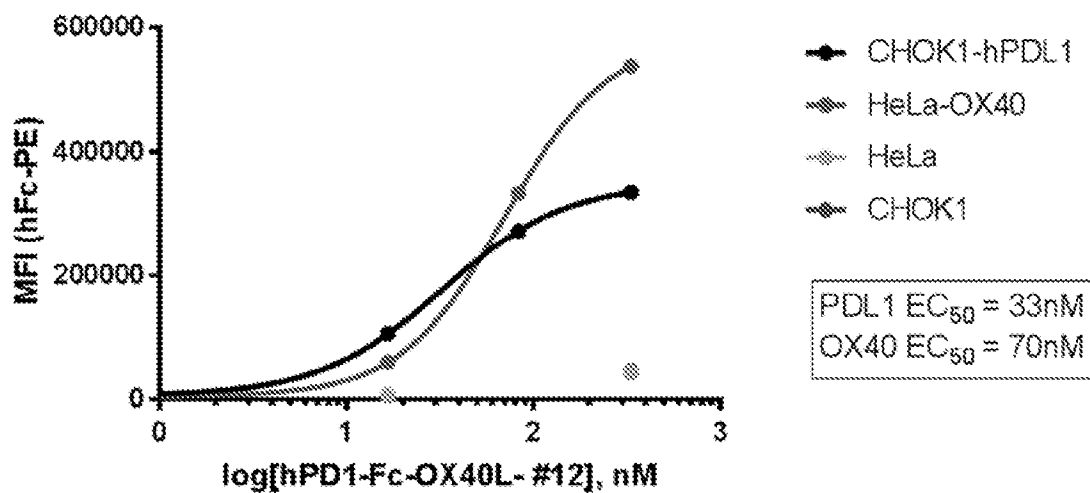
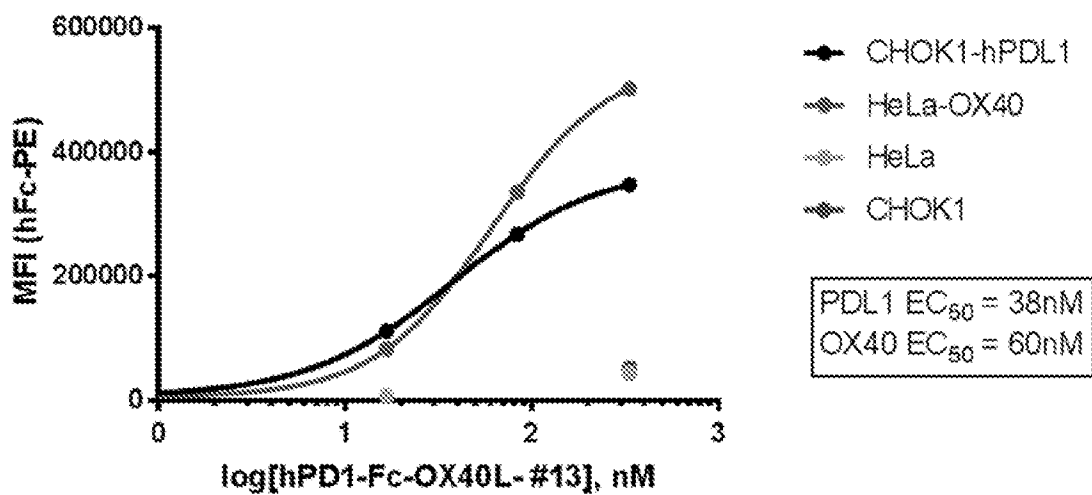


FIG. 24L



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FIG. 24M

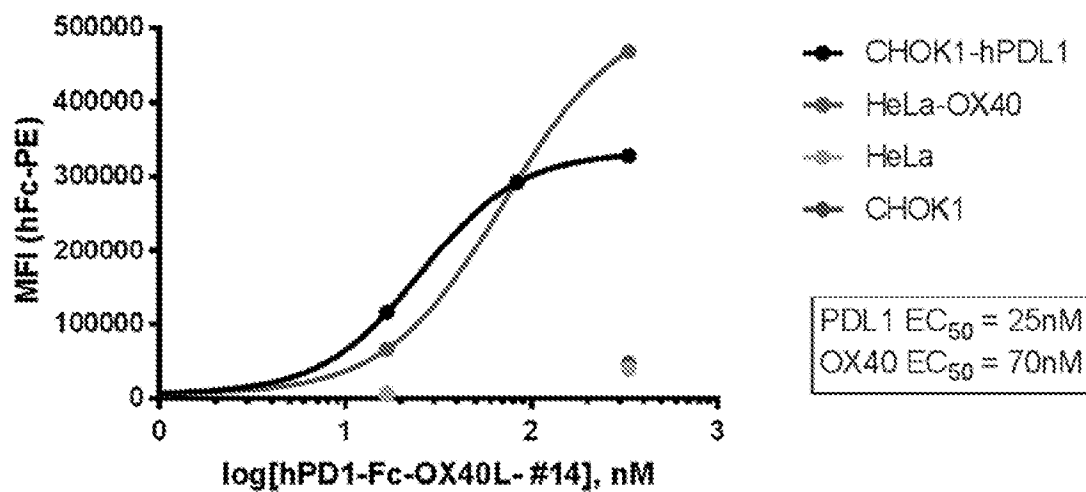
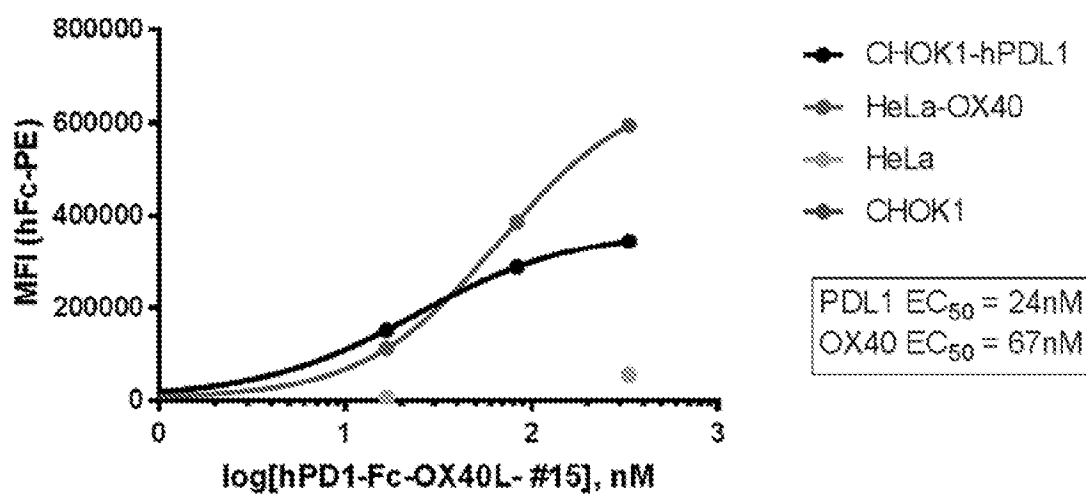


FIG. 24N



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FIG. 24O

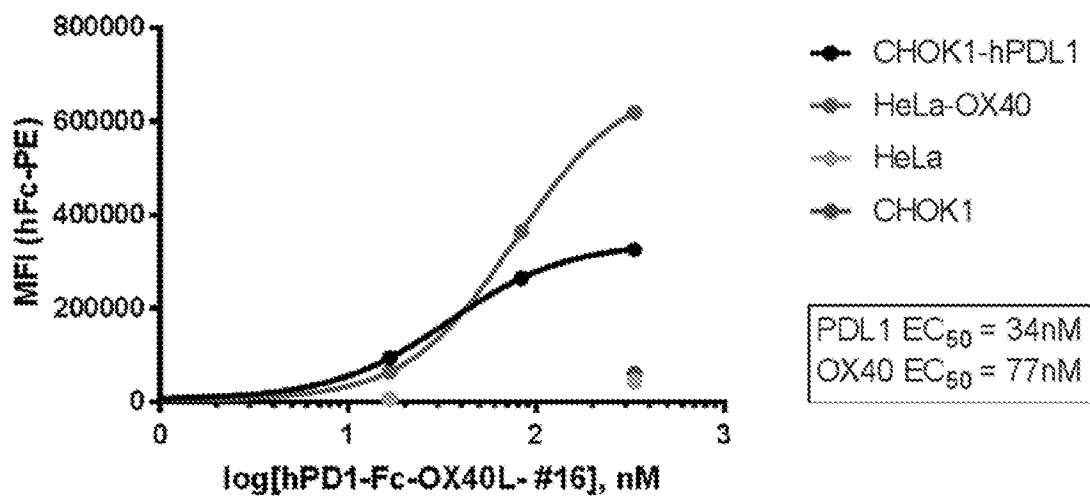
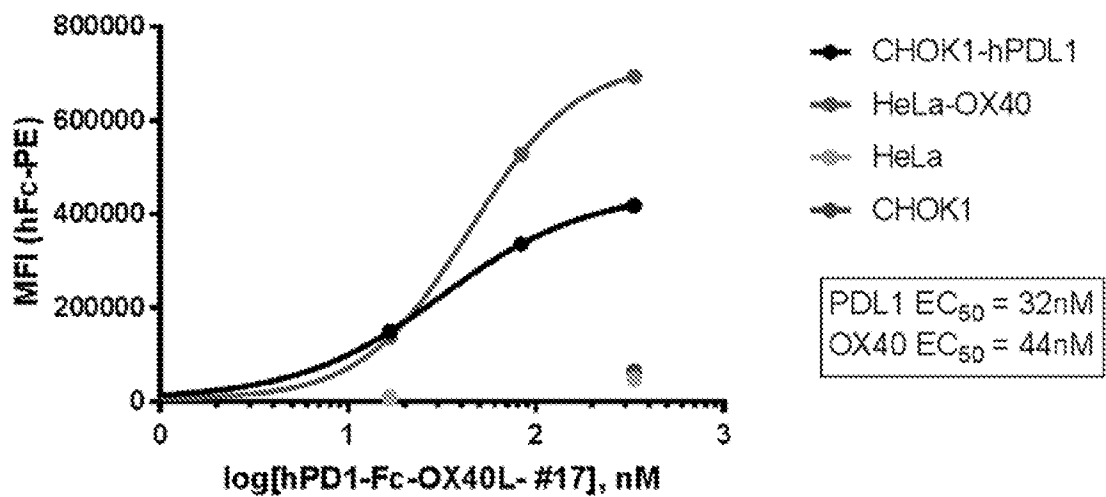


FIG. 24P





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FIG. 25A

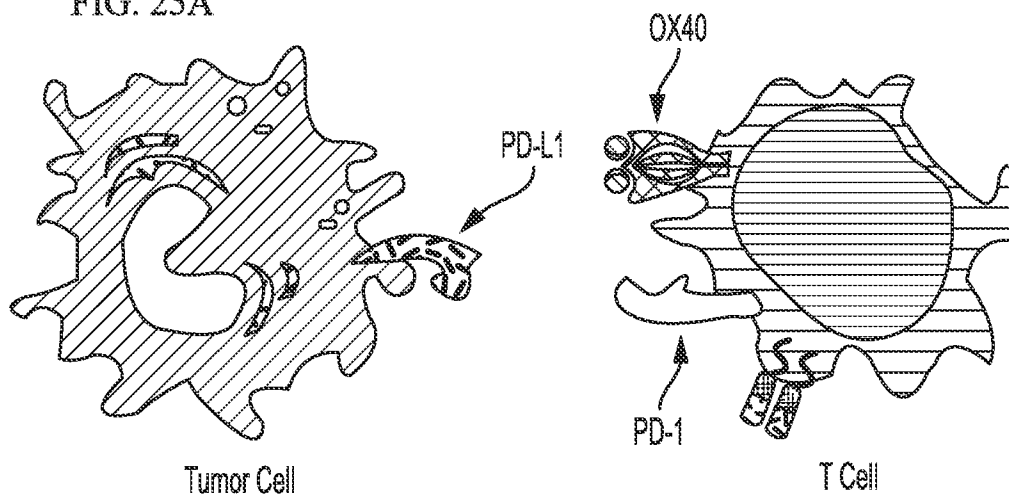


FIG. 25B

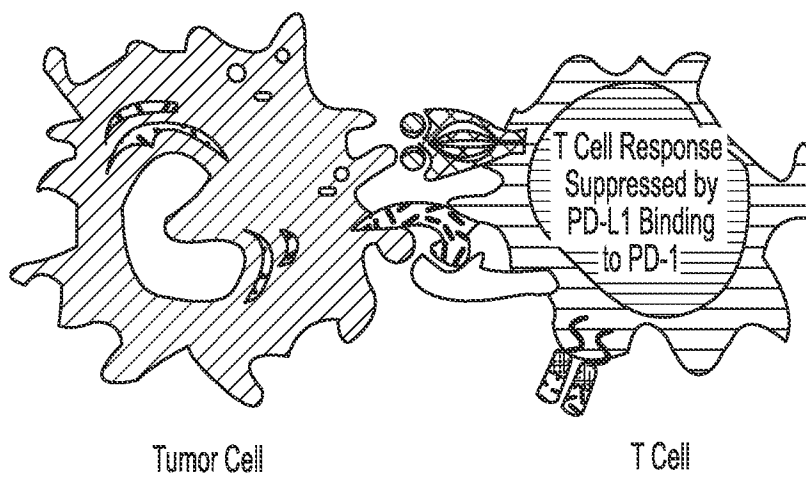
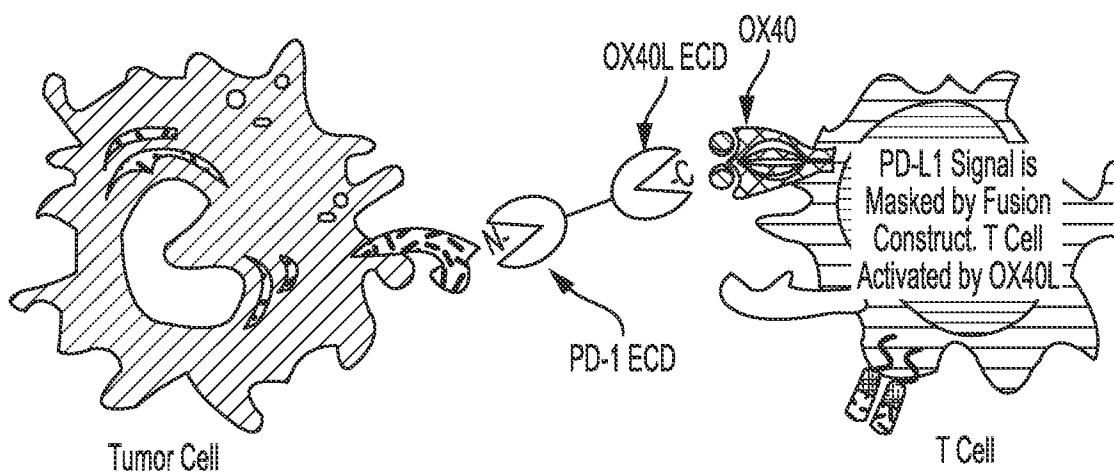
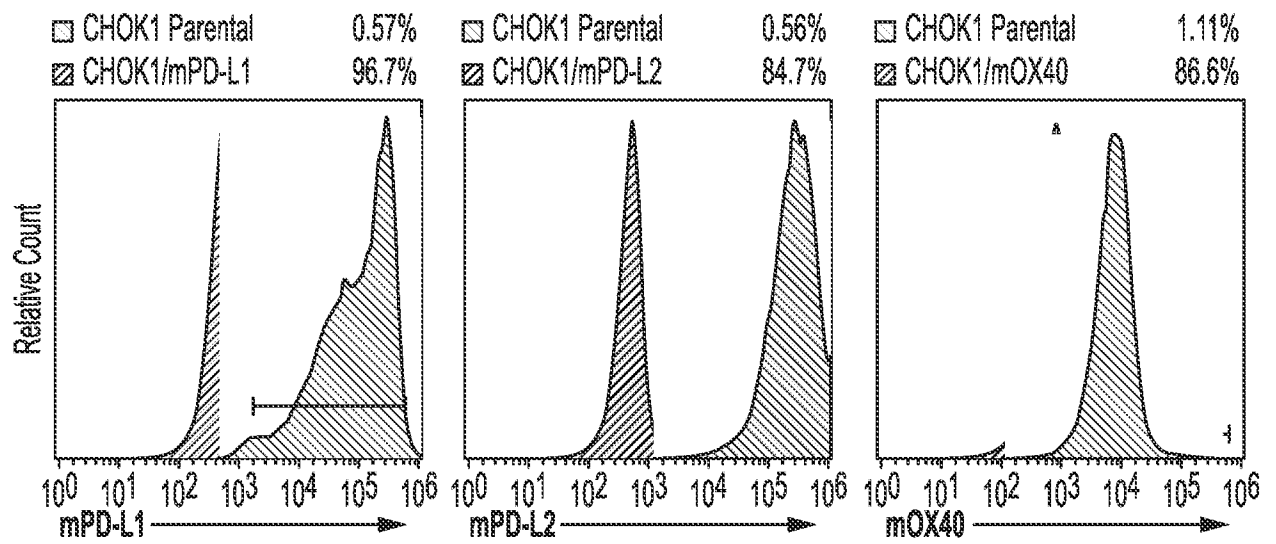


FIG. 25C



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FIG. 26A



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FIG. 26B

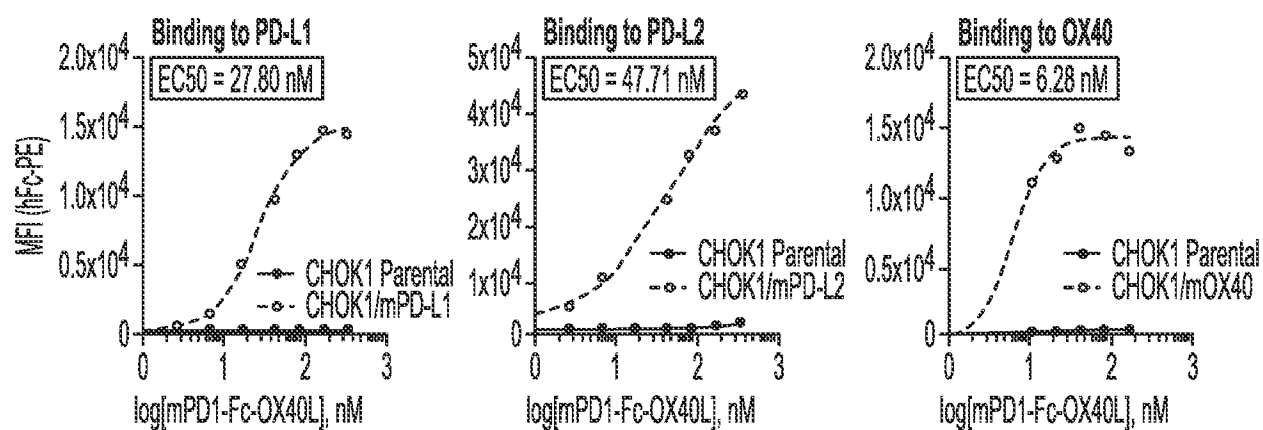


FIG. 26C

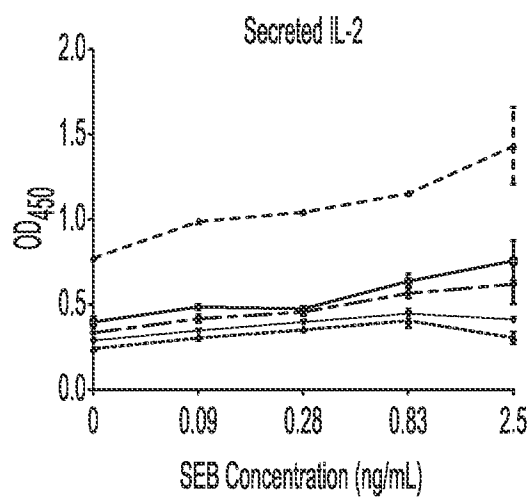


FIG. 26D

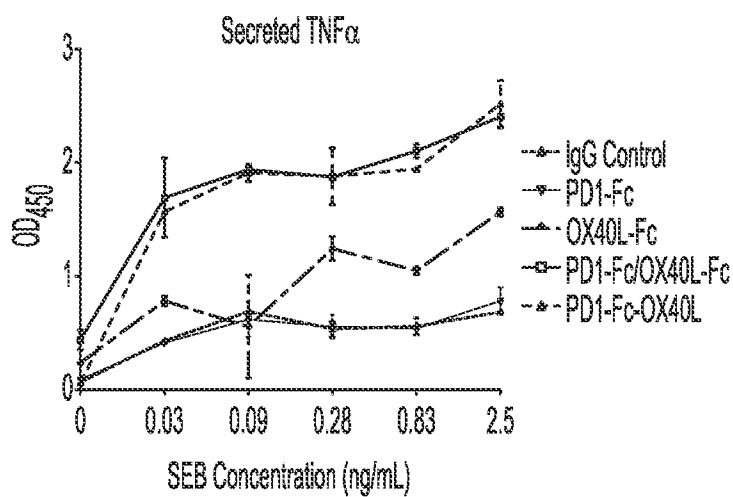
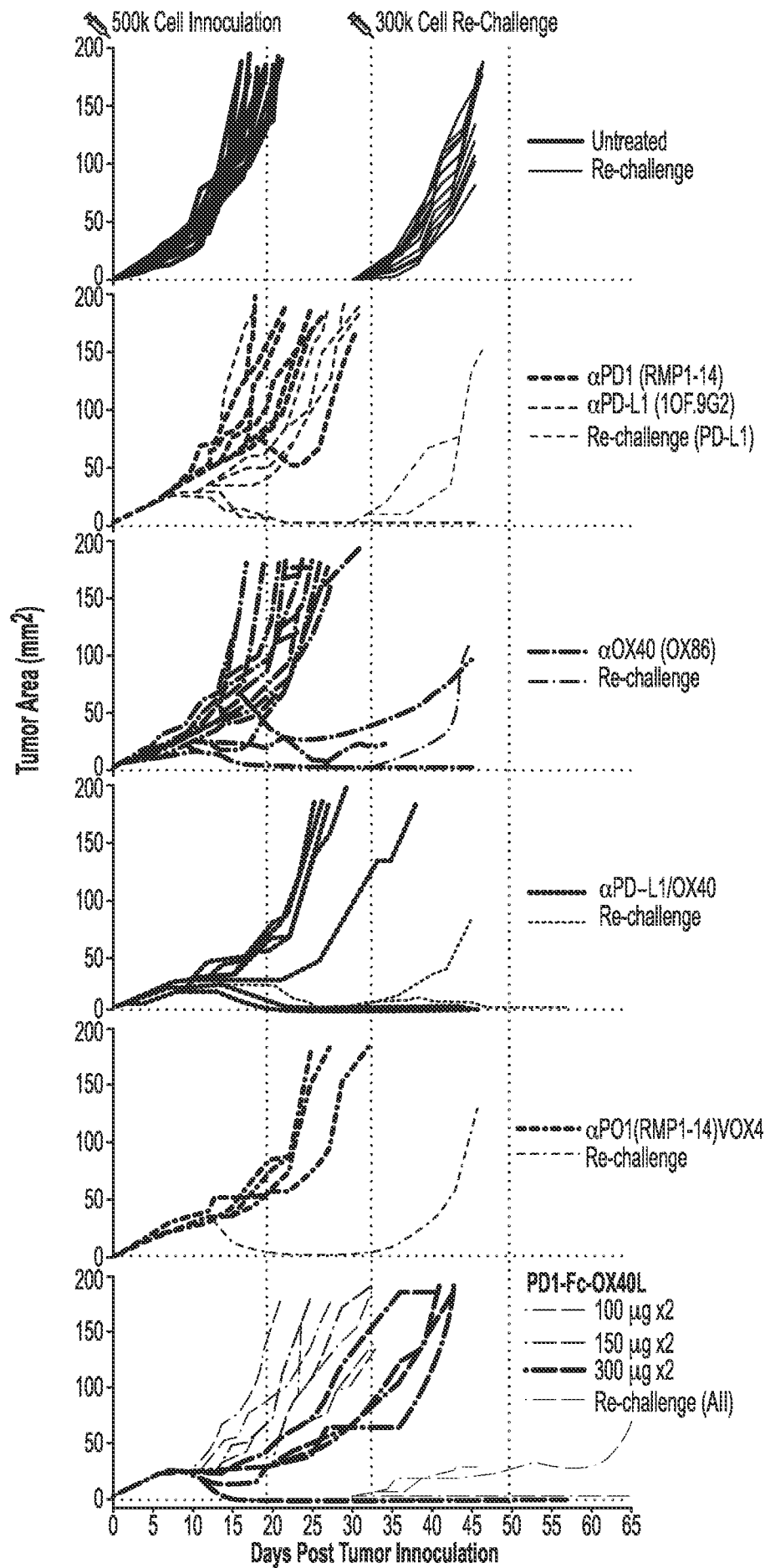
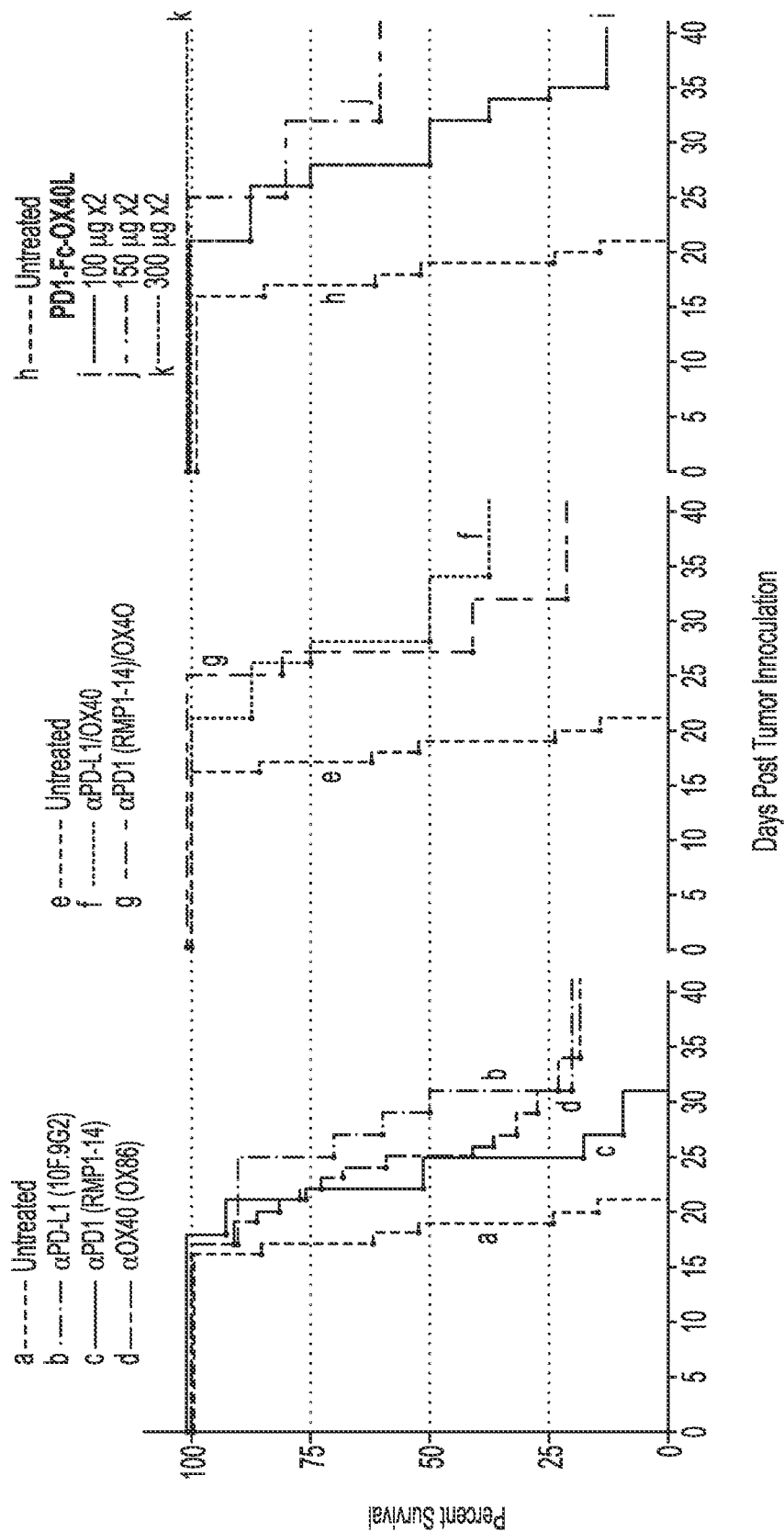


FIG. 27A



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FIG. 27B



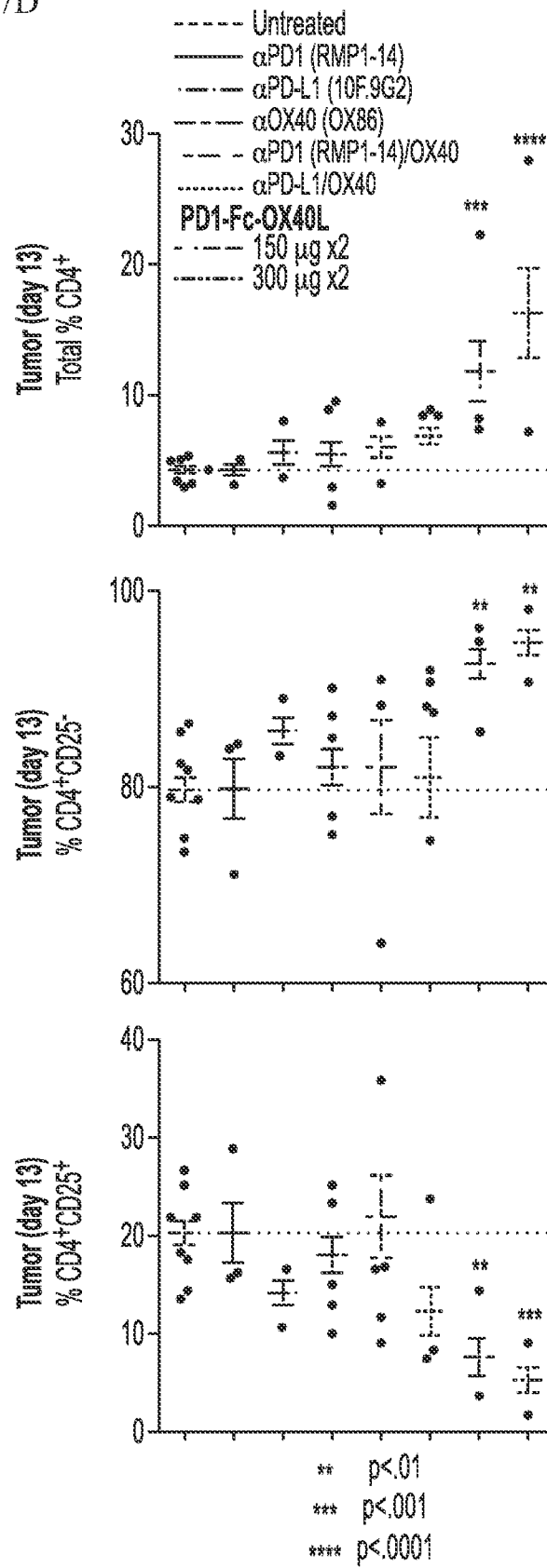
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FIG. 27C

CT26 Tumor Survival Statistics	
Mantel-Cox p-value	
Untr. vs OX40	<.0001
Untr. vs PD-L1	<.0001
Untr. vs PD1 (RMP1-14)	<.0001
Untr. vs OX40/PD-L1	<.0001
Untr. vs OX40/PD1 (RMP1-14)	.0001
Untr. vs mPD1-Fc-OX40L (100 µg x2)	<.0001
Untr. vs mPD1-Fc-OX40L (150 µg x2)	.0001
Untr. vs mPD1-Fc-OX40L (300 µg x2)	<.0001

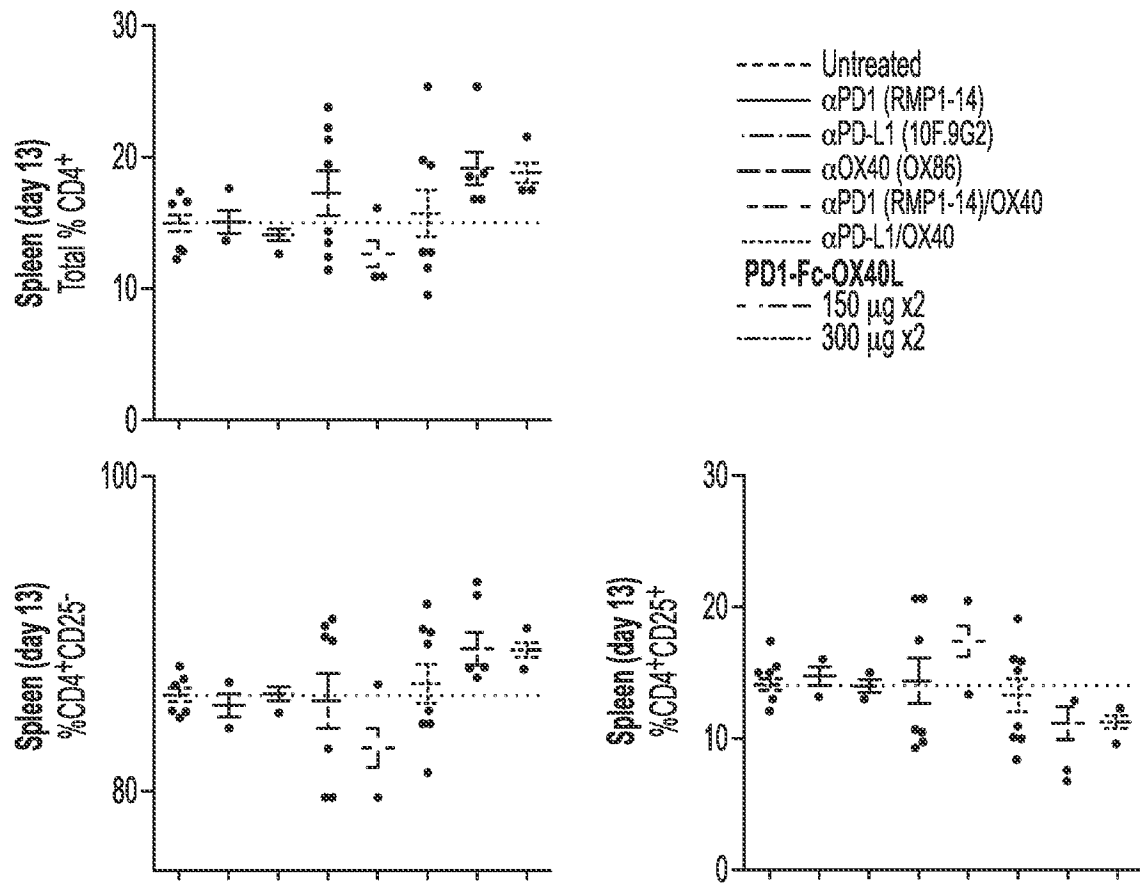
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FIG. 27D



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FIG. 27E





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FIG. 27F

Group	Total, N	Short-Term, N (Immune Profiling)	Long-Term, N (Tumor Growth/Survival)	% Rejection (Primary Tumor)	% Rejection (Re-challenge)	Serum Cytokine Total N	Response %
Untreated	33	12	21	0.0	0.0	0/20	0
$\alpha$ PD1 (RMP1-14)	14	12	2	0.0	N/A	0/9	0
$\alpha$ PD-L1 (10F.9G2)	14	12	2	20.0	0.0	4/8	50
$\alpha$ OX40 (OX86)	29	8	21	19.1	0.0	12/20	60
$\alpha$ PD-L1/OX40	17	9	8	37.5	66.6	10/10	100
$\alpha$ PD1 (RMP1-14)/OX40	10	5	5	20.0	0.0	3/5	60
PD1-Fc-OX40L (100 $\mu$ g x2)	8	/	8	0.0	NA	2/8	25
PD1-Fc-OX40L (150 $\mu$ g x2)	12	7	5	60.0	71.4	5/12	42
PD1-Fc-OX40L (300 $\mu$ g x2)	11	5	6	16.7	75.0	7/11	64

FIG. 27G

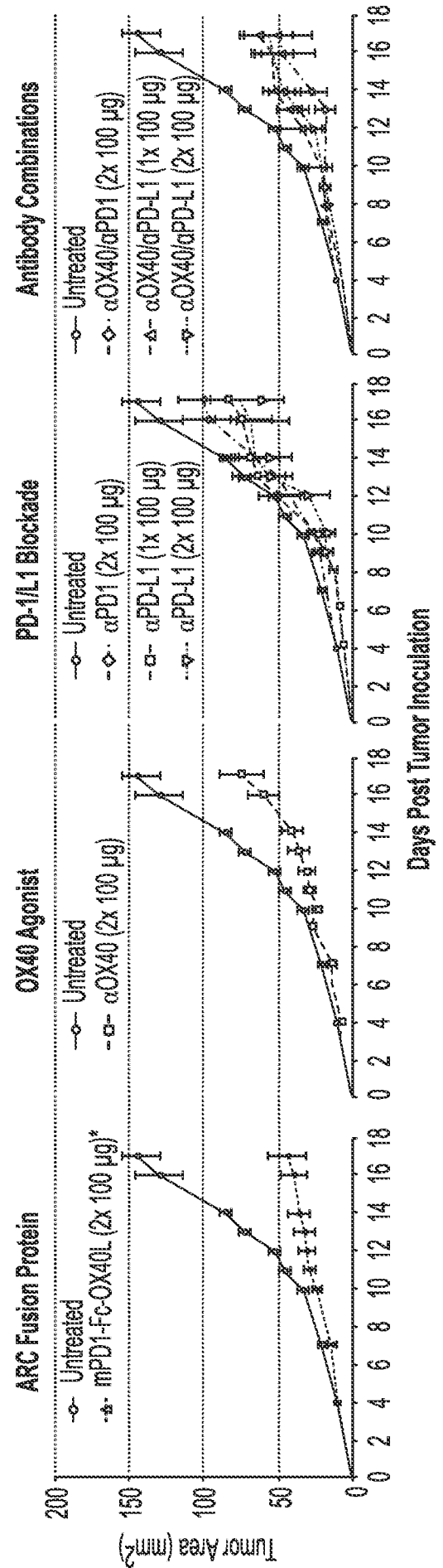


FIG. 27H

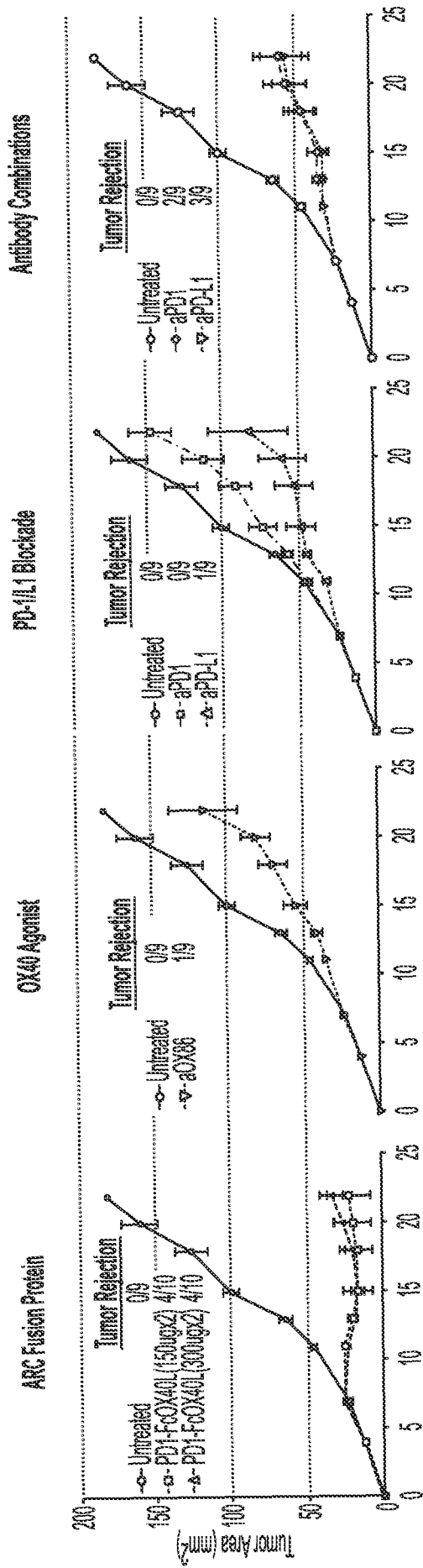


FIG. 28A

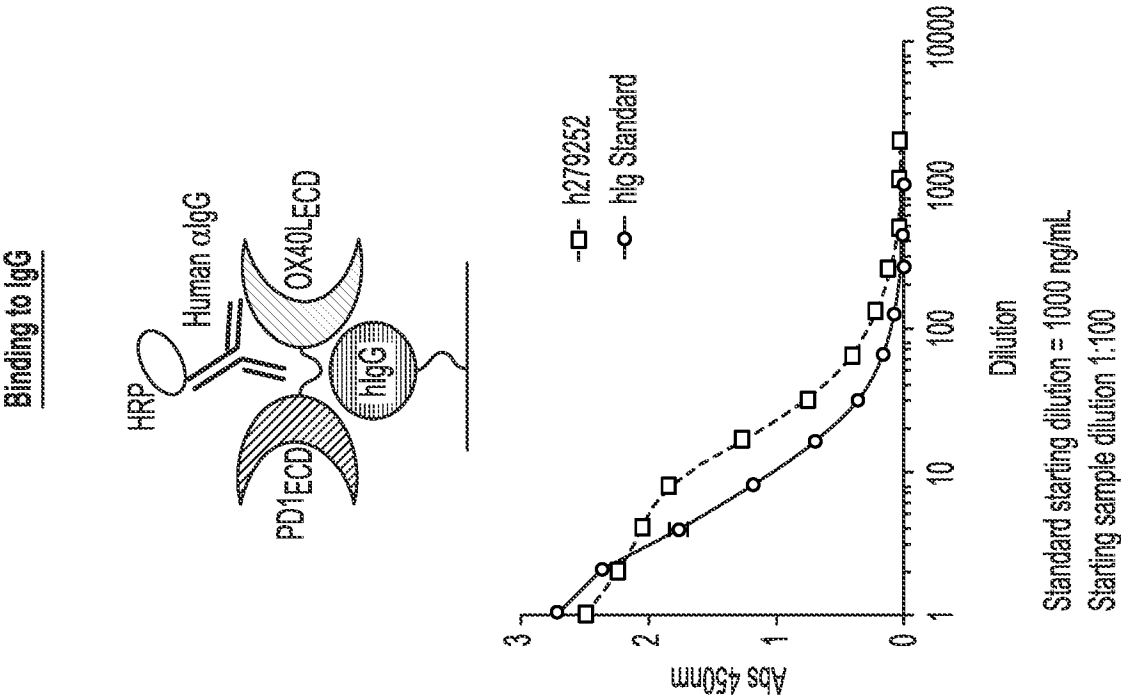
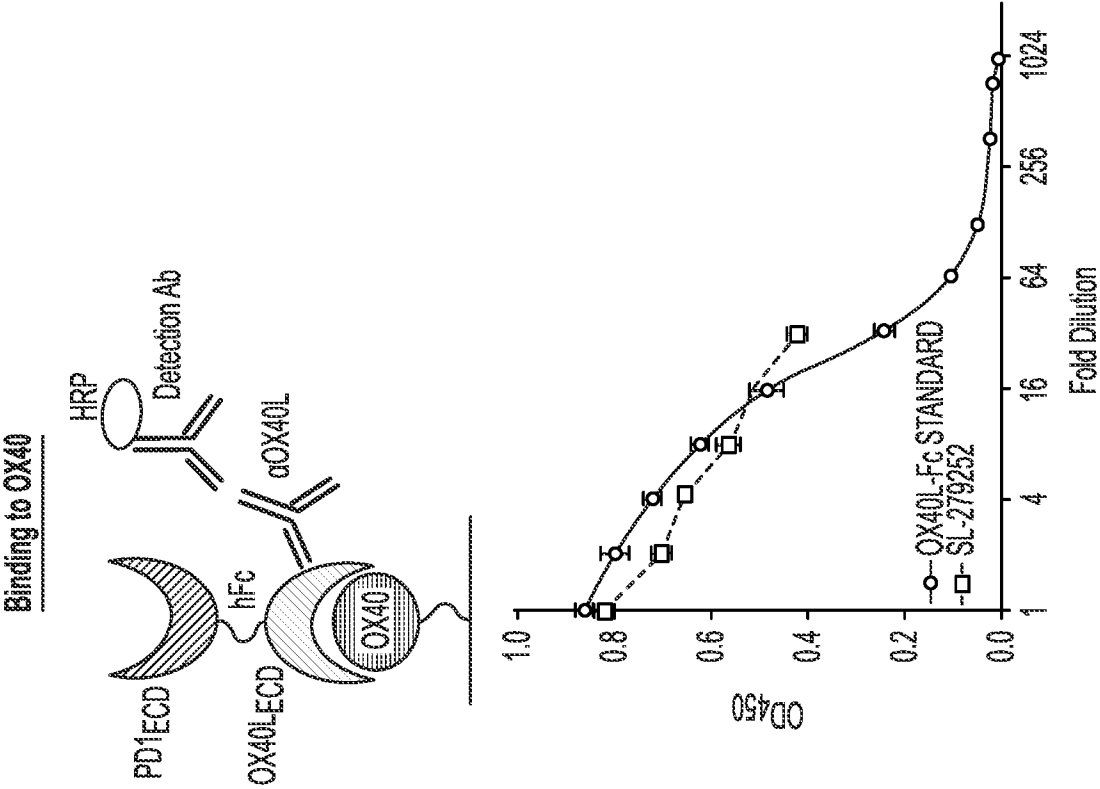


FIG. 28B



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FIG. 28C

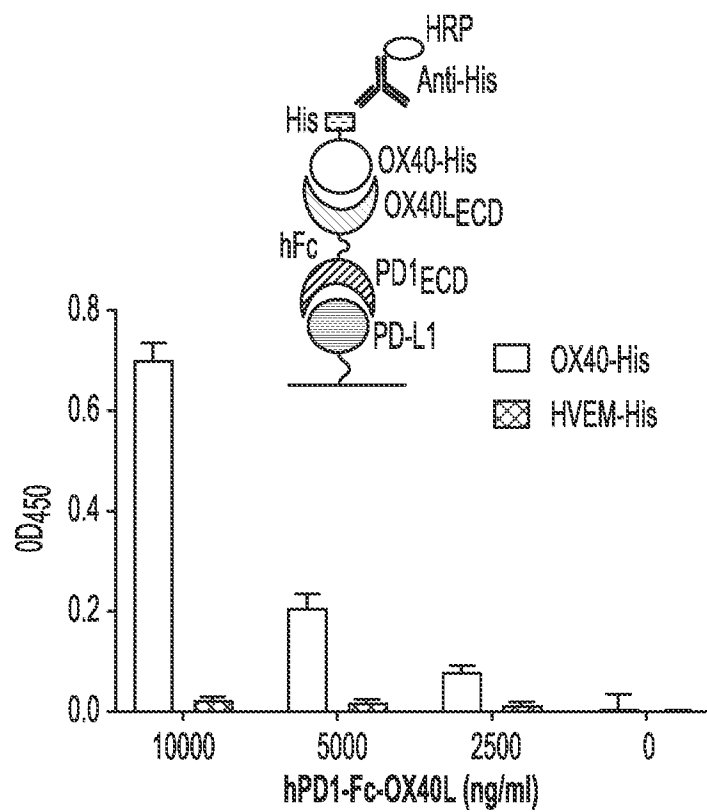
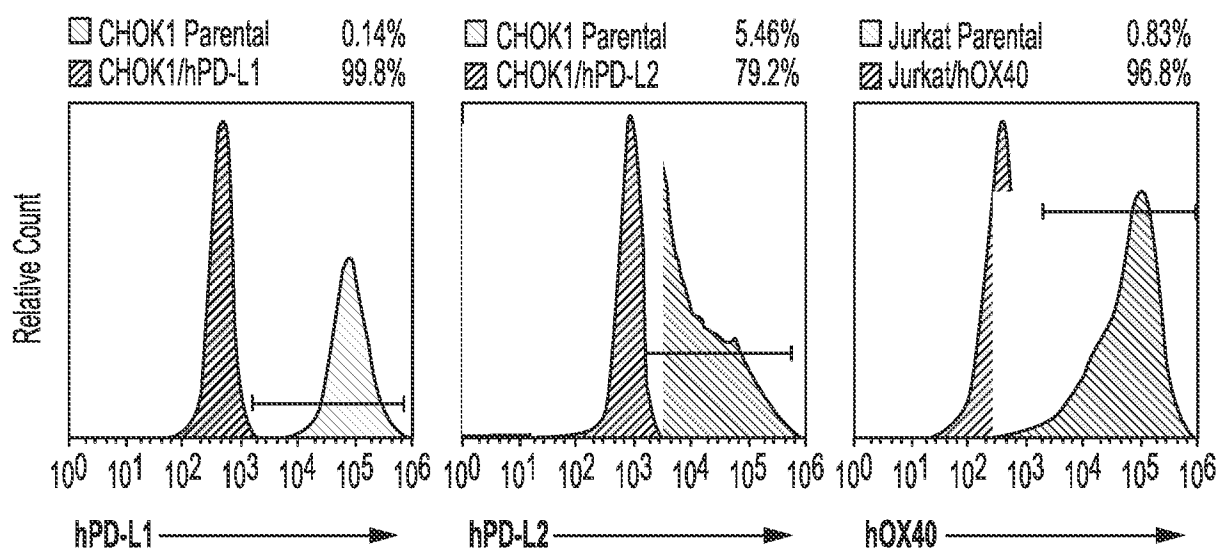


FIG. 29A



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FIG. 29B

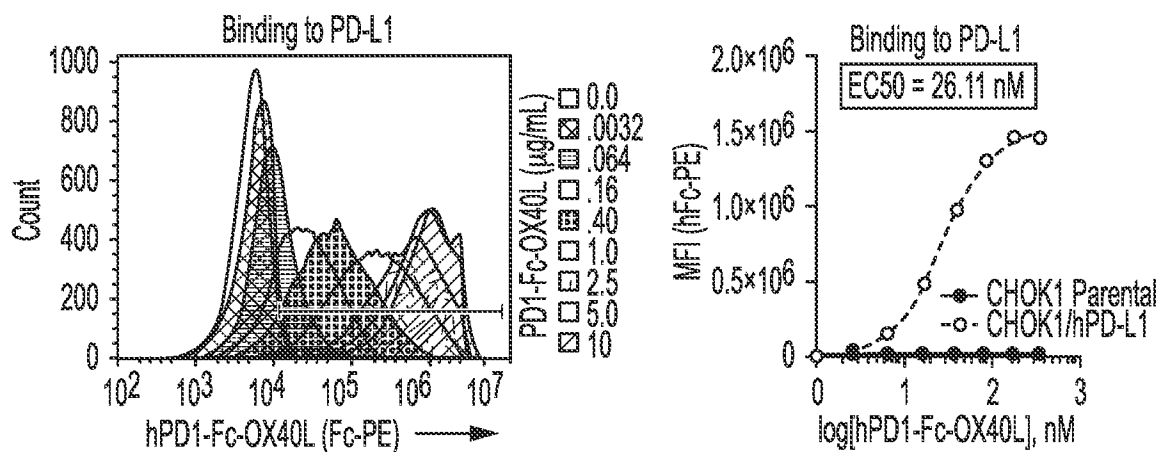


FIG. 29C

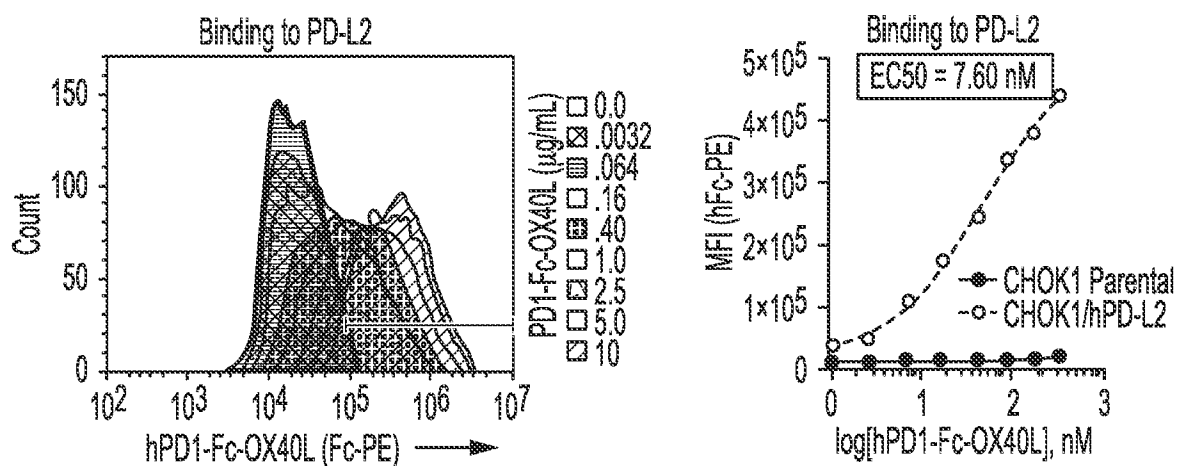
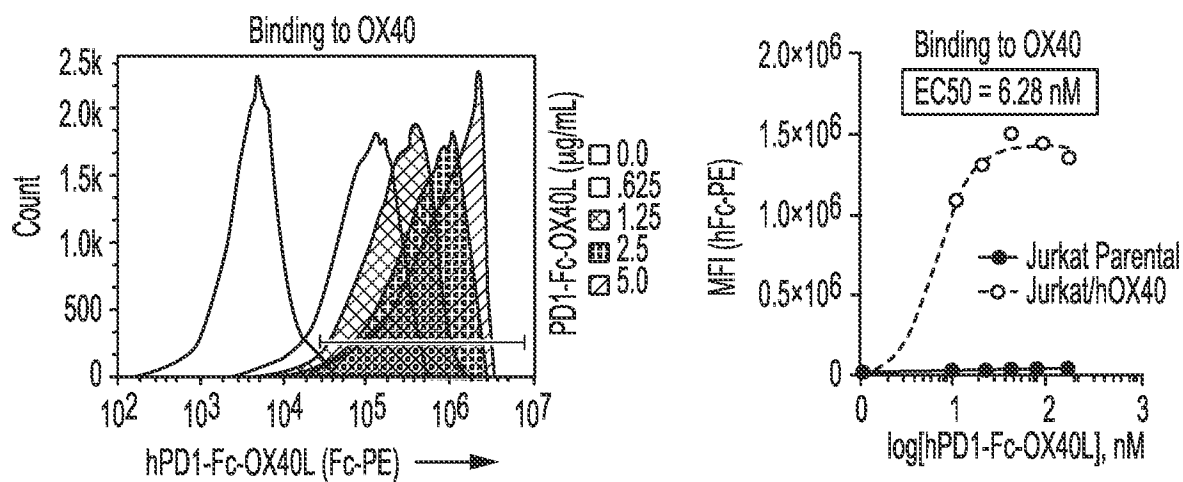
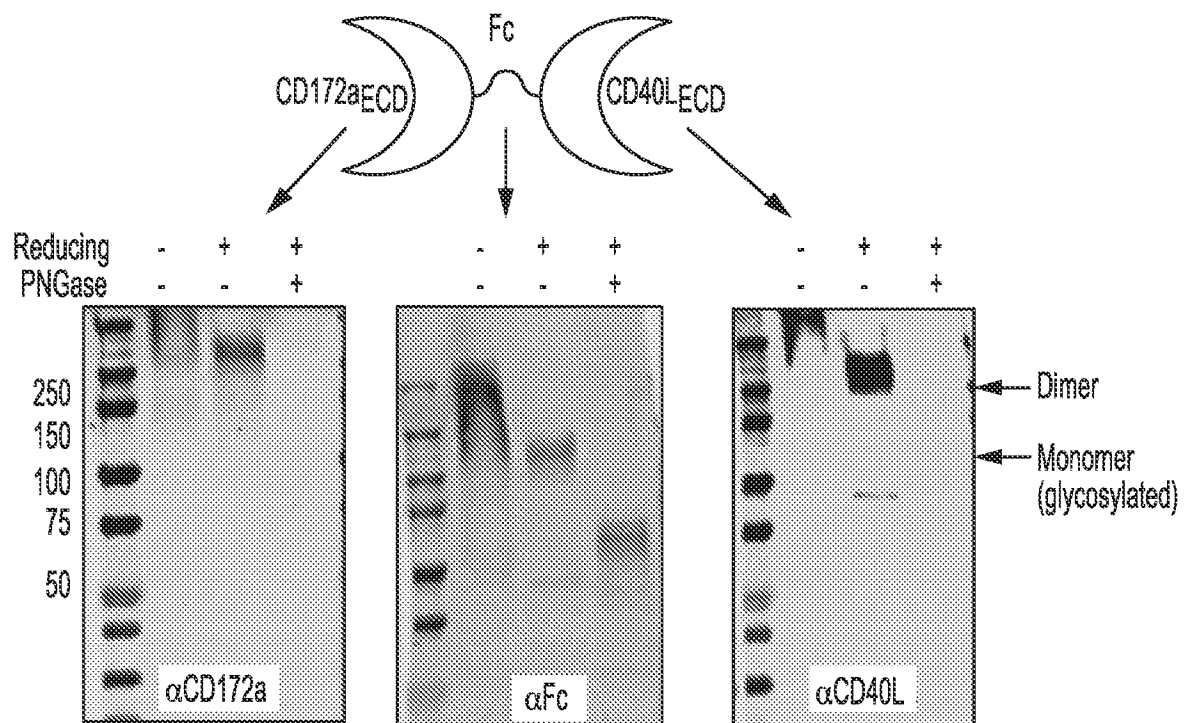


FIG. 29D



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FIG. 30



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FIG. 31A

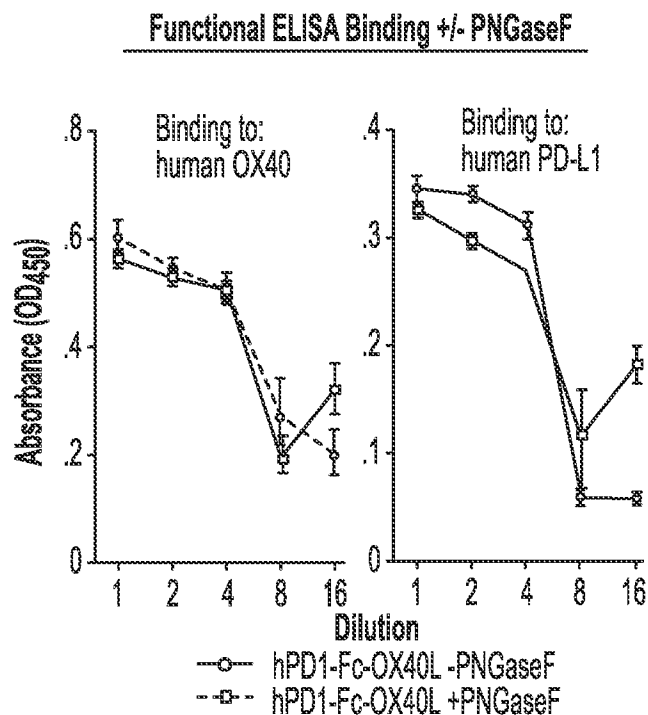


FIG. 31B

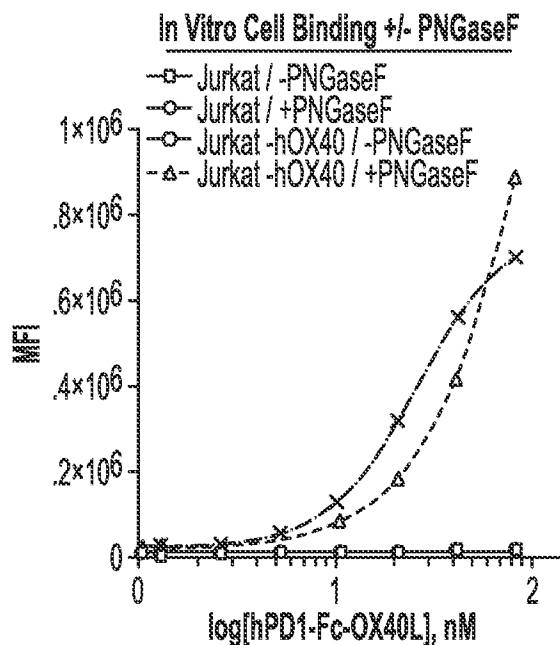


FIG. 32A

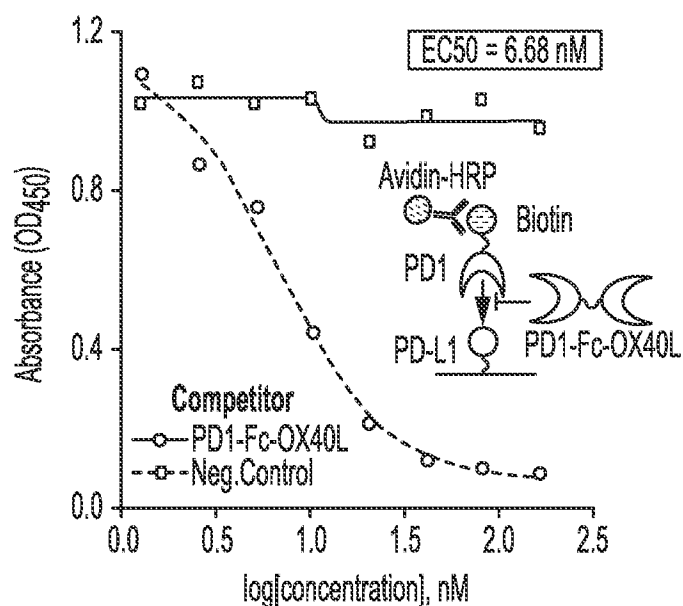


FIG. 32C

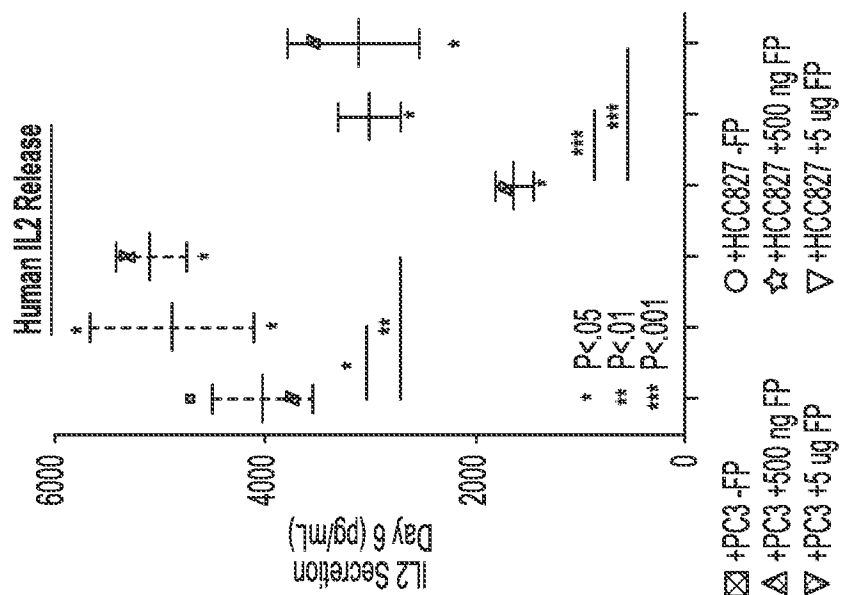
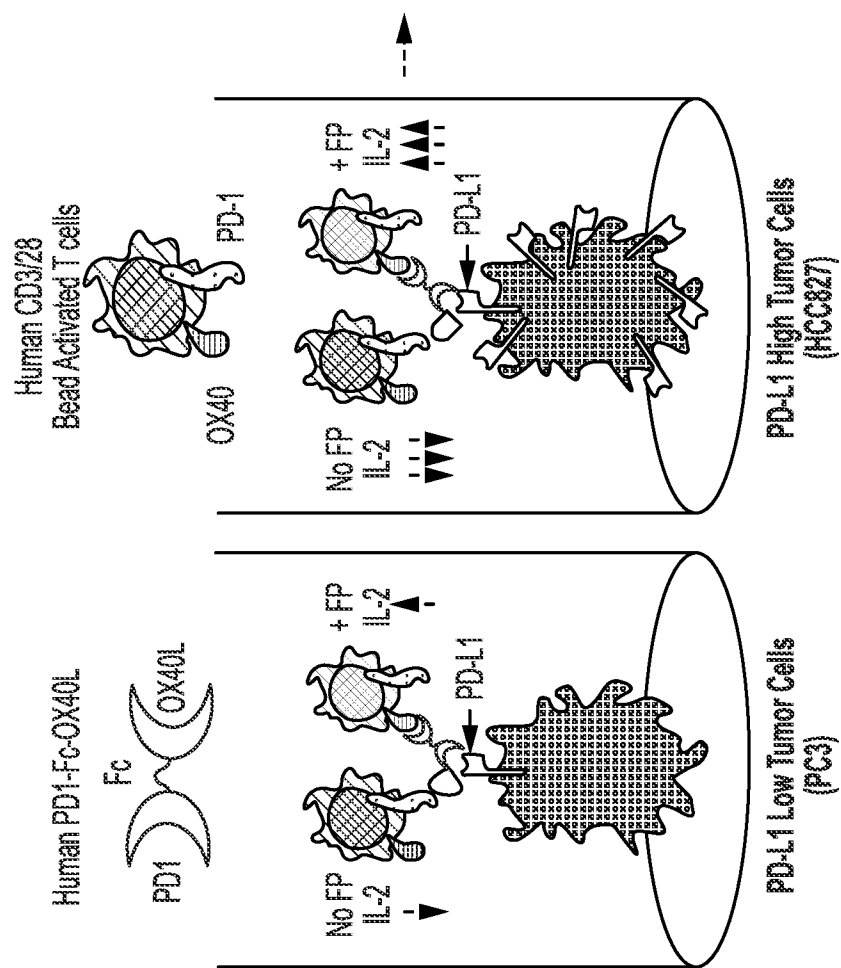


FIG. 32B





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FIG. 32D

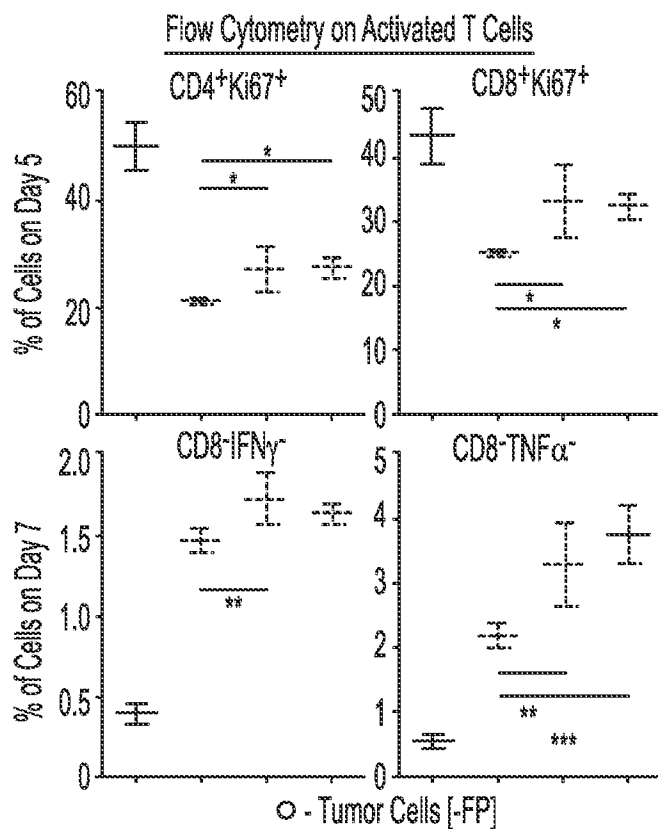


FIG. 32E

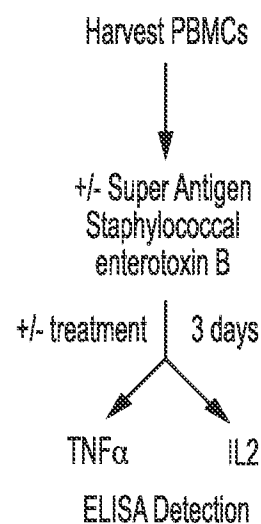


FIG. 32F

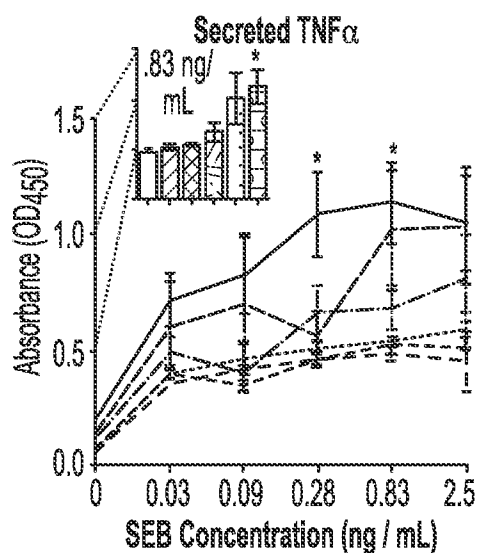
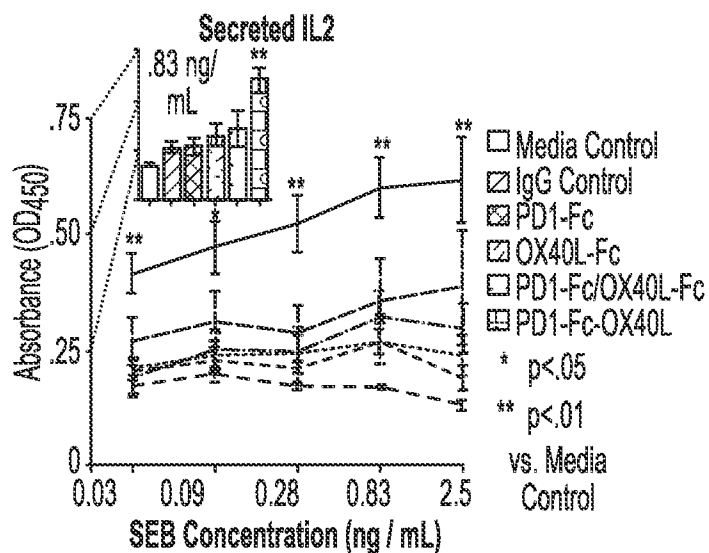


FIG. 32G



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FIG. 33A

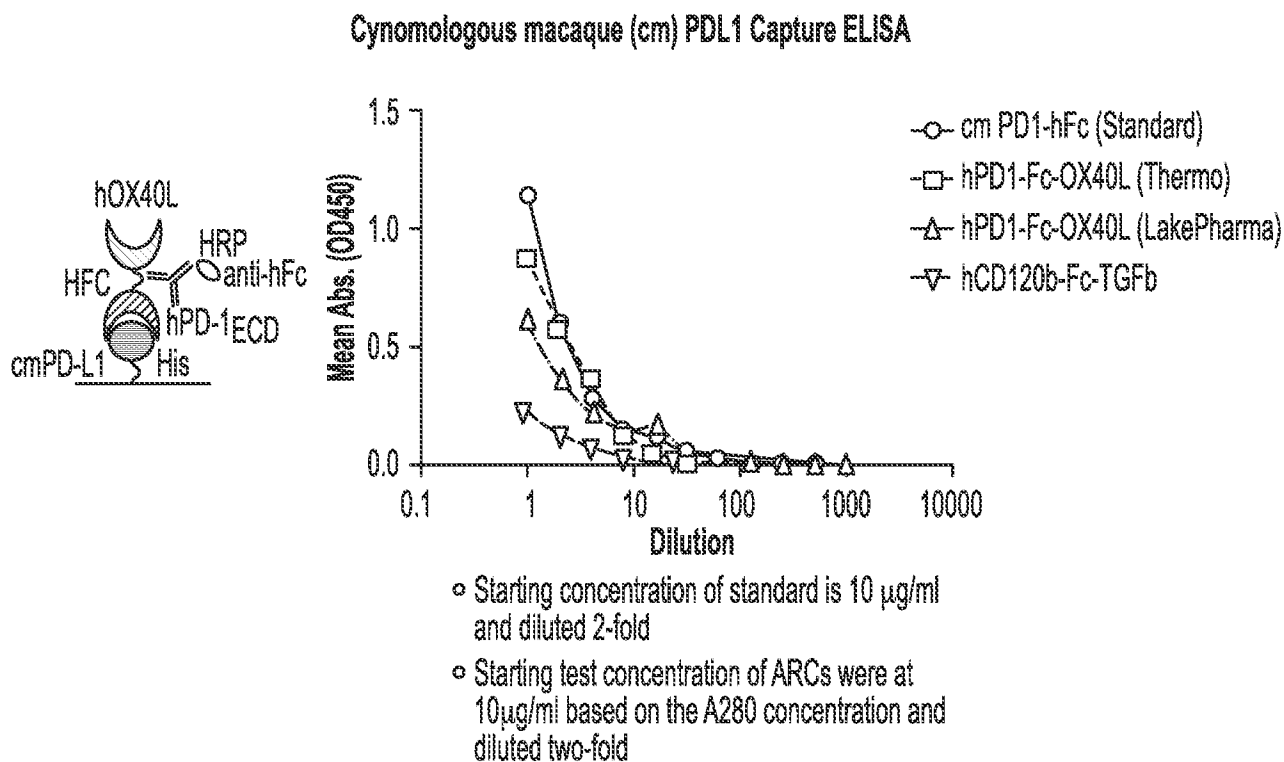


FIG. 33B

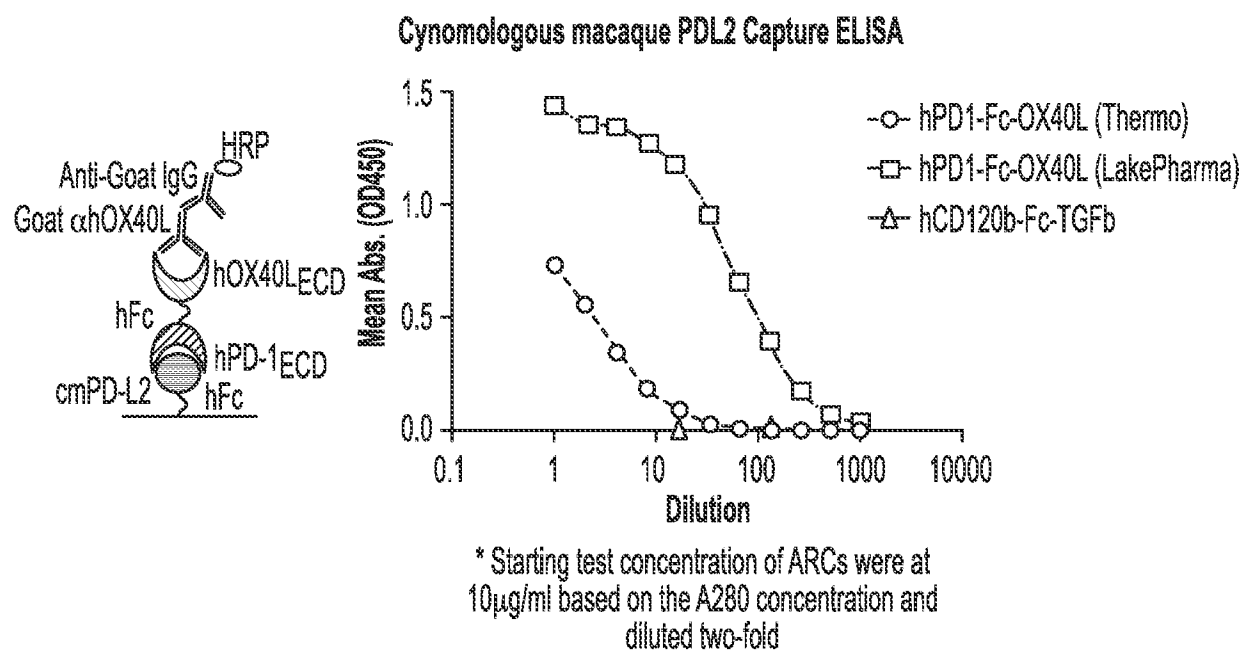
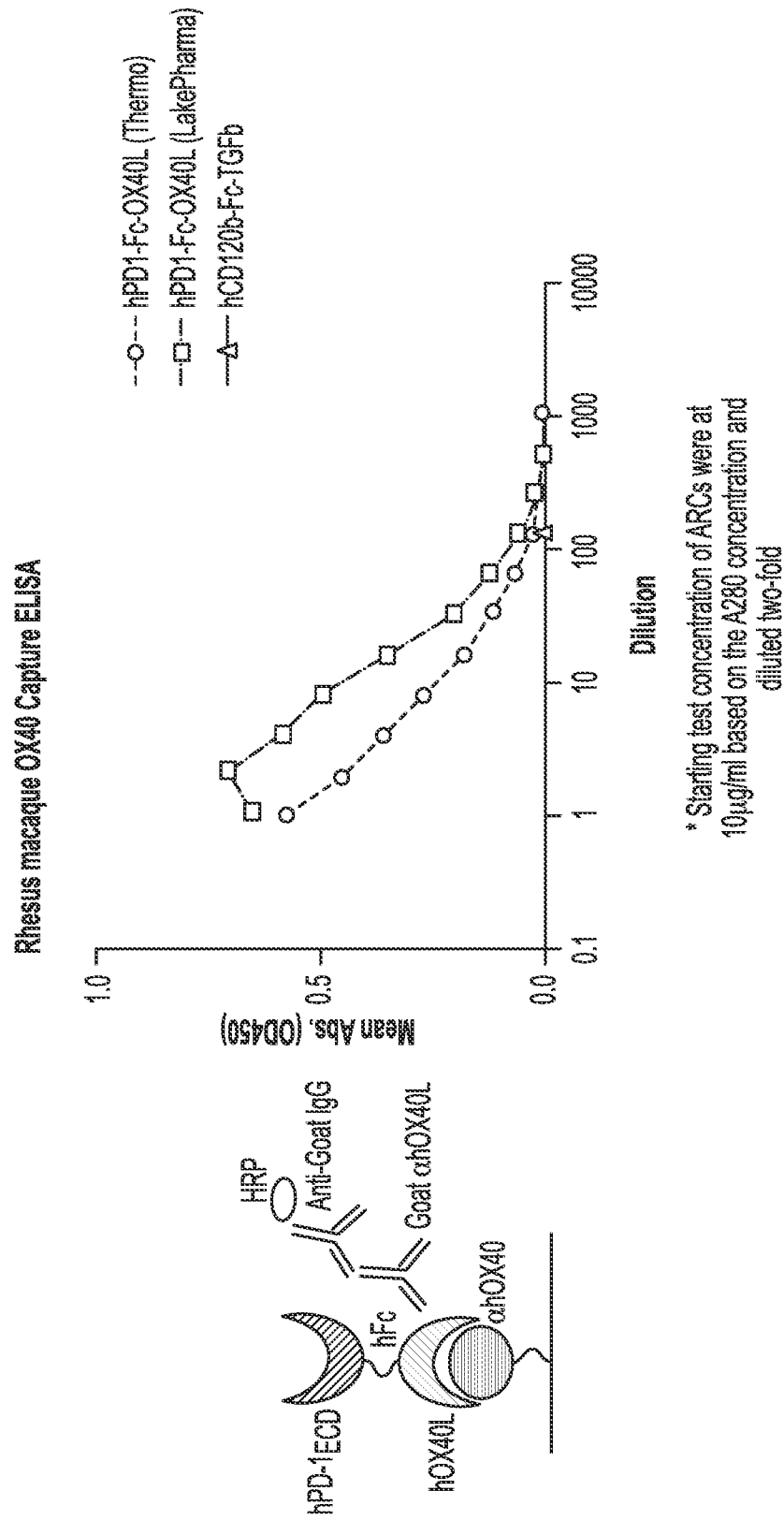


FIG. 33C



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FIG. 34

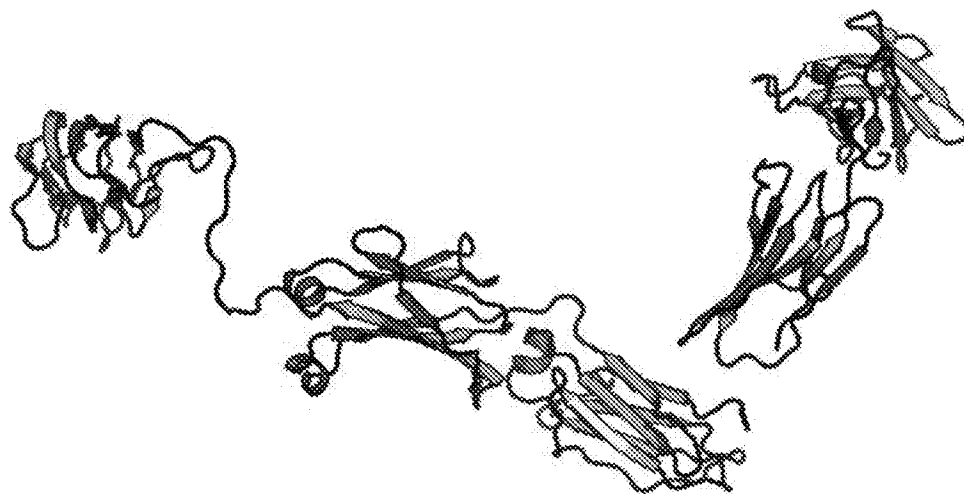
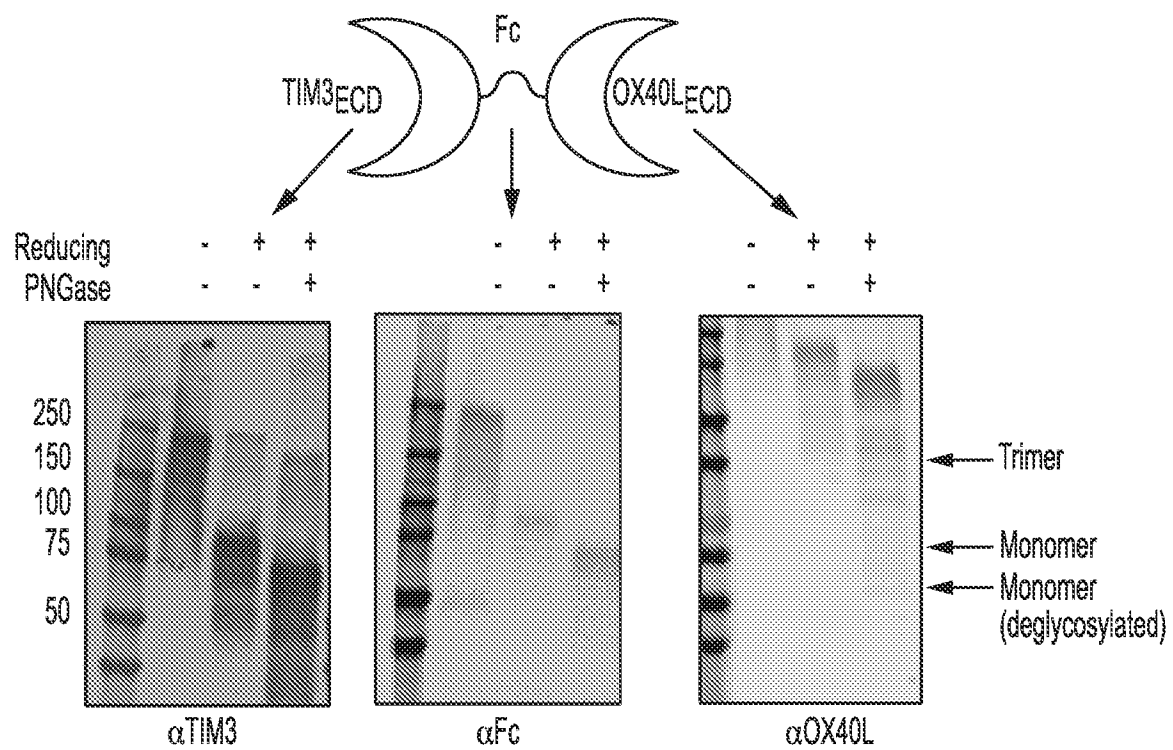
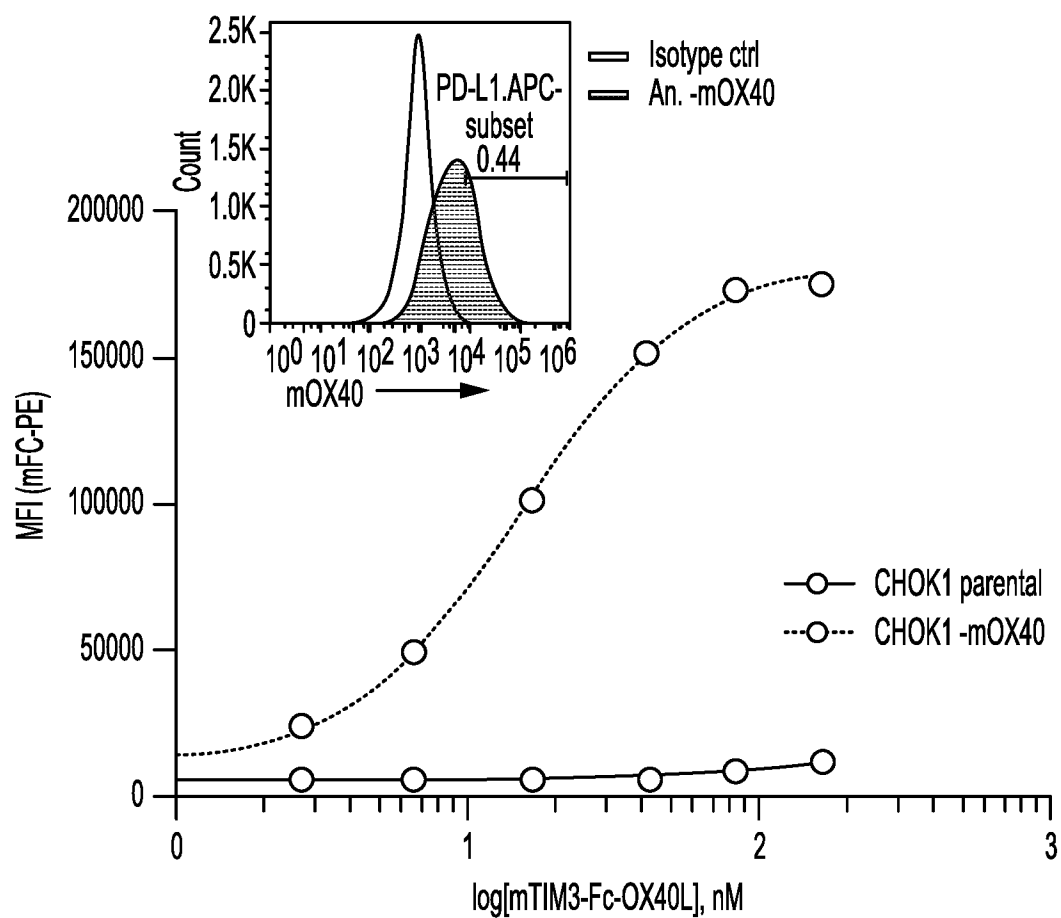


FIG. 35



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FIG. 36



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FIG. 37A

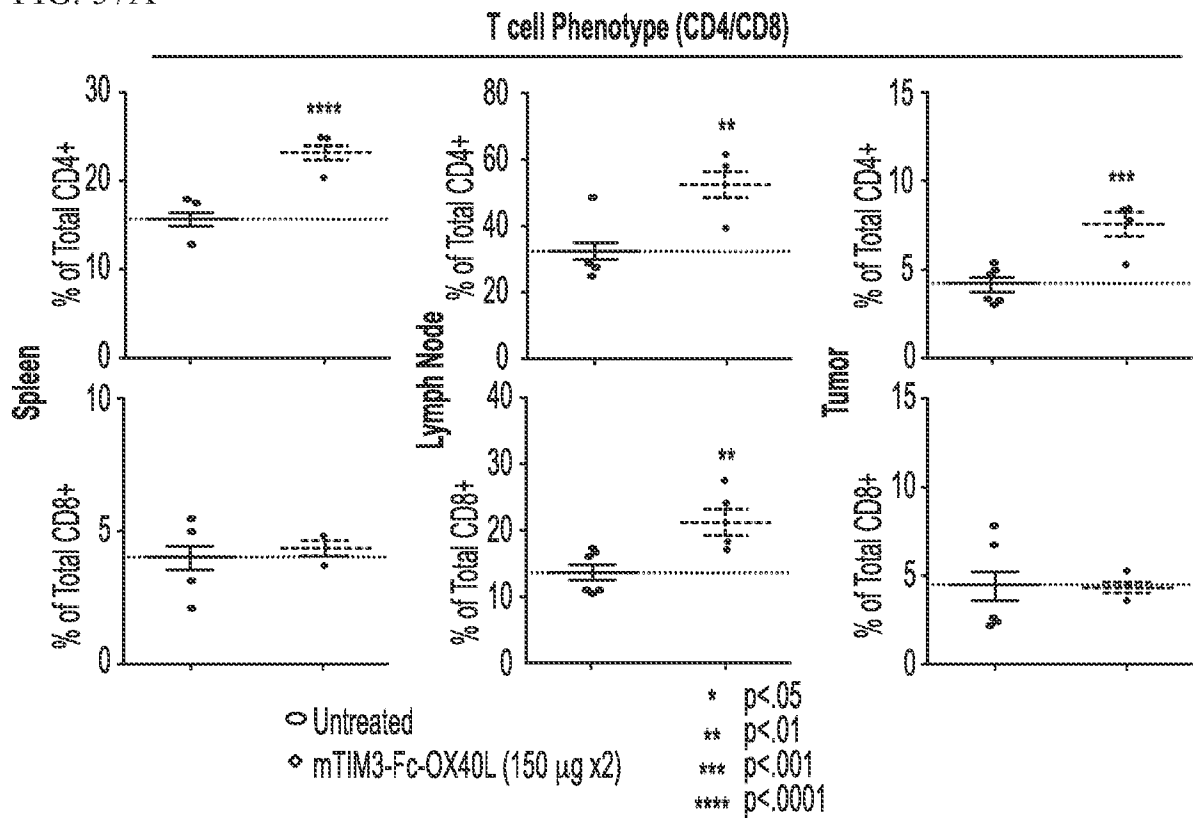


FIG. 37B

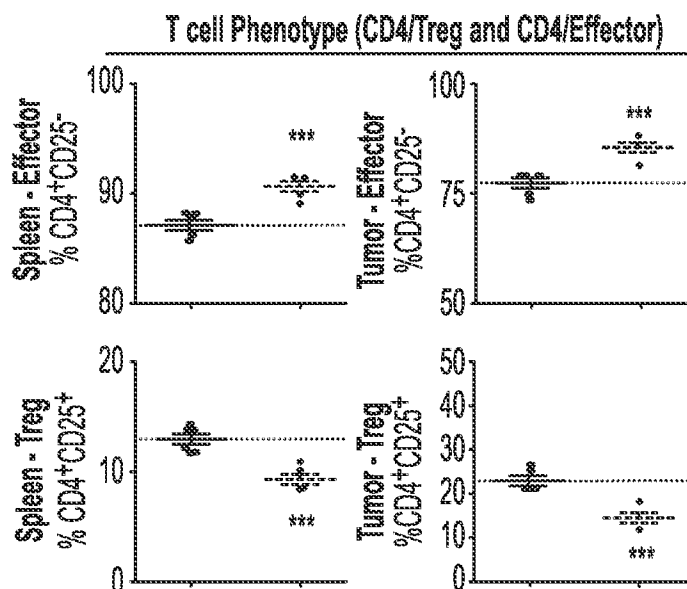
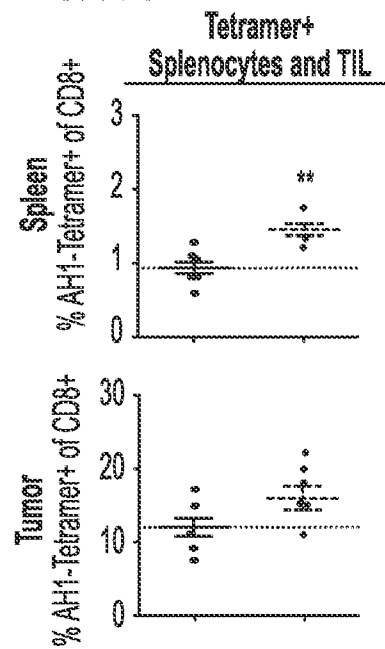


FIG. 37C



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FIG. 38A

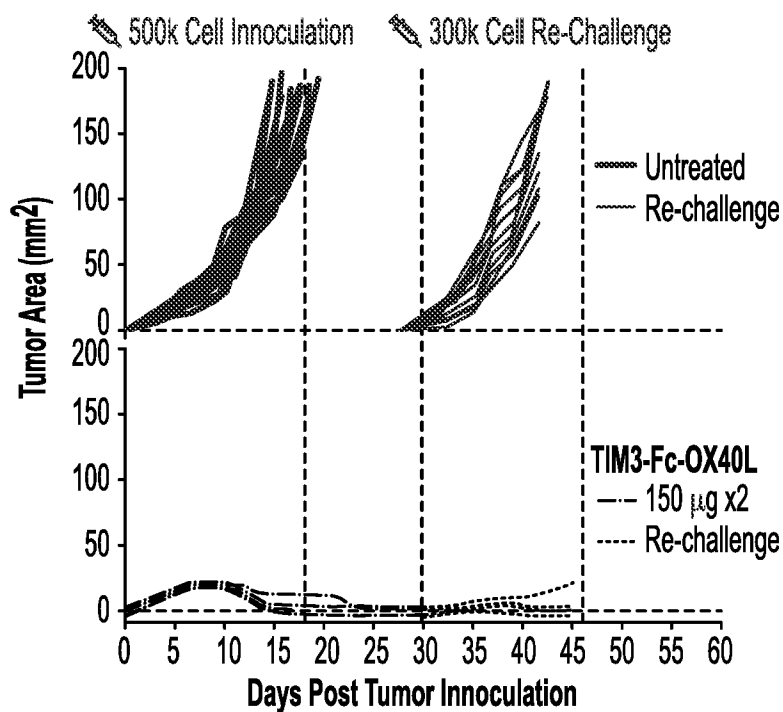


FIG. 38B

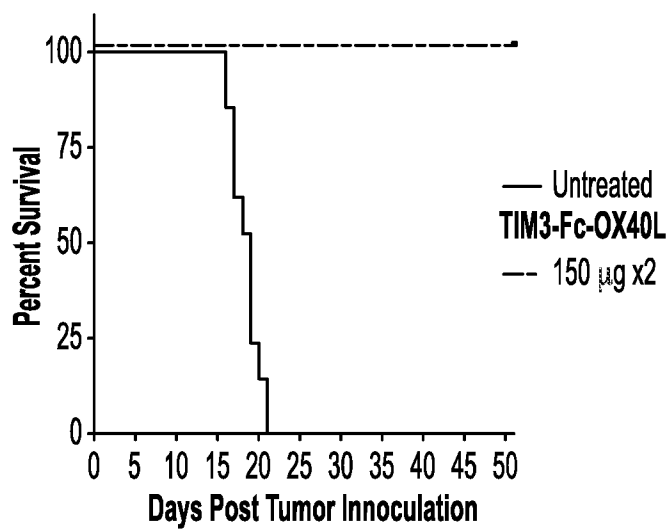


FIG. 38C

Group	Total, N	Short-Term, N (Immune Profiling)	Long-Term, N (Tumor Growth/Survival)	% Rejection (Primary Tumor)	% Rejection (Re-challenge)
Untreated	33	12	21	0.0	0.0
TIM3-Fc-OX40L (150µg x2)	9	5	4	100.0	75

FIG. 39

Joining Linker 1	Fc	Joining Linker 2	Linker Module = Joining Linker 1 + Fc + Joining Linker 2
SKYGPPCPSCP (SEQ ID NO: 48)	APEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVSQAE DPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIV LHQDWLSGKEYCKVSSKGLPSSIEKTIISNATGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSSWQEGNVFSC SVLHEALHNHYTQKSLSLGLK (SEQ ID NO: 45)	IEGRMD (SEQ ID NO: 51)	SKYGPPCPSCPAPAEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIVLHQDWLS GKEYCKVSSKGLPSSIEKTIISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLT VDKSSWQEGNVFSCSVLHEALHNHYTQKSLSLGLKIEGRMD (SEQ ID NO: 95)
SKYGPPCPSCP (SEQ ID NO: 48)	APEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVSQAE DPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIV PHSDWLSGKEYCKVSSKGLPSSIEKTIISNATGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSSWQEGNVFSC SVLHEALHNHYTQKSLSLGLK (SEQ ID NO: 46)	IEGRMD (SEQ ID NO: 51)	SKYGPPCPSCPAPAEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIVPHSDWLS GKEYCKVSSKGLPSSIEKTIISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLT VDKSSWQEGNVFSCSVLHEALHNHYTQKSLSLGLKIEGRMD (SEQ ID NO: 96)
SKYGPPCPSCP (SEQ ID NO: 48)	APEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVSQAE DPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIV LHQDWLSGKEYCKVSSKGLPSSIEKTIISNATGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSC SVLHEALHNHYTQKSLSLGLK (SEQ ID NO: 47)	IEGRMD (SEQ ID NO: 51)	SKYGPPCPSCPAPAEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIVLHQDWLS GKEYCKVSSKGLPSSIEKTIISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLGLKIEGRMD (SEQ ID NO: 97)
SKYGPPCPSCP (SEQ ID NO: 49)	APEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVSQAE DPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIV LHQDWLSGKEYCKVSSKGLPSSIEKTIISNATGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSSWQEGNVFSC SVLHEALHNHYTQKSLSLGLK (SEQ ID NO: 45)	IEGRMD (SEQ ID NO: 51)	SKYGPPCPSCPAPAEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIVLHQDWLS GKEYCKVSSKGLPSSIEKTIISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLT VDKSSWQEGNVFSCSVLHEALHNHYTQKSLSLGLKIEGRMD (SEQ ID NO: 98)
SKYGPPCPSCP (SEQ ID NO: 49)	APEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVSQAE DPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIV PHSDWLSGKEYCKVSSKGLPSSIEKTIISNATGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSSWQEGNVFSC SVLHEALHNHYTQKSLSLGLK (SEQ ID NO: 46)	IEGRMD (SEQ ID NO: 51)	SKYGPPCPSCPAPAEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIVPHSDWLS GKEYCKVSSKGLPSSIEKTIISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLT VDKSSWQEGNVFSCSVLHEALHNHYTQKSLSLGLKIEGRMD (SEQ ID NO: 99)
SKYGPPCPSCP (SEQ ID NO: 49)	APEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVSQAE DPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIV LHQDWLSGKEYCKVSSKGLPSSIEKTIISNATGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSC SVLHEALHNHYTQKSLSLGLK (SEQ ID NO: 47)	IEGRMD (SEQ ID NO: 51)	SKYGPPCPSCPAPAEFLGGPSVFLFPKPKDQIMISRTPEVTCVVDVS QEDPEVQFNWYVDGVEVHNATKPREEQFNSTYRVVSVLTIVLHQDWLS GKEYCKVSSKGLPSSIEKTIISNATGQPREPQVYTLPPSQEEMTKNQV SLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLGLKIEGRMD (SEQ ID NO: 100)



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/020040

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00; C07K 14/705 (2018.01)

CPC - A61K 39/0011; C07K 14/70503; C07K 14/70596; C07K 2319/32; C07K 2319/74 (2018.02)

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

USPC - 424/192.1; 435/69.7; 514/19.3 (keyword delimited)

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

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016/0340430 A1 (THE JOHNS HOPKINS UNIVERSITY) 24 November 2016 (24.11.2016) entire document	1, 4
Y		2, 3, 11-13
Y	MARCUS et al. "Recognition of tumors by the innate immune system and natural killer cells," <i>Advances In Immunology</i> , 01 January 2015 (01.01.2015), Vol. 122, Pgs. 01 128. entire document	2, 11-13
Y	DE VISSER et al. "The interplay between innate and adaptive immunity regulates cancer development," <i>Cancer Immunology, Immunotherapy</i> , 12 May 2005 (12.05.2005), Vol. 54, No. 11, Pgs. 1143-1152. entire document	3
A	WO 2016/166139 A1 (EBERHARD KARLS UNIVERSITÄT TÜBINGEN) 20 October 2016 (20.10.2016) entire document	1-4, 11-13
A	WO 2013/000234 A1 (HUABO BIOPHARM CO., LTD et al) 03 January 2013 (03.01.2013) entire document	1-4, 11-13
P, A	US 2017/0095531 A1 (HEAT BIOLOGICS, INC.) 06 April 2017 (06.04.2017) entire document	1-4, 11-13

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

\* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

23 April 2018

Date of mailing of the international search report

23 MAY 2018

Name and mailing address of the ISA/US

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

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/020040

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

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

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 32-35  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
Claims 32-35 have been held as omnibus claims, as they refer to an invention "as described herein".
  
3. ☒ Claims Nos.: 5-10, 14-31  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

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