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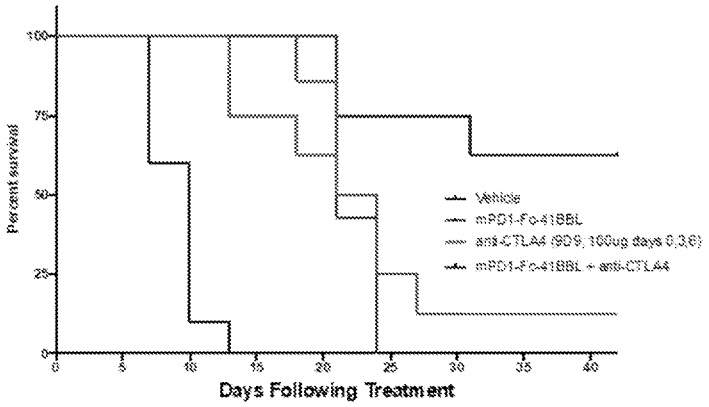
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FIG. 6B



(57) Abstract: The present invention relates to, *inter alia*, combinations of compositions which include chimeric proteins that find use in methods for treating disease, such as immunotherapies for cancer and autoimmunity.

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COMBINATION THERAPIES COMPRISING PD-1-BASED CHIMERIC PROTEINS

PRIORITY

This application claims the benefit of, and priority to, U.S. Provisional Application No. 62/724,600, filed Aug 29, 2018; U.S. Provisional Application No. 62/734,951, filed Sept 21, 2018; U.S. Provisional Application No. 62/793,235, filed January 16, 2019; U.S. Provisional Application No. 62/832,830, filed April 11, 2019; U.S. Provisional Application No. 62/890,217, filed August 22, 2019; the contents of each of which is herein incorporated by reference in its entirety.

TECHNICAL FIELD

The present invention relates to, *inter alia*, combinations of compositions which include chimeric proteins that find use in methods for treating disease, such as immunotherapies for cancer and autoimmunity.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

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BACKGROUND

The immune system is central to the body's response to cancer cells and disease-causing foreign entities. Many cancers, however, have developed mechanisms to avoid the immune system by, for instance, delivering or propagating immune inhibitory signals. Additionally, many anti-cancer therapeutics do not directly stimulate and/or activate the immune response. Current combination immunotherapy with bispecific antibodies, linked scFv's, or T cell engagers have not been able to both block checkpoints (immune inhibitory signals) and agonize (stimulate) TNF receptors. This is likely because these molecules lose target avidity when engineered to bind multiple targets with monovalent antigen binding arms. Thus, there remains a need to develop therapeutics that, at least, are endowed with multiple functionalities but still retain target avidity – for instance, reverse immune inhibitory signals and stimulating an anti-cancer immune response.

SUMMARY

Accordingly, in various aspects, the present invention provides compositions and methods that are useful for cancer immunotherapy. For instance, the present invention, in part, relates to methods for treating cancer comprising administering (either simultaneously or sequentially) at least one antibody directed to an immune checkpoint molecule, a stimulator of interferon genes (STING) agonist, and/or one or more heterologous chimeric proteins, in which each

heterologous chimeric protein is capable of blocking immune inhibitory signals and/or stimulating immune activating signals.

An aspect of the present invention provides a method for treating a cancer in a subject in need thereof. The first pharmaceutical composition comprises an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). The second pharmaceutical composition comprising an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

Another aspect of the present invention provides a method for treating a cancer in a subject. The method comprising a step of providing the subject a pharmaceutical composition comprising an immunotherapy. The immunotherapy is selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain. In this aspect, the subject has undergone or is undergoing treatment with an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).

Yet another aspect of the present invention provides a method for treating a cancer in a subject. The method comprising a step of providing the subject a pharmaceutical composition comprising an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). In this aspect, the subject has undergone or is undergoing treatment with an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a

portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

In an aspect, the present invention provides a method for treating a cancer in a subject in need thereof. The method comprising steps of providing the subject a first pharmaceutical composition and a second pharmaceutical composition. The first pharmaceutical composition comprises a stimulator of interferon genes (STING) agonist. The second pharmaceutical composition comprising an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

In another aspect, the present invention provides a method for treating a cancer in a subject. The method comprising a step of providing the subject a pharmaceutical composition comprising an immunotherapy. The immunotherapy is selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein

comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain. In this aspect, the subject has undergone or is undergoing treatment with a stimulator of interferon genes (STING) agonist.

In yet another aspect, the present invention provides a method for treating a cancer in a subject. The method comprises a step of providing the subject a pharmaceutical composition comprising a stimulator of interferon genes (STING) agonist. In this aspect, the subject has undergone or is undergoing treatment with an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

Any aspect or embodiment disclosed 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 Type I transmembrane proteins (**FIG. 1A** and **FIG. 1B**, left proteins) and Type II transmembrane proteins (**FIG. 1A** and **FIG. 1B**, right proteins). A Type I transmembrane protein and a Type II transmembrane protein may be engineered such that their transmembrane and intracellular domains are omitted and the transmembrane proteins' extracellular domains are adjoined using a linker sequence to generate a single chimeric protein. As shown in **FIG. 1C** and **FIG. 1D**, the extracellular domain of a Type I transmembrane protein, *e.g.,* PD-1, and the extracellular domain of a Type II transmembrane protein, *e.g.,* CD40L, GITRL, and 4-1BBL, are combined into a single chimeric protein. **FIG. 1C** depicts the linkage of the Type I transmembrane protein and the Type II transmembrane protein by omission of the transmembrane and intracellular domains of each protein, and where the liberated extracellular domains from each protein have been adjoined by a linker sequence. The extracellular domains in this depiction may include the entire amino acid sequence of the Type I protein (*e.g.,* PD-1) and/or Type II protein (*e.g.,* CD40L, GITRL, and 4-1BBL) which is typically localized outside the cell membrane, or any portion thereof which retains binding to the intended receptor or ligand. Moreover, the heterologous chimeric protein used in a method of the present invention comprises sufficient overall flexibility and/or physical distance between domains such that a first

extracellular domain (shown at the left end of the heterologous chimeric protein in **FIG. 1C** and **FIG. 1D)** is sterically capable of binding its receptor/ligand and/or a second extracellular domain (shown at the right end of the heterologous chimeric protein in **FIG. 1C** and **FIG. 1D**) is sterically capable of binding its receptor/ligand. **FIG. 1D** depicts adjoined extracellular domains in a linear chimeric protein wherein each extracellular domain of the heterologous chimeric protein is facing "outward".

FIG. 2 shows immune inhibitory and immune stimulatory signaling that is relevant to the present invention (from Mahoney, *Nature Reviews Drug Discovery* 2015:14;561-585).

FIG. 3A is table showing anti-tumor treatment schedules for the *in vivo* experiments disclosed **FIG. 3B** to **FIG. 3D** and **FIG. 4A** to **FIG. 4B. FIG. 3B** shows *in vivo* reductions in tumor volume size for control treatments and **FIG. 3C** and **FIG. 3D** show *in vivo* reductions in tumor volume size resulting from methods of cancer treatments according to the present invention. For **FIG. 3B**, at time point 18 days, the curves are from top to bottom: vehicle, anti-PD1 (RMP1-14) antibody, anti-OX40 (OX86) antibody, anti-CTLA4 (9D9) antibody, and the PD1-Fc-GITRL chimeric protein. For **FIG. 3C**, at time point 18 days, the curves are from top to bottom: vehicle, anti-CTLA4 antibody then anti-PD1 antibody, anti-CTLA4 antibody then anti-OX40 antibody, and anti-CTLA4 then the PD1-Fc-GITRL chimeric protein. For **FIG. 3D**, the top curve is vehicle and the bottom curve is the PD1-Fc-GITRL chimeric protein then an anti-CTLA4 antibody.

FIG. 4A to **FIG. 4C** show *in vivo* anti-tumor activity of the PD-1-Fc-GITRL chimeric protein when administered in combination with an anti-CTLA-4 antibody. For **FIG. 4B**, at time point 30 days, the curves are from top to bottom: the PD1-Fc-GITRL chimeric protein and an anti-CTLA4 antibody; the PD1-Fc-GITRL chimeric protein; and the anti-CTLA4 antibody. Before time point 15 days, the vehicle mice had all died.

FIG. 5A to **FIG. 5C** show *in vivo* anti-tumor activity of the PD-1-Fc-CD40L chimeric protein when administered in combination with an anti-CTLA-4 antibody. For **FIG. 5A**, at time point 5 days, the curves are from top to bottom: a PD1-Fc fusion protein, vehicle, the PD1-Fc fusion protein with the CD40L-Fc fusion protein, an anti-CTLA4 antibody, the CD40L-Fc fusion protein, the PD1-Fc-CD40L chimeric protein, and the PD1-Fc-CD40L chimeric protein with the anti-CTLA4 antibody. For FIG. 5B, at approximately time point 10 days, the curves are from top to bottom: the PD1-Fc-CD40L chimeric protein treatment overlapping the PD1-Fc-CD40L chimeric protein with the anti-CTLA4 antibody co-treatment, the anti-CTLA4 antibody; at the PD1-Fc fusion protein with the CD40L-Fc fusion protein. Before approximately time point 10 days, the CD40L-Fc fusion protein mice had all died. Before time point 8 days, the vehicle and the PD1-Fc fusion protein mice had all died.

FIG. 6A to **FIG. 6C** show *in vivo* anti-tumor activity of the PD-1-Fc-4-1BBL chimeric protein when administered in combination with an anti-CTLA-4 antibody. For **FIG. 6B**, at time point 20 days, the curves are from top to bottom: the PD1-Fc-4-1BBL chimeric protein with an anti-CTLA4 antibody, the PD1-Fc-4-1BBL chimeric protein, and the anti-CTLA4 antibody. Before time point 15 days, the vehicle mice had all died. **FIG. 6D** includes data demonstrating

improvements obtained from the combination treatments relative to monotherapies.

FIG. 7A shows *in vivo* reductions in tumor volume size for control treatments and **FIG. 7B** show *in vivo* reductions in tumor volume size resulting from methods of cancer treatments according to the present invention. For **FIG. 7A**, at approximately time point 20 days, the curves are from top to bottom: vehicle (IP), vehicle (IT), an anti-PD1 (RMP1-14) antibody, the PD-1-Fc-GITRL chimeric protein, DMXAA, and the anti-OX40 (OX86) antibody. For **FIG. 7B**, at approximately time point 20 days, the curves are from top to bottom: vehicle (IP), vehicle (IT), DMXAA then the anti-PD1 antibody, DMXAA then the anti-OX40 antibody, and DMXAA then the PD-1-Fc-GITRL chimeric protein.

DETAILED DESCRIPTION

The present invention is based, in part, on the discovery of methods for treating cancer comprising administering (either simultaneously or sequentially) at least one antibody directed to an immune checkpoint molecule, e.g., CTLA-4, a stimulator of interferon genes (STING) agonist, and/or one or more heterologous chimeric proteins, in which each heterologous chimeric protein is capable of blocking immune inhibitory signals and/or stimulating immune activating signals.

Importantly, since the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention disrupt, block, reduces, inhibit, and/or sequester the transmission of immune inhibitory signals, *e.g.,* originating from a cancer cell that is attempting to avoid its detection and/or destruction and/or enhance, increase, and/or stimulate the transmission of an immune stimulatory signal to an anti-cancer immune cell, the methods can provide an anti-tumor effect by multiple distinct pathways. By treating cancer *via* multiple distinct pathways, the methods of the present invention are more likely to provide any anti-tumor effect in a patient and/or to provide an enhanced anti-tumor effect in a patient. Moreover, since the methods operate by multiple distinct pathways, they can be efficacious, at least, in patients who do not respond, respond poorly, or become resistant to treatments that target one of the pathways. Thus, a patient who is a poor responder to treatments acting *via* one of the two pathways, can receive a therapeutic benefit by targeting multiple pathways.

Antibodies

The methods of the present invention comprise methods for treating cancer, which, in embodiments, comprise administering an immunotherapy comprising an antibody capable of binding an immune checkpoint molecule.

The antibody may be selected from one or more of a monoclonal antibody, polyclonal antibody, antibody fragment, Fab, Fab', Fab'-SH, F(ab')2, Fv, single chain Fv, diabody, linear antibody, bispecific antibody, multispecific antibody, chimeric antibody, humanized antibody, human antibody, and fusion protein comprising the antigen-binding portion of an antibody. In embodiments, the antibody is a monoclonal antibody, *e.g.,* a humanized monoclonal antibody.

In embodiments, the antibody capable of binding an immune checkpoint molecule binds CTLA-4. Illustrative antibodies capable of binding CTLA-4 include YERVOY (ipilimumab), 9D9, tremelimumab (formerly ticilimumab, CP-675,206;

MedImmune), AGEN1884, and RG2077.

STING Agonists

The methods of the present invention comprise methods for treating cancer, which, in embodiments, comprise administering a pharmaceutical composition comprising a stimulator of interferon genes (STING) agonist.

In embodiments, the STING agonist is selected from the group consisting of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), MIW815(ADU-S100), CRD5500, MK-1454, and any STING agonist described in US20140341976, US20180028553, US20180230178, US9549944, WO2017106740, WO2018045204, or WO2018098203, the contents of which are incorporated herein by reference in their entireties.

Heterologous Chimeric Proteins

The present invention comprises methods for treating cancer, which, in embodiments, comprise administering a pharmaceutical composition comprising a heterologous chimeric protein capable of blocking immune inhibitory signals and/or stimulating immune activating signals.

Heterologous chimeric proteins used in methods of the present invention comprise a general structure of: N terminus – (a) – (b) – (c) – C terminus, where (a) is a first domain comprising an extracellular domain of Type I transmembrane protein (e.g., PD-1), (b) is a linker adjoining the first domain and the second domain, *e.g.,* the linker comprising at least one cysteine residue capable of forming a disulfide bond and/or comprising a hinge-CH2-CH3 Fc domain, and (c) is a second domain comprising an extracellular domain of a Type II transmembrane protein (e.g., CD40L, GITRL, and 4-1BBL); wherein the linker connects the first domain and the second domain.

Transmembrane proteins typically consist of an extracellular domain, one or a series of transmembrane domains, and an intracellular domain. Without wishing to be bound by theory, the extracellular domain of a transmembrane protein is responsible for interacting with a soluble receptor or ligand or a membrane-bound receptor or ligand (*i.e.,* a membrane of an adjacent cell) in the extracellular environment. Without wishing to be bound by theory, the transmembrane domain(s) is responsible for localizing the transmembrane protein to the plasma membrane. Without wishing to be bound by theory, the intracellular domain of a transmembrane protein is responsible for coordinating interactions with cellular signaling molecules to coordinate intracellular responses with the extracellular environment (or visa-versa).

In embodiments, an extracellular domain refers to a portion of a transmembrane protein which is sufficient for binding to a ligand or receptor and is effective in transmitting a signal to a cell. In embodiments, an extracellular domain is the entire amino acid sequence of a transmembrane protein which is normally present at the exterior of a cell or of the cell membrane. In embodiments, an extracellular domain is that portion of an amino acid sequence of a transmembrane protein which is external of a cell or of the cell membrane and is needed for signal transduction and/or ligand binding as may be assayed using methods know in the art (*e.g., in vitro* ligand binding and/or cellular activation assays).

There are generally two types of single-pass transmembrane proteins: Type I transmembrane proteins which have an extracellular amino terminus and an intracellular carboxy terminus (see, **FIG. 1A**, left protein) and Type II transmembrane proteins which have an extracellular carboxy terminus and an intracellular amino terminus (see, **FIG. 1A**, right protein). Type I and Type II transmembrane proteins can be either receptors or ligands. For Type I transmembrane proteins (*e.g.,* PD-1), 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 (see, **FIG. 1B**, left protein). For Type II transmembrane proteins (*e.g.,* CD40L, GITRL, and 4-1BBL), 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 (see, **FIG. 1B**, right protein). Thus, these two types of transmembrane proteins have opposite orientations to each other relative to the cell membrane.

Heterologous chimeric proteins used in methods of the present invention comprise an extracellular domain of a Type I transmembrane protein that is PD-1 and an extracellular domain of a Type II transmembrane protein selected from CD40L, GITRL, and 4-1BBL. Thus, a heterologous chimeric protein used in a method of the present invention comprises, at least, a first domain comprising the extracellular domain of PD-1, which is connected – directly or *via* a linker – to a second domain comprising the extracellular domain of CD40L, GITRL, or 4-1BBL. As illustrated in **FIG. 1C** and **FIG. 1D**, when the domains are linked in an amino-terminal to carboxy-terminal orientation, the first domain is located on the "left'" side of the heterologous chimeric protein and is "outward facing" and the second domain is located on "right" side of the heterologous chimeric protein and is "outward facing".

Other configurations of first and second domains are envisioned, *e.g.,* the first domain is inward facing and the second domain is outward facing, the first domain is outward facing and the second domain is inward facing, and the first and second domains are both inward facing. When both domains are "inward facing", the heterologous chimeric protein would have an amino-terminal to carboxy-terminal configuration comprising an extracellular domain of a Type II transmembrane protein, a linker, and an extracellular domain of Type I transmembrane protein. In such configurations, it may be necessary for the heterologous chimeric protein to include extra "slack", as described elsewhere herein, to permit binding domains of the heterologous chimeric protein to one or both of its receptors/ligands.

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises the extracellular domain of human PD-1 which comprises the following amino acid sequence:

LDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ
PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPT
AHPSPSPRPAGQFQ (SEQ ID NO: 57).

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises a variant of the extracellular domain of PD-1. As examples, the variant may have 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 SEQ ID NO: 57.

In embodiments, the first domain of a heterologous chimeric protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 57.

In embodiments, a heterologous chimeric protein comprises substantially the entire extracellular domain of PD-1.

One of ordinary skill may select variants of the known amino acid sequence of PD-1 by consulting the literature, *e.g.* Zhang *et al* "Structural and Functional Analysis of the Costimulatory Receptor Programmed Death-1" Immunity. 2004 Mar; 20(3):337-47; Lin *et al* "The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors", Proc Natl Acad Sci U S A. 2008 Feb 26;105(8):3011-6; Zak *et al* "Structure of the Complex of Human Programmed Death 1, PD-1, and Its Ligand PD-L1", Structure. 2015 Dec 1;23(12):2341-2348; and Cheng *et al* "Structure and Interactions of the Human Programmed Cell Death 1 Receptor", J Biol Chem. 2013 Apr 26;288(17):11771-85, each of which is incorporated by reference in its entirety.

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises the extracellular domain of human GITRL which comprises the following amino acid sequence:

ETAKEPCMAKFGPLPSKWQMASSEPPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDVAPFEV RLYKNKDMIQTLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGIILLANPQFIS (SEQ ID NO: 58).

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises a variant of the extracellular domain of GITRL. As examples, the variant may have 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 SEQ ID NO: 58.

In embodiments, the second domain of a heterologous chimeric protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 58.

In embodiments, a heterologous chimeric protein comprises substantially the entire extracellular domain of GITRL.

One of ordinary skill may select variants of the known amino acid sequence of GITRL by consulting the literature, *e.g.* Chattopadhyay *et al.* "Evolution of GITRL immune function: Murine GITRL exhibits unique structural and biochemical properties within the TNF superfamily." PNAS, Volume 105, Issue 2, 2008, pp. 635-640 and Zjou, *et al.* "Structural basis for ligand-mediated mouse GITR activation Structural basis for ligand-mediated mouse GITR activation." PNAS January 15, 2008. 105 (2) 641-645 each of which is incorporated by reference in its entirety.

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises the extracellular domain of human CD40L which comprises the following amino acid sequence:

HRRLDKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFE
MQKGDQNPQIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTF
CSNREASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVNVTD
PSQVSHGTGFTSFGLLKL (SEQ ID NO: 60).

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises a variant of the extracellular domain of CD40L. As examples, the variant may have 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 SEQ ID NO: 60.

In embodiments, the second domain of a heterologous chimeric protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 60.

In embodiments, a heterologous chimeric protein comprises substantially the entire extracellular domain of CD40L.

One of ordinary skill may select variants of the known amino acid sequence of CD40L by consulting the literature, *e.g.* An, *et al*. "Crystallographic and Mutational Analysis of the CD40-CD154 Complex and Its Implications for Receptor Activation", The Journal of Biological Chemistry 286, 11226-11235, which is incorporated by reference in its entirety.

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises the extracellular domain of human 4-1BBL which comprises the following amino acid sequence:

ACPWAVSGARASPGSAASPRLREGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDP GLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAA LALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPE IPAGLPSPRSE (SEQ ID NO: 61).

In embodiments, a heterologous chimeric protein used in methods of the present invention comprises a variant of the extracellular domain of 4-1BBL. As examples, the variant may have 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 SEQ ID NO: 61.

In embodiments, the second domain of a heterologous chimeric protein comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 61.

In embodiments, a heterologous chimeric protein comprises substantially the entire extracellular domain of 4-1BBL.

One of ordinary skill may select variants of the known amino acid sequence of 4-1BBL by consulting the literature, *e.g.,* Goodwin *et al*., "Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor." Eur. J. Immunol. 23 (10), 2631-2641 (1993); Alderson *et al*., "Molecular and biological characterization of human 4-1BB and its ligand." Eur. J. Immunol. 24 (9), 2219-2227 (1994); and Arch and Thompson "4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappaB." Mol. Cell. Biol. 18 (1), 558-565 (1998), each of which is incorporated by reference in its entirety, which is incorporated by reference in its entirety.

In embodiments, a heterologous chimeric protein comprising a first domain which comprises a variant of the extracellular domain of PD-1 and/or a second domain which comprises a variant of the extracellular domain of GITRL.

In embodiments, a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, a heterologous chimeric protein comprising a first domain which comprises a variant of the extracellular domain of PD-1 and/or a second domain which comprises a variant of the extracellular domain of CD40L.

In embodiments, a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, a heterologous chimeric protein comprising a first domain which comprises a variant of the extracellular domain of PD-1 and/or a second domain which comprises a variant of the extracellular domain of 4-1BBL.

In embodiments, a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

In any herein-disclosed aspect and embodiment, the heterologous chimeric protein may comprise an amino acid sequence having one or more amino acid mutations relative to any of the protein sequences disclosed 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, based on 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 α-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 β-alanine, GABA and δ-Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu,

ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general).

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

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

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

In embodiments, each extracellular domain (or variant thereof) of the heterologous chimeric protein binds to its cognate receptor or ligand 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 heterologous chimeric protein binds to a cognate receptor or ligand 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, each extracellular domain (or variant thereof) of the heterologous chimeric protein binds to its cognate receptor or ligand with a K_D of less than about 1 μM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 150 nM, about 130 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 55 nM, about 50 nM, about 45 nM, about 40 nM, about 35 nM, about 30 nM, about 25 nM, about 20 nM, about 15 nM, about 10 nM, or about 5 nM, or about 1 nM (as measured, for example, by surface plasmon resonance or biolayer interferometry). In embodiments, the heterologous chimeric protein binds to human CSF1 with a K_D of less than about 1 nM, about 900 pM, about 800 pM, about 700 pM, about 600 pM, about 500 pM, about 400 pM, about 300 pM, about 200 pM, about 100 pM, about 90 pM, about 80 pM, about 70 pM, about 60 pM about 55 pM about 50 pM about 45 pM, about 40 pM, about 35 pM, about 30 pM, about 25 pM, about 20 pM, about 15 pM, or about 10 pM, or about 1 pM (as measured, for example, by surface plasmon resonance or biolayer interferometry).

As used herein, a variant of an extracellular domain is capable of binding the receptor/ligand of a native extracellular domain. For example, a variant may include one or more mutations in an extracellular domain which do not affect its binding affinity to its receptor/ligand; alternately, the one or more mutations in an extracellular domain may improve binding affinity for the receptor/ligand; or the one or more mutations in an extracellular domain may reduce binding affinity for the receptor/ligand, yet not eliminate binding altogether. In embodiments, the one or more mutations are located outside the binding pocket where the extracellular domain interacts with its receptor/ligand. In embodiments,

the one or more mutations are located inside the binding pocket where the extracellular domain interacts with its receptor/ligand, as long as the mutations do not eliminate binding altogether. Based on the skilled artisan's knowledge and the knowledge in the art regarding receptor-ligand binding, s/he would know which mutations would permit binding and which would eliminate binding.

In embodiments, the chimeric protein exhibits enhanced stability, high-avidity binding characteristics, prolonged off-rate for target binding and protein half-life relative to single-domain fusion protein or antibody controls.

A heterologous chimeric protein used in a method of the present invention may comprise more than two extracellular domains. For example, the heterologous chimeric protein may comprise three, four, five, six, seven, eight, nine, ten, or more extracellular domains. A second extracellular domain may be separated from a third extracellular domain *via* a linker, as disclosed herein. Alternately, a second extracellular domain may be directly linked (*e.g., via* a peptide bond) to a third extracellular domain. In embodiments, a heterologous chimeric protein includes extracellular domains that are directly linked and extracellular domains that are indirectly linked *via* a linker, as disclosed herein.

Heterologous chimeric proteins of the present invention and/or heterologous chimeric proteins used in methods of the present invention have a first domain which is sterically capable of binding its ligand/receptor and/or a second domain which is sterically capable of binding its ligand/receptor. This means that there is sufficient overall flexibility in the heterologous chimeric protein and/or physical distance between an extracellular domain (or portion thereof) and the rest of the heterologous chimeric protein such that the ligand/receptor binding domain of the extracellular domain is not sterically hindered from binding its ligand/receptor. This flexibility and/or physical distance (which is herein referred to as "slack") may be normally present in the extracellular domain(s), normally present in the linker, and/or normally present in the heterologous chimeric protein (as a whole). Alternately, or additionally, the heterologous chimeric protein may be modified by including one or more additional amino acid sequences (*e.g.,* the joining linkers described below) or synthetic linkers (*e.g.,* a polyethylene glycol (PEG) linker) which provide additional slack needed to avoid steric hindrance.

Linkers

In embodiments, the heterologous chimeric protein used in a method of the present invention comprises a linker.

In embodiments, the linker comprising at least one cysteine residue capable of forming a disulfide bond. The at least one cysteine residue is capable of forming a disulfide bond between a pair (or more) of chimeric proteins. Without wishing to be bound by theory, such disulfide bond forming is responsible for maintaining a useful multimeric state of chimeric proteins. This allows for efficient production of the heterologous chimeric proteins; it allows for desired activity *in vitro* and *in vivo.*

Importantly*, inter alia*, stabilization in a linker region including one or more disulfide bonds provides for improved chimeric proteins that can maintain a stable and producible multimeric state.

In a heterologous chimeric protein used in a method of the present invention, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, or an antibody sequence.

In embodiments, the linker is derived from naturally-occurring multi-domain proteins or is an empirical linker 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 comprises a polypeptide. In embodiments, the polypeptide 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 embodiments, 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 comprises 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_{H1} 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_{H2} domain and includes residues in C_{H2}. *Id.* The core hinge region of wild-type human IgG1 contains the sequence CPPC (SEQ ID NO: 24) 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 a heterologous chimeric protein used in a method of the present invention, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG4. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from a human IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of any one of SEQ ID NO: 1 to SEQ ID NO: 3, *e.g.,* at least 95% identical to the amino acid sequence of SEQ ID NO: 2. In embodiments, the linker comprises one or more joining linkers, such joining linkers independently selected from SEQ ID NO: 4 to SEQ ID NO: 50 (or a variant thereof). In embodiments, the linker comprises two or more joining linkers each joining linker independently selected from SEQ ID NO: 4 to SEQ ID NO: 50 (or a variant thereof); wherein one joining linker is N terminal to the hinge-CH2-CH3 Fc domain and another joining linker is C terminal to the hinge-CH2-CH3 Fc domain.

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 heterologous chimeric proteins used in methods of the present invention.

In embodiments, the Fc domain in a 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 embodiments, the amino acid substitution at amino acid residue 250 is a substitution with glutamine. In embodiments, the amino acid substitution at amino acid residue 252 is a substitution with tyrosine, phenylalanine, tryptophan or threonine. In embodiments, the amino acid substitution at amino acid residue 254 is a substitution with threonine. In embodiments, the amino acid substitution at amino acid residue 256 is a substitution with serine, arginine, glutamine, glutamic acid, aspartic acid, or threonine. In embodiments, the amino acid substitution at amino acid residue 308 is a substitution with threonine. In embodiments, the amino acid substitution at amino acid residue 309 is a substitution with proline. In embodiments, the amino acid substitution at amino acid residue 311 is a substitution with serine. In embodiments, the amino acid substitution at amino acid residue 385 is a substitution with arginine, aspartic acid, serine, threonine, histidine, lysine, alanine or glycine. In embodiments, the amino acid substitution at amino acid residue 386 is a substitution with threonine, proline, aspartic acid, serine, lysine, arginine, isoleucine, or methionine. In embodiments, the amino acid substitution at amino acid residue 387 is a substitution with arginine, proline, histidine, serine, threonine, or alanine. In embodiments, the amino acid substitution at amino acid residue 389 is a substitution with proline, serine or asparagine. In embodiments, the amino acid substitution at amino acid residue 416 is a substitution with serine. In embodiments, the amino acid substitution at amino acid residue 428 is a substitution with leucine. In embodiments, the amino acid substitution at amino acid residue 433 is a substitution with arginine, serine, isoleucine, proline, or glutamine. In embodiments, 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 embodiments, the IgG constant region includes a triple M252Y/S254T/T256E mutation or YTE mutation. In embodiments, the IgG constant region includes a triple H433K/N434F/Y436H mutation or KFH mutation. In embodiments, the IgG constant region includes an YTE and KFH mutation in combination.

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

Additional exemplary 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.

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.

In embodiments, the heterologous chimeric protein binds to FcRn with high affinity. In embodiments, the heterologous chimeric protein may bind to FcRn with a K_D of about 1 nM to about 80 nM. For example, the heterologous 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 embodiments, the heterologous chimeric protein may bind to FcRn with a K_D of about 9 nM. In embodiments, the heterologous chimeric protein does not substantially bind to other Fc receptors (*i.e.* other than FcRn) with effector function.

In embodiments, the Fc domain in a linker has the amino acid sequence of SEQ ID NO: 1 (see **Table 1**, below), 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: 1 to increase stability and/or half-life. For instance, in embodiments, the Fc domain in a linker comprises the amino acid sequence of SEQ ID NO: 2 (see **Table 1**, below), or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto. For instance, in embodiments, the Fc domain in a linker comprises the amino acid sequence of SEQ ID NO: 3 (see **Table 1**, below), or at least 90%, or 93%, or 95%, or 97%, or 98%, or 99% identity thereto.

Further, one or more joining linkers may be employed to connect an Fc domain in a linker (*e.g.,* one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 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: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, or variants thereof may connect an extracellular domain as disclosed herein and an Fc domain in a linker as disclosed herein. Optionally, any one of SEQ ID NO: 4 to SEQ ID NO: 50, or variants thereof are located between an extracellular domain as disclosed herein and an Fc domain as disclosed herein.

In embodiments, the heterologous chimeric proteins used in methods of the present invention may comprise variants of the joining linkers disclosed in **Table 1**, below. For instance, a linker may have 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 any one of SEQ ID NO: 4 to SEQ ID NO: 50.

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

Without wishing to be bound by theory, including a linker comprising at least a part of an Fc domain in a heterologous chimeric protein, helps avoid formation of insoluble and, likely, non-functional protein concatenated oligomersand/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.

In embodiments, a heterologous chimeric protein may comprise one or more joining linkers, as disclosed herein, and lack an Fc domain linker, as disclosed herein.

In embodiments, the first and/or second joining linkers are independently selected from the amino acid sequences of SEQ ID NO: 4 to SEQ ID NO: 50 and are provided in **Table 1** below:

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

SEQ ID NO.	Sequence
1	APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSSWQEGNVFSCSVMHEALHN HYTQKSLSLSLGK

2	APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTTPHSDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSSWQEGNVFSCSVLHEALHNH YTQKSLSLSLGK
3	APEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEKTISNATGQPREPQVYTLPPSQEEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNH YTQKSLSLSLGK
4	SKYGPPCPSCP
5	SKYGPPCPPCP
6	SKYGPP
7	IEGRMD
8	GGGVPRDCG
9	IEGRMDGGGGAGGGG
10	GGGSGGGS
11	GGGSGGGGSGGG
12	EGKSSGSGSESKST
13	GGSG
14	GGSGGGSGGGSG
15	EAAAKEAAAKEAAAK
16	EAAAREAAAREAAAREAAAR
17	GGGGSGGGGSGGGGSAS
18	GGGGAGGGG
19	GS or GGS or LE
20	GSGSGS
21	GSGSGSGSGS
22	GGGGSAS
23	APAPAPAPAPAPAPAPAPAP
24	CPPC
25	GGGGS
26	GGGGSGGGGS
27	GGGGSGGGGSGGGGS
28	GGGGSGGGGSGGGGSGGGGS
29	GGGGSGGGGSGGGGSGGGGSGGGGS
30	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
31	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
32	GGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS
33	GGSGGSGGGGSGGGGS
34	GGGGGGGG
35	GGGGGG
36	EAAAK
37	EAAAKEAAAK
38	EAAAKEAAAKEAAAK
39	AEAAAKEAAAKA
40	AEAAAKEAAAKEAAAKA
41	AEAAAKEAAAKEAAAKEAAAKA
42	AEAAAKEAAAKEAAAKEAAAKEAAAKA
43	AEAAAKEAAAKEAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKA
44	PAPAP

45	KESGSVSSEQLAQFRSLD
46	GSAGSAAGSGEF
47	GGGSE
48	GSESG
49	GSEGS
50	GEGGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGS

In embodiments, the joining linker substantially comprises 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 $(Gly_4Ser)_n$, where n is from about 1 to about 8, *e.g.,* 1, 2, 3, 4, 5, 6, 7, or 8 (SEQ ID NO: 25 to SEQ ID NO: 32, respectively). In embodiments, the joining linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO: 33). Additional illustrative joining linkers include, but are not limited to, linkers having the sequence LE, $(EAAAK)_n$ (n=1-3) (SEQ ID NO: 36 to SEQ ID NO: 38), $A(EAAAK)_nA$ (n = 2-5) (SEQ ID NO: 39 to SEQ ID NO: 42), $A(EAAAK)_4ALEA(EAAAK)_4A$ (SEQ ID NO: 43), PAPAP (SEQ ID NO: 44), KESGSVSSEQLAQFRSLD (SEQ ID NO: 45), GSAGSAAGSGEF (SEQ ID NO: 46), and $(XP)_n$, with X designating any amino acid, *e.g.,* Ala, Lys, or Glu. In embodiments, the joining linker is GGS. In embodiments, a joining linker has the sequence $(Gly)_n$ where n is any number from 1 to 100, for example: $(Gly)_8$ (SEQ ID NO: 34) and $(Gly)_6$ (SEQ ID NO: 35).

In embodiments, the joining linker is one or more of GGGSE (SEQ ID NO: 47), GSESG (SEQ ID NO: 48), GSEGS (SEQ ID NO: 49), GEGGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGS (SEQ ID NO: 50), and a joining linker of randomly placed G, S, and E every 4 amino acid intervals.

In embodiments, where a heterologous chimeric protein used in a method of the present invention comprises an extracellular domain (ECD) of a first transmembrane protein, one joining linker preceding an Fc domain, a second joining linker following the Fc domain, and an ECD of second transmembrane protein, the heterologous chimeric protein may comprise the following structure:

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

The combination of a first joining linker, an Fc Domain linker, and a second joining linker is referend to herein as a "modular linker". In embodiments, a heterologous chimeric protein used in a method of the present invention comprises a modular linker as shown in **Table 2:**

Table 2: Illustrative modular linkers

Joining Linker 1	Fc	Joining Linker 2	Modular Linker = Joining Linker 1 + Fc + Joining Linker 2
SKYGPPCPSCP (SEQ ID NO: 4)	APEFLGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSQEDPEVQFN WYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPRE PQVYTLPPSQEEMTKNQVSLTCL	IEGRMD (SEQ ID NO: 7)	SKYGPPCPSCPAPEFLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAK TKPREEQFNSTYRVVSVLTVLHQ DWLSGKEYKCKVSSKGLPSSIEK TISNATGQPREPQVYTLPPSQEE

	VKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKS SWQEGNVFSCSVMHEALHNHYT QKSLSLSLGK (SEQ ID NO: 1)		MTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDG SFFLYSRLTVDKSSWQEGNVFSC SVMHEALHNHYTQKSLSLSLGKIE GRMD (SEQ ID NO: 51)
SKYGPPCPSCP (SEQ ID NO: 4)	APEFLGGPSVFLFPPKPKDQLMIS RTPEVTCVVVDVSQEDPEVQFN WYVDGVEVHNAKTKPREEQFNS TYRVVSVLTTPHSDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPRE PQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKS SWQEGNVFSCSVLHEALHNHYT QKSLSLSLGK (SEQ ID NO: 2)	IEGRMD (SEQ ID NO: 7)	SKYGPPCPSCPAPEFLGGPSVFL FPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNA KTKPREEQFNSTYRVVSVLTTPH SDWLSGKEYKCKVSSKGLPSSIE KTISNATGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSD GSFFLYSRLTVDKSSWQEGNVFS CSVLHEALHNHYTQKSLSLSLGKI EGRMD (SEQ ID NO: 52)
SKYGPPCPSCP (SEQ ID NO: 4)	APEFLGGPSVFLFPPKPKDQLMIS RTPEVTCVVVDVSQEDPEVQFN WYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPRE PQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKS RWQEGNVFSCSVLHEALHNHYT QKSLSLSLGK (SEQ ID NO: 3)	IEGRMD (SEQ ID NO: 7)	SKYGPPCPSCPAPEFLGGPSVFL FPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNA KTKPREEQFNSTYRVVSVLTVLH QDWLSGKEYKCKVSSKGLPSSIE KTISNATGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSD GSFFLYSRLTVDKSRWQEGNVFS CSVLHEALHNHYTQKSLSLSLGKI EGRMD (SEQ ID NO: 53)
SKYGPPCPPCP (SEQ ID NO: 5)	APEFLGGPSVFLFPPKPKDTLMIS RTPEVTCVVVDVSQEDPEVQFN WYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPRE PQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKS SWQEGNVFSCSVMHEALHNHYT QKSLSLSLGK (SEQ ID NO: 1)	IEGRMD (SEQ ID NO: 7)	SKYGPPCPPCPAPEFLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAK TKPREEQFNSTYRVVSVLTVLHQ DWLSGKEYKCKVSSKGLPSSIEK TISNATGQPREPQVYTLPPSQEE MTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDG SFFLYSRLTVDKSSWQEGNVFSC SVMHEALHNHYTQKSLSLSLGKIE GRMD (SEQ ID NO: 54)
SKYGPPCPPCP (SEQ ID NO: 5)	APEFLGGPSVFLFPPKPKDQLMIS RTPEVTCVVVDVSQEDPEVQFN WYVDGVEVHNAKTKPREEQFNS TYRVVSVLTTPHSDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPRE PQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKS SWQEGNVFSCSVLHEALHNHYT QKSLSLSLGK (SEQ ID NO: 2)	IEGRMD (SEQ ID NO: 7)	SKYGPPCPPCPAPEFLGGPSVFL FPPKPKDQLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNA KTKPREEQFNSTYRVVSVLTTPH SDWLSGKEYKCKVSSKGLPSSIE KTISNATGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSD GSFFLYSRLTVDKSSWQEGNVFS CSVLHEALHNHYTQKSLSLSLGKI EGRMD (SEQ ID NO: 55)
SKYGPPCPPCP (SEQ ID NO: 5)	APEFLGGPSVFLFPPKPKDQLMIS RTPEVTCVVVDVSQEDPEVQFN	IEGRMD (SEQ ID NO: 7)	SKYGPPCPPCPAPEFLGGPSVFL FPPKPKDQLMISRTPEVTCVVVD

	WYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLSGKEYKC KVSSKGLPSSIEKTISNATGQPRE PQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNY KTTPPVLDSDGSFFLYSRLTVDKS RWQEGNVFSCSVLHEALHNHYT QKSLSLSLGK (SEQ ID NO: 3)		VSQEDPEVQFNWYVDGVEVHNA KTKPREEQFNSTYRVVSVLTVLH QDWLSGKEYKCKVSSKGLPSSIE KTISNATGQPREPQVYTLPPSQE EMTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSD GSFFLYSRLTVDKSRWQEGNVFS CSVLHEALHNHYTQKSLSLSLGKI EGRMD (SEQ ID NO: 56)

In embodiments, the heterologous chimeric proteins used in methods of the present invention may comprise variants of the modular linkers disclosed in **Table 2**, above. For instance, a linker may have 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 any one of SEQ ID NO: 51 to SEQ ID NO: 56.

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. Characteristics of illustrative joining linkers is shown below in **Table 3**:

Table 3: Characteristics of illustrative joining linkers

Joining Linker Sequence	Characteristics
SKYGPPCPPCP (SEQ ID NO: 5)	IgG4 Hinge Region
IEGRMD (SEQ ID NO: 7)	Linker
GGGVPRDCG (SEQ ID NO: 8)	Flexible
GGGSGGGS (SEQ ID NO: 10)	Flexible
GGGSGGGGSGGG (SEQ ID NO: 11)	Flexible
EGKSSGSGSESKST (SEQ ID NO: 12)	Flexible + soluble
GGSG (SEQ ID NO: 13)	Flexible
GGSGGGSGGGSG (SEQ ID NO: 14)	Flexible
EAAAKEAAAKEAAAK (SEQ ID NO: 15)	Rigid Alpha Helix
EAAAREAAAREAAAREAAAR (SEQ ID NO: 16)	Rigid Alpha Helix
GGGGSGGGGSGGGGSAS (SEQ ID NO: 17)	Flexible
GGGGAGGGG (SEQ ID NO: 18)	Flexible
GS (SEQ ID NO: 19)	Highly flexible

Joining Linker Sequence	Characteristics
GSGSGS (SEQ ID NO: 20)	Highly flexible
GSGSGSGSGS (SEQ ID NO: 21)	Highly flexible
GGGGSAS (SEQ ID NO: 22)	Flexible
APAPAPAPAPAPAPAPAPAP (SEQ ID NO: 23)	Rigid

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 heterologous chimeric protein used in a method of the present invention. In another example, the linker may function to target the heterologous chimeric protein to a particular cell type or location.

In embodiments, a heterologous chimeric protein used in a method of the present invention comprises only one joining linkers.

In embodiments, a heterologous chimeric protein used in a method of the present invention lacks joining linkers.

In embodiments, the linker is a synthetic linker such as polyethylene glycol (PEG).

In embodiments, a heterologous chimeric protein has a first domain which is sterically capable of binding its ligand/receptor and/or the second domain which is sterically capable of binding its ligand/receptor. Thus, there is enough overall flexibility in the heterologous chimeric protein and/or physical distance between an extracellular domain (or portion thereof) and the rest of the heterologous chimeric protein such that the ligand/receptor binding domain of the extracellular domain is not sterically hindered from binding its ligand/receptor. This flexibility and/or physical distance (which is referred to as "slack") may be normally present in the extracellular domain(s), normally present in the linker, and/or normally present in the heterologous chimeric protein (as a whole). Alternately, or additionally, an amino acid sequence (for example) may be added to one or more extracellular domains and/or to the linker to provide the slack needed to avoid steric hindrance. Any amino acid sequence that provides slack may be added. In embodiments, the added amino acid sequence comprises the sequence $(Gly)_n$ where n is any number from 1 to 100. Additional examples of addable amino acid sequence include the joining linkers described in **Table 1** and **Table 3**. In embodiments, a polyethylene glycol (PEG) linker may be added between an extracellular domain and a linker to provide the slack needed to avoid steric hindrance. Such PEG linkers are well known in the art.

In embodiments, a heterologous chimeric protein comprises a first domain comprising a portion of PD-1, a second domain comprising a portion of GITRL, and a linker. In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence. In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain, *e.g.,* from an IgG1 or from IgG4, including human IgG1 or IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3. Thus, in embodiments, when a heterologous chimeric

protein used in a method of the present invention comprises the extracellular domain of PD-1 (or a variant thereof), a linker comprising a hinge-CH2-CH3 Fc domain, and the extracellular domain of GITRL (or a variant thereof), it may be referred to herein as "PD-1-Fc-GITRL".

In embodiments, a PD-1-Fc-GITRL heterologous chimeric protein of the present invention and/or a PD-1-Fc-GITRL heterologous chimeric protein used in methods of the present invention comprises: (1) a first domain comprising the amino acid sequence of SEQ ID NO: 57, (b) a second domain comprises the amino acid sequence of SEQ ID NO: 58, and (c) a linker comprises an amino acid sequence that is at least 95% identical to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, a PD-1-Fc-GITRL heterologous chimeric protein of the present invention and/or a PD-1-Fc-GITRL heterologous chimeric protein used in methods of the present invention has the following amino acid sequence:

LDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ
PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPT
AHPSPSPRPAGQFQSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED
PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEK
TISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
SDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKIEGRMDQLETAKEPCMA
KFGPLPSKWQMASSEPPCVNKVSDWKLEILQNGLYLIYGQVAPNANYNDVAPFEVRLYKNKDMIQ
TLTNKSKIQNVGGTYELHVGDTIDLIFNSEHQVLKNNTYWGIILLANPQFIS (SEQ ID NO: 62).

In embodiments, a heterologous chimeric protein of the present invention and/or a heterologous chimeric protein used in methods of the present invention comprises a variant of a PD-1-Fc-GITRL heterologous chimeric protein. As examples, the variant may have 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 SEQ ID NO: 62.

In embodiments, a heterologous chimeric protein comprises a first domain comprising a portion of PD-1, a second domain comprising a portion of CD40L, and a linker. In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence. In embodiments, the linker comprises at least

one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain, e.g., from an IgG1 or from IgG4, including human IgG1 or IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3. Thus, in embodiments, when a heterologous chimeric protein used in a method of the present invention comprises the extracellular domain of PD-1 (or a variant thereof), a linker comprising a hinge-CH2-CH3 Fc domain, and the extracellular domain of CD40L (or a variant thereof), it may be referred to herein as "PD-1-Fc-4-CD40L".

In embodiments, a PD-1-Fc-CD40L heterologous chimeric protein of the present invention and/or a PD-1-Fc-CD40L heterologous chimeric protein used in methods of the present invention comprises: (1) a first domain comprising the amino acid sequence of SEQ ID NO: 57, (b) a second domain comprises the amino acid sequence of SEQ ID NO: 60, and (c) a linker comprises an amino acid sequence that is at least 95% identical to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, a PD-1-Fc-CD40L heterologous chimeric protein of the present invention and/or a PD-1-Fc-CD40L heterologous chimeric protein used in methods of the present invention has the following amino acid sequence:

LDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ
PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPT
AHPSPSPRPAGQFQSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED
PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEK
TISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
SDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKIEGRMDHRRLDKIEDER
NLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKGDQNPQIA
AHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSNREASSQAP
FIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSQVSHGTGFT
SFGLLKL (SEQ ID NO: 63).

In embodiments, a heterologous chimeric protein of the present invention and/or a heterologous chimeric protein used in methods of the present invention comprises a variant of a PD-1-Fc-CD40L heterologous chimeric protein. As examples, the variant may have 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 SEQ ID NO: 63.

In embodiments, a heterologous chimeric protein comprises a first domain comprising a portion of PD-1, a second domain comprising a portion of 4-1BBL, and a linker. In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence. In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain, e.g., from an IgG1 or from IgG4, including human IgG1 or IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3. Thus, in embodiments, when a heterologous chimeric protein used in a method of the present invention comprises the extracellular domain of PD-1 (or a variant thereof), a linker comprising a hinge-CH2-CH3 Fc domain, and the extracellular domain of CD40L (or a variant thereof), it may be referred to herein as "PD-1-Fc-4-1BBL".

In embodiments, a PD-1-Fc-4-1BBL heterologous chimeric protein of the present invention and/or a PD-1-Fc-4-1BBL heterologous chimeric protein used in methods of the present invention comprises: (1) a first domain comprising the amino acid sequence of SEQ ID NO: 57, (b) a second domain comprises the amino acid sequence of SEQ ID NO: 61, and (c) a linker comprises an amino acid sequence that is at least 95% identical to SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, a PD-1-Fc-4-1BBL heterologous chimeric protein of the present invention and/or a PD-1-Fc-4-1BBL heterologous chimeric protein used in methods of the present invention has the following amino acid sequence:

LDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQ
PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPT
AHPSPSPRPAGQFQSKYGPPCPPCPAPEFLGGPSVFLFPPKPKDQLMISRTPEVTCVVVDVSQED
PEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLSGKEYKCKVSSKGLPSSIEK
TISNATGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
SDGSFFLYSRLTVDKSRWQEGNVFSCSVLHEALHNHYTQKSLSLSLGKIEGRMDACPWAVSGAR
ASPGSAASPRLREGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTGGL
SYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASS
EARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSPRSE
(SEQ ID NO: 64).

In embodiments, a heterologous chimeric protein of the present invention and/or a heterologous chimeric protein used in methods of the present invention comprises a variant of a PD-1-Fc-4-1BBL heterologous chimeric protein. As examples, the variant may have 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 SEQ ID NO: 64.

Diseases, Methods of Treatment, and Mechanisms of Action

The methods comprise steps of administering to a subject in need thereof (either simultaneously or sequentially) an effective amount of at least one antibody directed to an immune checkpoint molecule, e.g., CTLA-4, a stimulator of interferon genes (STING) agonist, and/or one or more heterologous chimeric proteins, in which each heterologous chimeric protein is capable of blocking immune inhibitory signals and/or stimulating immune activating signals.

It is often desirable to disrupt, block, reduce, inhibit, and/or sequester the transmission of immune inhibitory signals and, simultaneously or contemporaneously, enhance, increase, and/or stimulate the transmission of an immune stimulatory signal to an anti-cancer immune cell, to boost an immune response, for instance to enhance a patient's anti-tumor immune response.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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 antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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 patient's T cells are activated and/or stimulated by the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention, with the activated T cells being capable of dividing and/or secreting cytokines.

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 hematogeneous 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; thus, the cancer may comprise cells that were originally skin, colon, breast, or prostate tissue, 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention treat a subject that has a treatment-refractory cancer. In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention treat a subject that is refractory to one or more immune-modulating agents. For example, in embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and/or MPDL328OA (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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention. 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention targets a cancer cell. In embodiments, the cancer cell expresses one or more of targets or binding partners of the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention.

In embodiments, the present methods provide treatment with the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins in a patient who is refractory to an additional agent, such

"additional agents" being disclosed elsewhere herein, inclusive, without limitation, of the various chemotherapeutic agents disclosed herein.

The activation of regulatory T cells is critically influenced by costimulatory and co-inhibitory 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 co-inhibitors and their receptors belong to the B7 and CD28 families.

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, LTbR, CD27, CD30, 4-1BB or TNFRSF25 using either receptor agonist antibodies or using a heterologous chimeric protein comprising the ligands for such receptors (OX40L, LIGHT, CD70, CD30L, 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+FoxP3+ 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+ or CD8+ 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 heterologous chimeric protein comprising 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.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins are capable of, or find use in methods involving, enhancing, restoring, promoting and/or stimulating immune modulation. In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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: pro-inflammatory cytokine production or T cell migration or T cell tumor infiltration.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention enhance recognition of tumor antigens by CD8+ T cells, particularly those T cells that have infiltrated into the tumor microenvironment. In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention induce CD19 expression and/or increases the number of CD19 positive cells (*e.g.,* CD19 positive B cells). In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention induce IL-15Rα expression and/or increases the number of IL-15Rα positive cells (*e.g.,* IL-15Rα positive dendritic cells).

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of, or find use in methods involving, inhibiting and/or causing a decrease in immunosuppressive cells (*e.g.,* myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor associated neutrophils (TANs), M2 macrophages, and tumor associated macrophages (TAMs)), and particularly within 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are able to increase the serum levels of various cytokines or chemokines including, but not limited to, one or more of IFNγ, TNFα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17A, IL-17F, IL-22, CCL2, CCL3, CCL4, CXCL8, CXCL9, CXCL10, CXCL11 and CXCL12. In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of enhancing IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-22, TNFα or IFNγ in the serum of a treated subject. In embodiments, administration of the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention is capable of enhancing TNFα secretion. In a specific embodiment, administration of the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention is capable

of enhancing superantigen mediated TNFα secretion by leukocytes. Detection of such a cytokine response may provide a method to determine the optimal dosing regimen for the indicated antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention.

The antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of increasing or preventing a decrease in a sub-population of CD4+ and/or CD8+ T cells.

The antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of enhancing tumor-killing activity by T cells.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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, inflammatory diseases, 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. 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 refer to receptors expressed on immune cells that prevent or inhibit uncontrolled immune responses. In contrast, an anti-tumor CD8+ and/or CD4+ T cell refers to T cells that can mount an immune response to a tumor.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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.,* αβ TCR, $CD3^+$, $CD8^+$, $CD45RO^+$); $CD4^+$ effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$, $CCR7^+$, CD62Lhi, $IL\text{-}7R/CD127^+$); $CD8^+$ effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD8^+$, $CCR7^+$, CD62Lhi, $IL\text{-}7R/CD127^+$); effector memory T cells (*e.g.,* CD62Llow, $CD44^+$, TCR, $CD3^+$, $IL\text{-}7R/CD127^+$, $IL\text{-}15R^+$, CCR7low); central memory T cells (*e.g.,* $CCR7^+$, $CD62L^+$, $CD27^+$; or CCR7hi, $CD44^+$, CD62Lhi, TCR, $CD3^+$, IL-7R/$CD127^+$, $IL\text{-}15R^+$); $CD62L^+$ 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 αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}12R^+$, $IFN\gamma R^+$, $CXCR3^+$), TH2 effector T cells (*e.g.,* $CCR3^+$, $CCR4^+$ and $CCR8^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}4R^+$, $IL\text{-}33R^+$, $CCR4^+$, $IL\text{-}17RB^+$, $CRTH2^+$); TH9 effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$); TH17 effector T cells (*e.g.,* αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}23R^+$, $CCR6^+$, $IL\text{-}1R^+$);

$CD4^+CD45RO^+CCR7^+$ effector T cells, $CD4^+CD45RO^+CCR7(^-)$ effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN-γ. Illustrative regulatory T cells include $ICOS^+$ regulatory T cells, $CD4^+CD25^+FOXP3^+$ regulatory T cells, $CD4^+CD25^+$ regulatory T cells, $CD4^+CD25^-$ regulatory T cells, CD4+CD25high regulatory T cells, $TIM\text{-}3^+PD\text{-}1^+$ regulatory T cells, lymphocyte activation gene-3 (LAG-3)$^+$ regulatory T cells, CTLA-4/$CD152^+$ regulatory T cells, neuropilin-1 (Nrp-1)$^+$ regulatory T cells, $CCR4^+CCR8^+$ regulatory T cells, CD62L (L-selectin)$^+$ regulatory T cells, CD45RBlow regulatory T cells, CD127low regulatory T cells, LRRC32/$GARP^+$ regulatory T cells, $CD39^+$ regulatory T cells, $GITR^+$ regulatory T cells, LAP^+ regulatory T cells, $1B11^+$ regulatory T cells, $BTLA^+$ 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), $CD8^+$ regulatory T cells, $CD8^+CD28^-$ regulatory T cells and/or regulatory T-cells secreting IL-10, IL-35, TGF-β, TNF-α, Galectin-1, IFN-γ and/or MCP1.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention cause an increase in effector T cells (*e.g.,* CD4+CD25- T cells).

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention cause a decrease in regulatory T cells (*e.g.,* CD4+CD25+ T cells).

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention generate a memory response which may 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention is later able to attack tumor cells and/or prevent development of tumors when rechallenged after an initial treatment with the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention. Accordingly, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention stimulate 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 antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of causing activation of antigen presenting cells. In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable enhancing the ability of antigen presenting cells to present antigen.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of, and can be used in methods comprising, transiently stimulating effector T cells for 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 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.

The heterologous chimeric proteins used in methods of the present invention unexpectedly provide binding of the extracellular domain components to their respective binding partners with slow off rates (Kd 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 longer positive signal effect, *e.g.,* increase in or activation of immune stimulatory signals. For example, the heterologous chimeric proteins used in methods of the present invention, *e.g., via* the long off rate binding allows sufficient signal transmission to provide immune cell proliferation, allow for anti-tumor attack, allows sufficient signal transmission to provide release of stimulatory signals, *e.g.,* cytokines.

The heterologous chimeric proteins used in methods of the present invention are capable of forming a stable synapse between cells. The stable synapse of cells promoted by the heterologous chimeric proteins (*e.g.,* between cells bearing negative signals) 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 heterologous chimeric proteins. 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 heterologous chimeric proteins. Such properties could have the combined advantage of reducing off-target toxicities associated with systemic distribution of the heterologous chimeric proteins.

In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention are capable of providing a sustained immunomodulatory effect.

The antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention provide synergistic therapeutic effects (*e.g.,* anti-tumor effects) as it allows for improved site-specific interplay of two immunotherapy agents. In embodiments, the antibodies directed to immune checkpoint molecules, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention provide the potential for reducing off-site and/or systemic toxicity.

In embodiments, the heterologous chimeric proteins used in methods of the present invention exhibit enhanced safety profiles. In embodiment, the heterologous chimeric proteins used in methods of the present invention exhibit reduced

toxicity profiles. For example, administration of the heterologous chimeric proteins used in methods of the present invention may result in reduced side effects such as one or more of diarrhea, inflammation (*e.g.,* of the gut), or weight loss, which occur following administration of antibodies directed to the ligand(s)/receptor(s) targeted by the extracellular domains of the heterologous chimeric proteins used in methods of the present invention used in methods of the present invention. In embodiments, the heterologous chimeric proteins used in methods of the present invention provides improved safety, as compared to antibodies directed to the ligand(s)/receptor(s) targeted by the extracellular domains of the heterologous chimeric proteins used in methods of the present invention used in methods of the present invention, yet, without sacrificing efficacy.

In embodiments, the heterologous chimeric proteins used in methods of the present invention provide reduced side effects, *e.g.,* GI complications, relative to current immunotherapies, *e.g.,* antibodies directed to ligand(s)/receptor(s) targeted by the extracellular domains of the heterologous chimeric proteins used in methods of the present invention used in methods of the present invention. 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.

Methods of treatment

An aspect of the present invention provides a method for treating a cancer in a subject in need thereof. The first pharmaceutical composition comprises an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). The second pharmaceutical composition comprising an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

In embodiments, the first pharmaceutical composition and the second pharmaceutical composition are provided simultaneously, the first pharmaceutical composition is provided after the second pharmaceutical composition is

provided, or the first pharmaceutical composition is provided before the second pharmaceutical composition is provided.

In embodiments, the dose of the first pharmaceutical composition is less than the dose of the first pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the second pharmaceutical composition.

In embodiments, the dose of the second pharmaceutical composition provided is less than the dose of the second pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the first pharmaceutical composition.

In embodiments, the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the first pharmaceutical composition.

In embodiments, the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment the second pharmaceutical composition.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or human IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, the heterologous chimeric protein comprises: (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of GITRL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; (a) a first

domain comprising a portion of PD-1, (b) a second domain comprising a portion of 4-1BBL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; or (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of CD40L, and (c) a linker comprising a hinge-CH2-CH3 Fc domain.

In embodiments, the antibody that is capable of binding CTLA-4 is selected from the group consisting of YERVOY (ipilimumab), 9D9, tremelimumab (formerly ticilimumab, CP-675,206; MedImmune), AGEN1884, and RG2077.

In embodiments, the cancer is or is related 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 subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment. In embodiments, the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

Another aspect of the present invention provides a method for treating a cancer in a subject. The method comprising a step of providing the subject a pharmaceutical composition comprising an immunotherapy. The immunotherapy is selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular

domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain. In this aspect, the subject has undergone or is undergoing treatment with an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).

In embodiments, the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with an antibody that is capable of binding CTLA-4.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, the heterologous chimeric protein comprises: (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of GITRL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of 4-1BBL, and (c) a linker comprising

a hinge-CH2-CH3 Fc domain; or (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of CD40L, and (c) a linker comprising a hinge-CH2-CH3 Fc domain.

In embodiments, the antibody that is capable of binding CTLA-4 is selected from the group consisting of YERVOY (ipilimumab), 9D9, tremelimumab (formerly ticilimumab, CP-675,206; MedImmune), AGEN1884, and RG2077.

In embodiments, the cancer is or is related 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 subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment. In embodiments, the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

Yet another aspect of the present invention provides a method for treating a cancer in a subject. The method comprising a step of providing the subject a pharmaceutical composition comprising an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). In this aspect, the subject has undergone or is undergoing treatment with an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a

portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

In embodiments, the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with the immunotherapy.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or human IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, the heterologous chimeric protein comprises: (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of GITRL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of 4-1BBL, and (c) a linker comprising

a hinge-CH2-CH3 Fc domain; or (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of CD40L, and (c) a linker comprising a hinge-CH2-CH3 Fc domain.

In embodiments, the antibody that is capable of binding CTLA-4 is selected from the group consisting of YERVOY (ipilimumab), 9D9, tremelimumab (formerly ticilimumab, CP-675,206; MedImmune), AGEN1884, and RG2077.

In embodiments, the cancer is or is related 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 subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment. In embodiments, the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

In an aspect, the present invention provides a method for treating a cancer in a subject in need thereof. The method comprising steps of providing the subject a first pharmaceutical composition and a second pharmaceutical composition. The first pharmaceutical composition comprises a stimulator of interferon genes (STING) agonist. The second pharmaceutical composition comprising an immunotherapy selected from: (i) a heterologous chimeric protein

comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

In embodiments, the first pharmaceutical composition and the second pharmaceutical composition are provided simultaneously, the first pharmaceutical composition is provided after the second pharmaceutical composition is provided, or the first pharmaceutical composition is provided before the second pharmaceutical composition is provided.

In embodiments, the dose of the first pharmaceutical composition is less than the dose of the first pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the second pharmaceutical composition.

In embodiments, the dose of the second pharmaceutical composition provided is less than the dose of the second pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the first pharmaceutical composition.

In embodiments, the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the first pharmaceutical composition.

In embodiments, the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the second pharmaceutical composition.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or human IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, the heterologous chimeric protein comprises: (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of GITRL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of 4-1BBL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; or (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of CD40L, and (c) a linker comprising a hinge-CH2-CH3 Fc domain.

In embodiments, the STING agonist is selected from the group consisting of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), MIW815(ADU-S100), CRD5500 or MK-1454 and any STING agonist described in US20140341976, US20180028553, US20180230178, US9549944, WO2017106740, WO2018045204, or WO2018098203, the contents of which are incorporated herein by reference in their entireties.

In embodiments, the cancer is or is related 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 subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment. In embodiments, the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

In another aspect, the present invention provides a method for treating a cancer in a subject. The method comprising a step of providing the subject a pharmaceutical composition comprising an immunotherapy. The immunotherapy is selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain. In this aspect, the subject has undergone or is undergoing treatment with a stimulator of interferon genes (STING) agonist.

In embodiments, the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with a STING agonist.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, the heterologous chimeric protein comprises: (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of GITRL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of 4-1BBL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; or (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of CD40L, and (c) a linker comprising a hinge-CH2-CH3 Fc domain.

In embodiments, the STING agonist is selected from the group consisting of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), MIW815(ADU-S100), CRD5500 or MK-1454 and any STING agonist described in US20140341976, US20180028553, US20180230178, US9549944, WO2017106740, WO2018045204, or WO2018098203, the contents of which are incorporated herein by reference in their entireties.

In embodiments, the cancer is or is related 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 subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment. In embodiments, the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

In yet another aspect, the present invention provides a method for treating a cancer in a subject. The method comprises a step of providing the subject a pharmaceutical composition comprising a stimulator of interferon genes (STING) agonist. In this aspect, the subject has undergone or is undergoing treatment with an immunotherapy selected from: (i) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and (c) a linker linking the first domain and the second domain; (ii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and (c) a linker linking the first domain and the second domain; and (iii) a heterologous chimeric protein comprising: (a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand, (b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and (c) a linker linking the first domain and the second domain.

In embodiments, the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with the immunotherapy.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

4 In embodiments, the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

In embodiments, the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

In embodiments, the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain. In embodiments, the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or human IgG4. In embodiments, the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

In embodiments, the heterologous chimeric protein comprises: (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of GITRL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of 4-1BBL, and (c) a linker comprising a hinge-CH2-CH3 Fc domain; or (a) a first domain comprising a portion of PD-1, (b) a second domain comprising a portion of CD40L, and (c) a linker comprising a hinge-CH2-CH3 Fc domain.

In embodiments, the STING agonist is selected from the group consisting of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), MIW815(ADU-S100), CRD5500 or MK-1454 and any STING agonist described in US20140341976, US20180028553, US20180230178, US9549944, WO2017106740, WO2018045204, or WO2018098203, the contents of which are incorporated herein by reference in their entireties.

In embodiments, the cancer is or is related 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 subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment. In embodiments, the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

In addition to the above aspects and embodiments, the present invention further comprises combining the above methods with one or more other anti-cancer therapies.

The other anti-cancer therapy may comprise radiotherapy.

The other anti-cancer therapy may be surgery to excise the cancer, *i.e.,* tumor.

The other anti-cancer therapy may include a cell-based immuno-oncology therapy, *e.g.,* chimeric antigen receptor T cell (CAR-T), including wherein the CAR-T secretes the chimeric protein either continuously or in response to specific tumor antigen recognition.

The other anti-cancer therapy may include administration of one more chemotherapeutic agents.

In aspects and embodiments of the present invention, the one or more chemotherapeutic agent selected from 5-FU (Fluorouracil), Abemaciclib, Abiraterone Acetate, Abitrexate (Methotrexate), Abraxane (Paclitaxel Albumin-stabilized Nanoparticle Formulation), ABVD, ABVE, ABVE-PC, AC, Acalabrutinib, AC-T, ADE, Adriamycin (Doxorubicin), Afatinib Dimaleate, Afinitor (Everolimus), Afinitor Difsperz (Everolimus), Akynzeo (Netupitant and Palonosetron), Aldara (Imiquimod), Aldesleukin, Alecensa (Alectinib), Alectinib, Alimta (PEMETREXED), Aliqopa (Copanlisib Hydrochloride), Alkeran (Melphalan), Aloxi (Palonosetron Hydrochloride), Alunbrig (Brigatinib), Ambochlorin (Chlorambucil), Amboclorin (Chlorambucil), Amifostine, Aminolevulinic Acid, Anastrozole, Aprepitant, Aredia (Pamidronate), Arimidex (Anastrozole), Aromasin (Exemestane), Arranon (Nelarabine), Arsenic Trioxide, Asparaginase Erwinia chrysanthemi, Axicabtagene Ciloleucel, Axitinib, Azacitidine, BEACOPP, Becenum (Carmustine), Beleodaq (Belinostat), Belinostat, Bendamustine Hydrochloride, BEP, Bexarotene, Bicalutamide, BiCNU (Carmustine), Blenoxane (Bleomycin),

Bortezomib, Bosulif (Bosutinib), Bosutinib, Brigatinib, BuMel, Busulfan, Busulfex (Busulfan)C, Cabazitaxel, Cabometyx (Cabozantinib), Cabozantinib-S-Malate, CAF, Calquence (Acalabrutinib), Camptosar (Irinotecan Hydrochloride), Capecitabine, CAPOX, Caprelsa (Vandetanib), Carac (Fluorouracil--Topical), Carboplatin, CARBOPLATIN-TAXOL, Carfilzomib, Carmubris (Carmustine), Carmustine, Casodex (Bicalutamide), CeeNU (Lomustine), CEM, Ceritinib, Cerubidine (Daunorubicin), Cervarix (Recombinant HPV Bivalent Vaccine), CEV, Chlorambucil, CHLORAMBUCIL-PREDNISONE, CHOP, Cisplatin, Cladribine, Clafen (Cyclophosphamide), Clofarabine, Clofarex (Clofarabine), Clolar (Clofarabine), CMF, Cobimetinib, Cometriq (Cabozantinib), Copanlisib Hydrochloride, COPDAC, COPP, COPP-ABV, Cosmegen (Dactinomycin), Cotellic (Cobimetinib), Crizotinib, CVP, Cyclophosphamide, Cyfos (Ifosfamide), Cytarabine, Cytarabine Liposome, Cytosar-U (Cytarabine), Cytoxan (Cyclophosphamide), Cytoxan (Cytoxan), Dabrafenib, Dacarbazine, Dacogen (Decitabine), Dactinomycin, Dasatinib, Daunorubicin Hydrochloride, Daunorubicin Hydrochloride and Cytarabine Liposome, DaunoXome (Daunorubicin Lipid Complex), Decadron (Dexamethasone), Decitabine, Defibrotide Sodium, Defitelio (Defibrotide Sodium), Degarelix, Denileukin Diftitox, DepoCyt (Cytarabine Liposome), Dexamethasone, Dexamethasone Intensol (Dexamethasone), Dexpak Taperpak (Dexamethasone), Dexrazoxane Hydrochloride, Docefrez (Docetaxel), Docetaxel, Doxil (Doxorubicin Hydrochloride Liposome), Doxorubicin Hydrochloride, Doxorubicin Hydrochloride Liposome, Dox-SL (Doxorubicin Hydrochloride Liposome), Droxia (Hydroxyurea), DTIC (Decarbazine), DTIC-Dome (Dacarbazine), Efudex (Fluorouracil--Topical), Eligard (Leuprolide), Elitek (Rasburicase), Ellence (Ellence (epirubicin)), Eloxatin (Oxaliplatin), Elspar (Asparaginase), Eltrombopag Olamine, Emcyt (Estramustine), Emend (Aprepitant), Enasidenib Mesylate, Enzalutamide, Epirubicin Hydrochloride, EPOCH, Eribulin Mesylate, Erivedge (Vismodegib), Erlotinib Hydrochloride, Erwinaze (Asparaginase Erwinia chrysanthemi), Ethyol (Amifostine), Etopophos (Etoposide Phosphate), Etoposide, Etoposide Phosphate, Eulexin (Flutamide), Evacet (Doxorubicin Hydrochloride Liposome), Everolimus, Evista (Raloxifene Hydrochloride), Evomela (Melphalan Hydrochloride), Exemestane, Fareston (Toremifene), Farydak (Panobinostat), Faslodex (Fulvestrant), FEC, Femara (Letrozole), Filgrastim, Firmagon (Degarelix), FloPred (Prednisolone), Fludara (Fludarabine), Fludarabine Phosphate, Fluoroplex (Fluorouracil), Fluorouracil, Flutamide, Folex (Methotrexate), Folex PFS (Methotrexate), FOLFIRI, FOLFIRINOX, FOLFOX, Folotyn (Pralatrexate), FUDR (FUDR (floxuridine)), FU-LV, Fulvestrant, Gardasil (Recombinant HPV Quadrivalent Vaccine), Gardasil 9 (Recombinant HPV Nonavalent Vaccine), Gefitinib, Gemcitabine Hydrochloride, GEMCITABINE-CISPLATIN, GEMCITABINE-OXALIPLATIN, Gemzar (Gemcitabine), Gilotrif (Afatinib Dimaleate), Gilotrif (Afatinib), Gleevec (Imatinib Mesylate), Gliadel (Carmustine), Glucarpidase, Goserelin Acetate, Halaven (Eribulin Mesylate), Hemangeol (Propranolol Hydrochloride), Hexalen (Altretamine), HPV Bivalent Vaccine, Recombinant, HPV Nonavalent Vaccine, Recombinant, HPV Quadrivalent Vaccine, Recombinant, Hycamtin (Topotecan Hydrochloride), Hycamtin (Topotecan), Hydrea (Hydroxyurea), Hydroxyurea, Hyper-CVAD, Ibrance (Palbociclib), Ibrutinib, ICE, Iclusig (Ponatinib), Idamycin PFS (Idarubicin), Idarubicin Hydrochloride, Idelalisib, Idhifa (Enasidenib), Ifex (Ifosfamide), Ifosfamide, Ifosfamidum (Ifosfamide), Imatinib Mesylate, Imbruvica (Ibrutinib), Imiquimod, Imlygic (Talimogene Laherparepvec), Inlyta (Axitinib), Iressa

(Gefitinib), Irinotecan Hydrochloride, Irinotecan Hydrochloride Liposome, Istodax (Romidepsin), Ixabepilone, Ixazomib Citrate, Ixempra (Ixabepilone), Jakafi (Ruxolitinib Phosphate), Jakafi (Ruxolitinib), JEB, Jevtana (Cabazitaxel), Keoxifene (Raloxifene Hydrochloride), Kepivance (Palifermin), Kisqali (Ribociclib), Kyprolis (Carfilzomib), Lanreotide Acetate, Lanvima (Lenvatinib), Lapatinib Ditosylate, Lenalidomide, Lenvatinib Mesylate, Lenvima (Lenvatinib Mesylate), Letrozole, Leucovorin Calcium, Leukeran (Chlorambucil), Leukine (Sargramostim), Leuprolide Acetate, Leustatin (Cladribine), Levulan (Aminolevulinic Acid), Linfolizin (Chlorambucil), LipoDox (Doxorubicin Hydrochloride Liposome), Lomustine, Lonsurf (Trifluridine and Tipiracil), Lupron (Leuprolide), Lynparza (Olaparib), Lysodren (Mitotane), Marqibo (Vincristine Sulfate Liposome), Marqibo Kit (Vincristine Lipid Complex), Matulane (Procarbazine), Mechlorethamine Hydrochloride, Megace (Megestrol), Megestrol Acetate, Mekinist (Trametinib), Melphalan, Melphalan Hydrochloride, Mercaptopurine, Mesnex (Mesna), Metastron (Strontium-89 Chloride), Methazolastone (Temozolomide), Methotrexate, Methotrexate LPF (Methotrexate), Methylnaltrexone Bromide, Mexate (Methotrexate), Mexate-AQ (Methotrexate), Midostaurin, Mitomycin C, Mitoxantrone Hydrochloride, Mitozytrex (Mitomycin C), MOPP, Mostarina (Prednimustine), Mozobil (Plerixafor), Mustargen (Mechlorethamine), Mutamycin (Mitomycin), Myleran (Busulfan), Mylosar (Azacitidine), Nanoparticle Paclitaxel (Paclitaxel Albumin-stabilized Nanoparticle Formulation), Navelbine (Vinorelbine), Nelarabine, Neosar (Cyclophosphamide), Neratinib Maleate, Nerlynx (Neratinib), Netupitant and Palonosetron Hydrochloride, Neulasta (filgrastim), Neulasta (pegfilgrastim), Neupogen (filgrastim), Nexavar (Sorafenib), Nilandron (Nilutamide), Nilotinib, Nilutamide, Ninlaro (Ixazomib), Nipent (Pentostatin), Niraparib Tosylate Monohydrate, Nolvadex (Tamoxifen), Novantrone (Mitoxantrone), Nplate (Romiplostim), Odomzo (Sonidegib), OEPA, OFF, Olaparib, Omacetaxine Mepesuccinate, Oncaspar (Pegaspargase), Oncovin (Vincristine), Ondansetron Hydrochloride, Onivyde (Irinotecan Hydrochloride Liposome), Ontak (Denileukin Diftitox), Onxol (Paclitaxel), OPPA, Orapred (Prednisolone), Osimertinib, Oxaliplatin, Paclitaxel, Paclitaxel Albumin-stabilized Nanoparticle Formulation, PAD, Palbociclib, Palifermin, Palonosetron Hydrochloride, Palonosetron Hydrochloride and Netupitant, Pamidronate Disodium, Panobinostat, Panretin (Alitretinoin), Paraplat (Carboplatin), Pazopanib Hydrochloride, PCV, PEB, Pediapred (Prednisolone), Pegaspargase, Pegfilgrastim, Pemetrexed Disodium, Platinol (Cisplatin), PlatinolAQ (Cisplatin), Plerixafor, Pomalyst (Pomalidomide), Ponatinib Hydrochloride, Pralatrexate, Prednisone, Procarbazine Hydrochloride, Proleukin (Aldesleukin), Promacta (Eltrombopag Olamine), Propranolol Hydrochloride, Purinethol (Mercaptopurine), Purixan (Mercaptopurine), Radium 223 Dichloride, Raloxifene Hydrochloride, Rasburicase, R-CHOP, R-CVP, Reclast (Zoledronic acid), Recombinant Human Papillomavirus (HPV) Bivalent Vaccine, Recombinant Human Papillomavirus (HPV) Nonavalent Vaccine, Recombinant Human Papillomavirus (HPV) Quadrivalent Vaccine, Regorafenib, Relistor (Methylnaltrexone Bromide), R-EPOCH, Revlimid (Lenalidomide), Rheumatrex (Methotrexate), Ribociclib, R-ICE, Rolapitant Hydrochloride, Romidepsin, Romiplostim, Rubex (Doxorubicin), Rubidomycin (Daunorubicin Hydrochloride), Rubraca (Rucaparib), Rucaparib Camsylate, Ruxolitinib Phosphate, Rydapt (Midostaurin), Sandostatin (Octreotide), Sandostatin LAR Depot (Octreotide), Sclerosol Intrapleural Aerosol (Talc), Soltamox (Tamoxifen), Somatuline Depot (Lanreotide Acetate), Sonidegib, Sorafenib Tosylate, Sprycel (Dasatinib),

STANFORD V, Sterapred (Prednisone), Sterapred DS (Prednisone), Sterile Talc Powder (Talc), Steritalc (Talc), Sterecyst (Prednimustine), Stivarga (Regorafenib), Sunitinib Malate, Supprelin LA (Histrelin), Sutent (Sunitinib Malate), Sutent (Sunitinib), Synribo (Omacetaxine Mepesuccinate), Tabloid (Thioguanine), TAC, Tafinlar (Dabrafenib), Tagrisso (Osimertinib), Talc, Talimogene Laherparepvec, Tamoxifen Citrate, Tarabine PFS (Cytarabine), Tarceva (Erlotinib), Targretin (Bexarotene), Tasigna (Decarbazine), Tasigna (Nilotinib), Taxol (Paclitaxel), Taxotere (Docetaxel), Temodar (Temozolomide), Temozolomide, Temsirolimus, Tepadina (Thiotepa), Thalidomide, Thalomid (Thalidomide), TheraCys BCG (BCG), Thioguanine, Thioplex (Thiotepa), Thiotepa, TICE BCG (BCG), Tisagenlecleucel, Tolak (Fluorouracil--Topical), Toposar (Etoposide), Topotecan Hydrochloride, Toremifene, Torisel (Temsirolimus), Totect (Dexrazoxane Hydrochloride), TPF, Trabectedin, Trametinib, Treanda (Bendamustine hydrochloride), Trelstar (Triptorelin), Trexall (Methotrexate), Trifluridine and Tipiracil Hydrochloride, Trisenox (Arsenic trioxide), Tykerb (lapatinib), Uridine Triacetate, VAC, Valrubicin, Valstar (Valrubicin Intravesical), Valstar (Valrubicin), VAMP, Vandetanib, Vantas (Histrelin), Varubi (Rolapitant), VeIP, Velban (Vinblastine), Velcade (Bortezomib), Velsar (Vinblastine Sulfate), Vemurafenib, Venclexta (Venetoclax), Vepesid (Etoposide), Verzenio (Abemaciclib), Vesanoid (Tretinoin), Viadur (Leuprolide Acetate), Vidaza (Azacitidine), Vinblastine Sulfate, Vincasar PFS (Vincristine), Vincrex (Vincristine), Vincristine Sulfate, Vincristine Sulfate Liposome, Vinorelbine Tartrate, VIP, Vismodegib, Vistogard (Uridine Triacetate), Voraxaze (Glucarpidase), Vorinostat, Votrient (Pazopanib), Vumon (Teniposide), Vyxeos (Daunorubicin Hydrochloride and Cytarabine Liposome), W, Wellcovorin (Leucovorin Calcium), Wellcovorin IV (Leucovorin), Xalkori (Crizotinib), XELIRI, Xeloda (Capecitabine), XELOX, Xofigo (Radium 223 Dichloride), Xtandi (Enzalutamide), Yescarta (Axicabtagene Ciloleucel), Yondelis (Trabectedin), Zaltrap (Ziv-Aflibercept), Zanosar (Streptozocin), Zarxio (Filgrastim), Zejula (Niraparib), Zelboraf (Vemurafenib), Zinecard (Dexrazoxane Hydrochloride), Ziv-Aflibercept, Zofran (Ondansetron Hydrochloride), Zoladex (Goserelin), Zoledronic Acid, Zolinza (Vorinostat), Zometa (Zoledronic acid), Zortress (Everolimus), Zydelig (Idelalisib), Zykadia (Ceritinib), Zytiga (Abiraterone Acetate), and Zytiga (Abiraterone).

In embodiments, any antibody directed to immune checkpoint molecules, e.g., any antibody that is capable of binding CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention disclosed herein may be used in combination with any of the anti-cancer therapy disclosed herein.

In embodiments, any antibody directed to immune checkpoint molecules, e.g., any antibody that is capable of binding CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention disclosed herein acts synergistically when co-administered with another anti-cancer therapy (*e.g.,* radiotherapy and/or a chemotherapeutic agent); resulting in, for example, the other anti-cancer therapy is administered at doses that are lower than the doses commonly employed when the other anti-cancer therapy is are used as monotherapy. In embodiments, the heterologous chimeric protein, as disclosed herein, reduces the number of administrations of the co-administered anti-cancer therapy.

In aspects and embodiments of the present invention, a patient in need of a cancer treatment comprising an antibody

directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention, as disclosed herein, is or is predicted to be poorly responsive or is non-responsive to an immunotherapy, *e.g.,* an anti-cancer immunotherapy, as disclosed herein. Moreover, in embodiments, a patient in need of an anti-cancer agent, as disclosed herein, is or may is predicted to be poorly responsive or non-responsive to an immune checkpoint immunotherapy. The immune checkpoint molecule may be selected from PD-1, PD-L1, PD-L2, ICOS, ICOSL, and CTLA-4. Moreover, in embodiments, a patient in need of an anti-cancer agent, as disclosed herein, is or may is predicted to be poorly responsive or non-responsive to an therapy directed to one or more of epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (Her2), and CD20.

In embodiments, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) disclosed 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 antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, aand/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) disclosed 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 disclosed herein.

The antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or other anti-cancer therapy) disclosed 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.

In aspects and embodiments of the present invention, the patient in need of treatment for an inflammatory disease or disorder, has been treated with, is contemporaneously treated with, or is subsequently treated with another agent for treating an inflammatory disease or disorder. Examples of such other agents include a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent (NSAID), and/or an immunosuppressive drug.

Examples of a NSAID include salicylic acid, acetyl salicylic acid, methyl salicylate, glycol salicylate, salicylmides, benzyl-2,5-diacetoxybenzoic acid, ibuprofen, fulindac, naproxen, ketoprofen, etofenamate, phenylbutazone, and indomethacin.

Examples of a steroidal anti-inflammatory agents includes corticosteroids selected from 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, fluclorolone 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, clocortelone, clescinolone, dichlorisone, difluprednate, flucloronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate.

A steroidal anti-inflammatory agent may likewise have activity as an immunosuppressive drug.

Other examples of immunosuppressive drug include 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), inteferons, opioids, TNF binding proteins, mycophenolates, and small biological agents (*e.g.,* fingolimod, myriocin).

In embodiments, a patient in need of an agent for treating an autoimmune disease or disorder, has been treated with, is contemporaneously treated with, or is subsequently treated with a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent, and/or an immunosuppressive drug, as disclosed elsewhere herein.

In embodiments, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or other agent for treating an inflammatory disease or disorder) disclosed 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 tunicamycin, etc. Additionally, the derivative can contain one or more non-classical amino acids.

The antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or other agent for treating an inflammatory disease or disorder) disclosed 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.

Pharmaceutical composition

The methods of the present invention include administering pharmaceutical compositions comprising a therapeutically effective amount of, at least one, antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention, as disclosed herein.

The antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) disclosed 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 disclosed herein are in the form of a pharmaceutically acceptable salt.

Further, any antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) disclosed herein can be administered to a subject as a component of a composition, *e.g.,* pharmaceutical composition, that comprises a pharmaceutically acceptable carrier or vehicle. Such pharmaceutical 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 embodiments, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent disclosed 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 disclosed herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents.

In embodiments, the compositions, *e.g.,* pharmaceutical compositions, disclosed herein are resuspended in a saline buffer (including, without limitation TBS, PBS, and the like).

In embodiments, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention may by conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In embodiments, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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.

The present invention includes the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) in various formulations of pharmaceutical composition, which 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 embodiments, 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 pharmaceutical compositions comprising the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (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 pharmaceutical compositions comprising the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (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 pharmaceutical

compositions 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 embodiments, any antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed herein is formulated in accordance with routine procedures as a pharmaceutical composition adapted for a mode of administration disclosed herein.

Administration, Dosing, and Treatment Regimens

Routes of administration include, for example: intradermal, intratumoral, 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.

As examples, administration results in the release of antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) disclosed herein into the bloodstream (*via* enteral or parenteral administration), or alternatively, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) is administered directly to the site of active disease.

Any antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed herein can be administered orally. Such antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (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 embodiments, for instance in the treatment of cancer, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (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 antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention (and/or additional agents) are administered intratumorally.

In embodiments, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric proteins used in methods of the present invention 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 antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention, disclosed 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, an antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention and an additional agent(s) 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 an antibody directed to immune checkpoint molecules, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an innate immune response and another antibody directed to immune checkpoint molecules, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an adaptive immune response. In such embodiments, the antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an innate immune response may be administered before, concurrently with, or subsequent to administration of the antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an adaptive immune response. For example, the antibodies directed to immune checkpoint molecules, e.g., CTLA-4, STING agonists, and/or heterologous chimeric proteins used in methods of the present invention 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an innate immune response and the antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an adaptive response are administered 1 week apart, or administered on alternate weeks (*i.e.,* administration of the antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention inducing an innate immune response is followed 1 week later with administration of the antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention which induces an adaptive immune response and so forth).

The dosage of any antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (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, 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).

An antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 (s*ee, 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed 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 antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention (and/or additional agents) disclosed herein can be administered continuously rather than intermittently throughout the dosage regimen.

Fusion Proteins, Nucleic Acids, and Cells

A heterologous chimeric protein used in a method of the present invention may be a recombinant fusion protein, *e.g.,* a single polypeptide having the extracellular domains disclosed herein. For example, in embodiments, the heterologous chimeric protein is translated as a single unit in a prokaryotic cell, a eukaryotic cell, or a cell-free expression system.

In embodiments, a heterologous chimeric protein is recombinant protein comprising multiple polypeptides, *e.g.,* multiple extracellular domains disclosed herein, that are combined (*via* covalent or non-covalent bonding) to yield a single unit, *e.g., in vitro* (*e.g.,* with one or more synthetic linkers disclosed herein).

In embodiments, a heterologous 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 heterologous chimeric protein is translated and a portion is chemically synthesized.

Constructs could be produced by cloning of the nucleic acids encoding the three fragments (the extracellular domain of a Type I transmembrane protein, followed by a linker sequence, followed by the extracellular domain of a Type II transmembrane 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 extracellular domain of the Type I transmembrane protein and the carboxy terminus of the complete sequence corresponded to the 'right' side of the molecule containing the extracellular domain of Type II transmembrane protein. In embodiments of chimeric proteins having one of the other configurations, as described elsewhere herein, a construct would comprise three nucleic acids such that the translated chimeric protein produced would have the desired configuration, *e.g.,* a dual inward-facing chimeric protein. Accordingly, in embodiments, the heterologous chimeric proteins used in methods of the present invention are engineered as such.

A heterologous chimeric protein used in a method of the present invention may be encoded by a nucleic acid cloned into an expression vector. 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 heterologous 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 λP_L. Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (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 heterologous 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 β-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 heterologous chimeric proteins in recombinant host cells.

In embodiments, expression vectors comprise a nucleic acid encoding the heterologous chimeric proteins, 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.

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

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 embodiments, the cell is a tumor cell. In another embodiment, the cell is a non-tumor cell. In embodiments, 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 embodiments, 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 heterologous 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. In other examples, the chimeric protein is

expressed by a chimeric antigen receptor containing cell or an in vitro expanded tumor infiltrating lymphocyte, under the control of a promoter which is sensitive to antigen recognition by the cell, and leads to local secretion of the chimeric protein in response to tumor antigen recognition. Particular examples can be found, for example, in U.S. Patent 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 that function in human cells are well known in the art; these 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; they 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 is 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 embodiments, the expression vectors for the expression of the heterologous chimeric proteins (and/or additional agents) are viral vectors. Many viral vectors useful for gene therapy are known (*see*, *e.g.,* Lundstrom, Trends Biotechnol., 21: 1 17, 122, 2003. Illustrative viral vectors include those selected from Antiviruses (LV), retroviruses (RV), adenoviruses (AV), adeno-associated viruses (AAV), and α 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 α viruses and adenoviruses. Illustrative types of α 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 embodiments, 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.

Expression vectors can be introduced into host cells for producing the heterologous chimeric proteins used in methods of the present invention. 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 heterologous chimeric proteins disclosed 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 (ATCC), or from commercial suppliers.

Cells that can be used for production of the heterologous chimeric proteins used in methods of the present invention *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, chimeric antigen receptor expressing T cells, tumor infiltrating 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, and fetal liver. 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 heterologous chimeric proteins contemplated herein may have unique characteristics as compared to traditional Fc containing macromolecules. In certain examples, the heterologous chimeric proteins may be purified using specific chromatography resins, or using chromatography methods that do not depend upon Protein A capture. In embodiments, the heterologous 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 heterologous chimeric proteins obtained herein may be additionally 'polished' using methods that are specified in the art. In embodiments, the heterologous 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 heterologous 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, which comprises 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.

In embodiments, the subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand. In embodiments, the subject has a cancer that is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment.

Kits and Medicaments

Aspects of the present invention provide kits that can simplify the administration of the pharmaceutical compositions and/or chimeric proteins disclosed herein.

An illustrative kit of the invention comprises any antibody directed to immune checkpoint molecules, e.g., CTLA-4, STING agonist, and/or heterologous chimeric protein used in methods of the present invention and/or pharmaceutical composition disclosed herein in unit dosage form. In embodiments, the unit dosage form is a container, such as a pre-filled syringe, which can be sterile, containing any agent disclosed 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 disclosed 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 disclosed herein. In embodiments, the kit comprises a container containing an effective amount of a composition of the invention and an effective amount of another composition, such those disclosed herein.

Aspects of the present invention include use of a heterologous chimeric protein as disclosed herein in the manufacture of a medicament, *e.g.,* a medicament for treatment of cancer and/or treatment of an inflammatory disease.

Any aspect or embodiment disclosed 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: Functional in vivo anti-tumor activity of Specific Combinations of Antibodies Directed to Immune Checkpoint Molecules and Chimeric Proteins

The *in vivo* ability of specific combinations of antibodies directed to immune checkpoint molecules and chimeric proteins to target and reduce tumor volume was determined.

BALB/C mice were inoculated with 500,000 CT26 tumor cells. Eight days after inoculation, there was no significant difference between starting tumor volumes among the mice, *i.e.,* volumes were approximately 100 mm^3. Eight days after inoculation treatment began according to the schedule shown in **FIG. 3A**. Specific combinations were included: anti-CTLA-4 (9D9); anti-PD-1 (RMP1-14); anti-OX40 (OX86); PD-1-Fc-GITRL (**FIG. 3B**); anti-CTLA-4 then anti-PD1; anti-CTLA-4 then anti-OX40; anti-CTLA-4 then PD-1-Fc-GITRL (**FIG. 3C**); PD-1-Fc-GITRL then anti-CTLA-4 (**FIG. 3D**). Tumor sizes were assayed every periodically until the 27th day after inoculation. Mice that rejected the tumor were re-challenged with a secondary tumor (300,000 CT26 tumor cells) on the opposing flank, and primary/secondary tumors continued to be measured.

As shown in the final column of **FIG. 3A**, relative to vehicle, all treatments were effective in promoting survival of tumor-bearing mice. However, only the combination comprising the PD1-Fc-GITRL chimeric protein provided before the CTLA3 antibody demonstrated 100% survival at day 27. The next greatest survival rate (75%) was observed when the PD1-Fc-GITRL chimeric protein provided after the CTLA3 antibody or when the PD1-Fc-GITRL chimeric protein was provided alone.

As shown in **FIG. 3B**, relative to vehicle, all single-component (treatments were effective in reducing tumor volume. Likewise, as shown in **FIG. 3C** and **FIG. 3D**, the combination treatments showed reduction in tumor volume over the course of the study.

In other sets of *in vivo* experiments, BALB/C mice were inoculated with CT26 cells into one flank. Mice were divided into three experimental groups, which were each subdivided into treatment groups. Experimental Group 1 included mice of the PD1-Fc-GITRL experiment, which were treated with an anti-CTLA4 antibody, the PD1-Fc-GITRL chimeric protein, or a combination of PD1-Fc-GITRL and anti-CTLA4. Experimental Group 2 included mice of the PD1-Fc-CD40L experiment, which were treated with a PD-1-Fc single-side fusion protein, an Fc-CD40L single-side fusion protein, both PD-1-Fc + Fc-CD40L single-side fusion proteins, an anti-CTLA4 antibody, the PD-1-Fc-CD40L chimeric protein, or a combination of PD-1-Fc-CD40L and anti-CTLA4. And, Experimental Group 3 included mice of the PD1-Fc-41BBL experiment, which were treated with an anti-CTLA4 antibody, the PD-1-Fc-4-1BBL chimeric protein, or a combination of PD-1-Fc-4-1BBL and anti-CTLA4). Mice that rejected the tumor were re-challenged with a secondary tumor on the opposing flank, and primary/secondary tumors continued to be measured. Each Experimental Group included Vehicle mice which were IP administered PBS.

On the eighth day after inoculation, mice of Experimental Group 1 were IP administered 100 μg of the anti-CTLA4 (clone 9D9) antibody, 300 μg of the PD-1-Fc-GITRL chimeric protein, or 300 μg of the PD-1-Fc-GITRL chimeric protein along with 100 μg of the anti-CTLA4 antibody. On the eleventh day after inoculation and on the thirteenth day after inoculation, the prior treatments were repeated. Tumor volumes were measured periodically and the number of surviving mice was determined.

FIG. 4A is a graph showing changes in tumor volume for each mouse of the four treatment groups of Experimental Group 1; **FIG. 4B** is a graph showing survivorship for mice in the four treatment groups of Experimental Group 1. **FIG. 4C** is a table including data relevant to the graphs of **FIG. 4A** and **FIG. 4B**. These data show that the combination of the PD-1-Fc-GITRL chimeric protein with an anti-CTLA-4 antibody improved survival and rejection.

Moreover, the Mantel-Cox significance analysis of survival curves, indicates that the improved survival provided by the combination of the PD-1-Fc-GITRL chimeric protein with an anti-CTLA-4 antibody is statistically significant ($p<.0001$) versus the anti-CTLA4 antibody alone treatment or versus the PD-1-Fc-GITRL alone treatment.

On the eighth day after inoculation, mice of Experimental Group 2 were IP administered 150 μg of the PD-1-Fc single-side fusion protein, 150 μg of the Fc-CD40L single-side fusion protein, 150 μg of each of the PD-1-Fc + Fc-CD40L single-side fusion protein, 100 μg of the anti-CTLA4 antibody, 300 μg of the PD-1-Fc-CD40L chimeric protein, or a combination of 300 μg of the PD-1-Fc-CD40L chimeric protein and 100 μg of the anti-CTLA4 antibody. On the tenth day after inoculation and on the twelfth day after inoculation, the prior treatments were repeated. Tumor volumes were measured periodically and the number of surviving mice was determined.

FIG. 5A is a graph showing average changes in tumor volume among mice of the seven treatment groups of Experimental Group 2; **FIG. 5B** is a graph showing survivorship for mice in the seven treatment groups of Experimental Group 2. **FIG. 5C** is a table including data relevant to the graphs of **FIG. 5A** and **FIG. 5B**. These data show that the combination of the PD-1-Fc-CD40L chimeric protein with an anti-CTLA-4 antibody improved survival and rejection.

On the eighth day after inoculation, mice of Experimental Group 3 were IP administered 100 μg of the anti-CTLA4 (clone 9D9) antibody, 300 μg of the PD-1-Fc-4-1BBL chimeric protein, or 300 μg of the PD-1-Fc-4-1BBL chimeric protein along with 100 μg of the anti-CTLA4 antibody. On the eleventh day after inoculation and on the thirteenth day after inoculation, the prior treatments were repeated. Tumor volumes were measured periodically and the number of surviving mice was determined.

FIG. 6A are graphs showing changes in tumor volume for each mouse of the four treatment groups of Experimental Group 3; **FIG. 6B** is a graph showing survivorship for mice in the four treatment groups of Experimental Group 3. **FIG. 6C** is a table including data relevant to the graphs of **FIG. 6A** and **FIG. 6B**. **FIG. 6D** includes data demonstrating improvements obtained from the combination treatments relative to monotherapies. Together, these data show that the combination of the PD-1-Fc-4-1BBL chimeric protein with an anti-CTLA-4 antibody improved survival and rejection.

Example 2: Functional in vivo anti-tumor activity of Specific Combinations of a STING Agonist and Chimeric Proteins

The *in vivo* ability of specific combinations of a stimulator of interferon genes (STING) agonist and chimeric proteins to target and reduce tumor volume was determined.

BALB/C mice were inoculated with 500,000 CT26 tumor cells. Eight days after inoculation, there was no significant difference between starting tumor volumes among the mice, *i.e.,* volumes were approximately 100 mm^3. Eight days

after inoculation treatment began according to the schedule shown in **FIG. 7A**. Specific combinations were included: DMXAA; anti-PD-1 (RMP1-14); anti-OX40 (OX86); PD1-Fc-GITRL; (**FIG. 7A**); DMXAA then anti-PD1; DMXAA then anti-OX40; and DMXAA then PD-1-Fc-GITRL (**FIG. 7B**). Here, DMXAA was administered intratumorally (IT) and the other agents were administered intraperitoneally (IP). Tumor sizes were assayed periodically until the 27th day after inoculation. Mice that rejected the tumor were re-challenged with a secondary tumor (300,000 CT26 tumor cells) on the opposing flank, and primary/secondary tumors continued to be measured.

As shown in the final column of **FIG. 3A**, only the combinations comprising the PD1-Fc-GITRL chimeric protein provided before or after the STING agonist (DMXAA) demonstrated 100% survival at day 27.

As shown in **FIG. 7A**, relative to vehicle, all single-component treatments were effective in reducing tumor volume. Likewise, as shown in **FIG. 7B**, the combination treatments showed reduction in tumor volume over the course of the study.

In any of the above-described Examples, the therapeutic activity of the treatments may further be assayed. In particular, changes in pharmacodynamic biomarkers showing tumor rejection will be determined by cytokine elevations in serum (*in vivo)* or changes in pharmacodynamic biomarkers *in vitro* in immune-related cells incubated with the super-antigen Staphylococcal enterotoxin B (SEB assay) or when cultured in AIM V media will be determined. Exemplary pharmacodynamic biomarkers include IFNγ, IL-2, IL-4, IL-5, IL-6, and IL-17A.

INCORPORATION BY REFERENCE

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

Specifically, additional teachings related to the present invention are found, in one or more of WO2018/157162; WO2018/157165; WO2018/157164; WO2018/157163; and WO2017/059168, the contents of each of which is incorporated herein by reference in its entirety.

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.

EQUIVALENTS

While the invention has been disclosed 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 disclosed specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

What is claimed is:

1. A method for treating a cancer in a subject in need thereof comprising:

providing the subject a first pharmaceutical composition comprising an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and

providing the subject a second pharmaceutical composition comprising an immunotherapy selected from:

(i) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and

(c) a linker linking the first domain and the second domain;

(ii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and

(c) a linker linking the first domain and the second domain; and

(iii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and

(c) a linker linking the first domain and the second domain.

2. The method of claim 1, wherein the first pharmaceutical composition and the second pharmaceutical composition are provided simultaneously.

3. The method of claim 1, wherein the first pharmaceutical composition is provided after the second pharmaceutical composition is provided.

4. The method of claim 1, wherein the first pharmaceutical composition is provided before the second pharmaceutical composition is provided.

5. The method of any one of claims 1 to 3, wherein the dose of the first pharmaceutical composition is less than the dose of the first pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the second pharmaceutical composition.

6. The method of any one of claims 1, 2, or 4, wherein the dose of the second pharmaceutical composition provided is less than the dose of the second pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the first pharmaceutical composition.

7. The method of any one of claims 1 to 6, wherein the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the first pharmaceutical composition.

8. The method of any one of claims 1 to 7, wherein the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the second pharmaceutical composition.

9. A method for treating a cancer in a subject comprising:

providing the subject a pharmaceutical composition comprising an immunotherapy selected from:

(i) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and

(c) a linker linking the first domain and the second domain;

(ii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and

(c) a linker linking the first domain and the second domain; and

(iii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and

(c) a linker linking the first domain and the second domain;

wherein the subject has undergone or is undergoing treatment with an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).

10. The method of claim 9, wherein the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with an antibody that is capable of binding CTLA-4.

11. A method for treating a cancer in a subject comprising:

providing the subject a pharmaceutical composition comprising an antibody that is capable of binding cytotoxic T lymphocyte-associated antigen 4 (CTLA-4),

wherein the subject has undergone or is undergoing treatment with an immunotherapy selected from:

(i) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and

(c) a linker linking the first domain and the second domain;

(ii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and

(c) a linker linking the first domain and the second domain; and

(iii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and

(c) a linker linking the first domain and the second domain.

12. The method of claim 11, wherein the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with the immunotherapy selected from (i) to (iii).

13. The method of any one of claims 1 to 12, wherein the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

14. The method of any one of claims 1 to 12, wherein the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

15. The method of any one of claims 1 to 12, wherein the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

16. The method of any one of claims 1 to 15, wherein the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

17. The method of any one of claims 1 to 16, wherein the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain.

18. The method of claim 17, wherein the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, *e.g.,* human IgG1 or human IgG4.

19. The method of claim 17 or claim 18, wherein the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

20. The method of claim 13, wherein the heterologous chimeric protein comprises:

(a) a first domain comprising a portion of PD-1,

(b) a second domain comprising a portion of GITRL, and

(c) a linker comprising a hinge-CH2-CH3 Fc domain.

21. The method of claim 14, wherein the heterologous chimeric protein comprises:

(a) a first domain comprising a portion of PD-1,

(b) a second domain comprising a portion of 4-1BBL, and

(c) a linker comprising a hinge-CH2-CH3 Fc domain.

22. The method of claim 15, wherein the heterologous chimeric protein comprises:

(a) a first domain comprising a portion of PD-1,

(b) a second domain comprising a portion of CD40L, and

(c) a linker comprising a hinge-CH2-CH3 Fc domain.

23. The method of any one of claims 1 to 22, wherein the antibody that is capable of binding CTLA-4 is selected from the group consisting of YERVOY (ipilimumab), 9D9, tremelimumab (formerly ticilimumab, CP-675,206; MedImmune), AGEN1884, and RG2077.

24. The method of any one of claims 1 to 23, wherein the cancer is or is related 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.

25. The method of any one of claims 1 to 24, wherein the subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand.

26. The method of any one of claims 1 to 25, wherein the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment.

27. The method of claim 25 or claim 26, wherein the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

28. A method for treating a cancer in a subject in need thereof comprising:

providing the subject a first pharmaceutical composition comprising a stimulator of interferon genes (STING) agonist and

providing the subject a second pharmaceutical composition comprising an immunotherapy selected from:

(i) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and

(c) a linker linking the first domain and the second domain;

(ii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and

(c) a linker linking the first domain and the second domain; and

(iii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and

(c) a linker linking the first domain and the second domain.

29. The method of claim 28, wherein the first pharmaceutical composition and the second pharmaceutical composition are provided simultaneously.

30. The method of claim 28, wherein the first pharmaceutical composition is provided after the second pharmaceutical composition is provided.

31. The method of claim 28, wherein the first pharmaceutical composition is provided before the second pharmaceutical composition is provided.

32. The method of any one of claims 28 to 30, wherein the dose of the first pharmaceutical composition is less than the dose of the first pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the second pharmaceutical composition.

33. The method of any one of claims 28, 29, or 31, wherein the dose of the second pharmaceutical composition provided is less than the dose of the second pharmaceutical composition provided to a subject who has not undergone or is not undergoing treatment with the first pharmaceutical composition.

34. The method of any one of claims 28 to 33, wherein the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the first pharmaceutical composition.

35. The method of any one of claims 28 to 34, wherein the subject has an increased chance of survival, without gastrointestinal inflammation and weight loss, and/or a reduction in tumor size or cancer prevalence when compared to a subject who has only undergone or is only undergoing treatment with the second pharmaceutical composition.

36. A method for treating a cancer in a subject comprising:

providing the subject a pharmaceutical composition comprising an immunotherapy selected from:

(i) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and

(c) a linker linking the first domain and the second domain;

(ii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and

(c) a linker linking the first domain and the second domain; and

(iii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and

(c) a linker linking the first domain and the second domain;

wherein the subject has undergone or is undergoing treatment with a stimulator of interferon genes (STING) agonist.

37. The method of claim 36, wherein the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with a STING agonist.

38. A method for treating a cancer in a subject comprising:

providing the subject a pharmaceutical composition comprising a stimulator of interferon genes (STING) agonist,

wherein the subject has undergone or is undergoing treatment with an immunotherapy selected from:

(i) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of GITRL, wherein the portion is capable of binding a GITRL receptor, and

(c) a linker linking the first domain and the second domain;

(ii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of 4-1BBL, wherein the portion is capable of binding a 4-1BBL receptor, and

(c) a linker linking the first domain and the second domain; and

(iii) a heterologous chimeric protein comprising:

(a) a first domain comprising a portion of the extracellular domain of PD-1, wherein the portion is capable of binding a PD-1 ligand,

(b) a second domain comprising a portion of the extracellular domain of CD40L, wherein the portion is capable of binding a CD40L receptor, and

(c) a linker linking the first domain and the second domain.

39. The method of any one of claims claim 38, wherein the dose of the pharmaceutical composition provided to the subject is less than the dose of the pharmaceutical composition that is provided to a subject who has not undergone or is not undergoing treatment with the immunotherapy selected from (i) to (iii).

40. The method of any one of claims 28 to 39, wherein the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of GITRL.

41. The method of any one of claims 28 to 39, wherein the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of 4-1BBL.

42. The method of any one of claims 28 to 39, wherein the immunotherapy comprises a heterologous chimeric protein comprising a first domain which comprises substantially the entire extracellular domain of PD-1 and/or a second domain which comprises substantially the entire extracellular domain of CD40L.

43. The method of any one of claims 28 to 42, wherein the linker is a polypeptide selected from a flexible amino acid sequence, an IgG hinge region, and an antibody sequence.

44. The method of any one of claims 28 to 43, wherein the linker comprises at least one cysteine residue capable of forming a disulfide bond and/or comprises a hinge-CH2-CH3 Fc domain.

45. The method of claim 44, wherein the linker comprises a hinge-CH2-CH3 Fc domain derived from IgG1 or IgG4, e.g., human IgG1 or human IgG4.

46. The method of claim 44 or claim 45, wherein the linker comprises an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 3.

47. The method of claim 40, wherein the heterologous chimeric protein comprises:

(a) a first domain comprising a portion of PD-1,

(b) a second domain comprising a portion of GITRL, and

(c) a linker comprising a hinge-CH2-CH3 Fc domain.

48. The method of claim 41, wherein the heterologous chimeric protein comprises:

(a) a first domain comprising a portion of PD-1,

(b) a second domain comprising a portion of 4-1BBL, and

(c) a linker comprising a hinge-CH2-CH3 Fc domain.

49. The method of claim 42, wherein the heterologous chimeric protein comprises:

(a) a first domain comprising a portion of PD-1,

(b) a second domain comprising a portion of CD40L, and

(c) a linker comprising a hinge-CH2-CH3 Fc domain.

50. The method of any one of claims 28 to 49, wherein the STING agonist is selected from the group consisting of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), MIW815(ADU-S100), CRD5500, or MK-1454.

51. The method of any one of claims 28 to 50, wherein the cancer is or is related 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.

52. The method of any one of claims 28 to 51, wherein the subject has a cancer that is poorly responsive or is refractory to treatment comprising an antibody that is capable of binding PD-1 or binding a PD-1 ligand.

53. The method of any one of claims 28 to 52, wherein the cancer is poorly responsive or is non-responsive to treatment with an antibody that is capable of binding PD-1 or binding a PD-1 ligand after 12 weeks or so of such treatment.

54. The method of claim 52 or claim 53, wherein the antibody that is capable of binding PD-1 or binding a PD-1 ligand is selected from the group consisting of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), cemiplimab (REGN-2810), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), Ibrutinib (PHARMACYCLICS/ABBVIE), atezolizumab (TECENTRIQ, GENENTECH), and MPDL328OA (ROCHE).

FIG. 1A

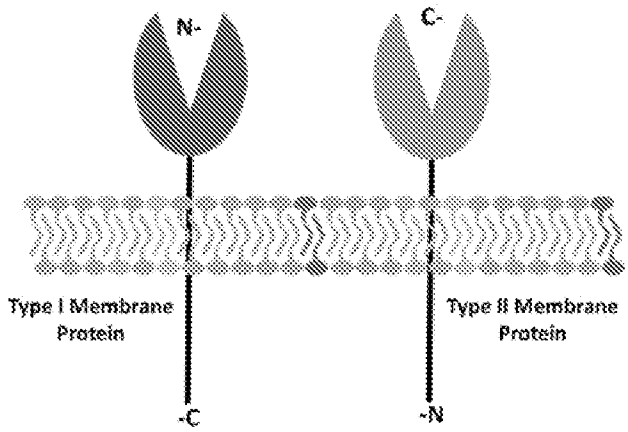


FIG. 1B

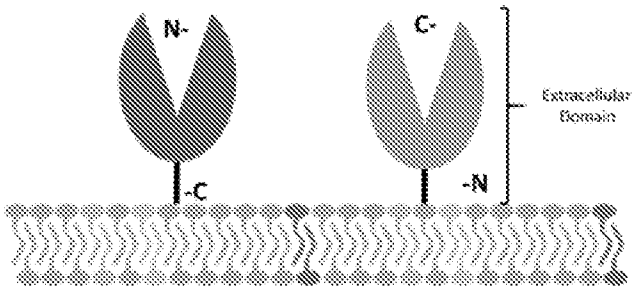


FIG. 1C

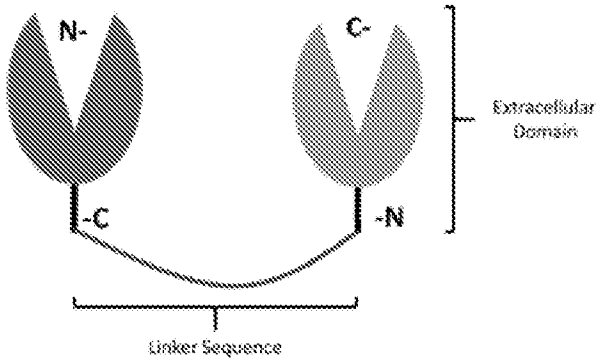


FIG. 1D

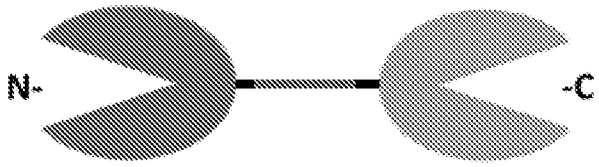


FIG. 2

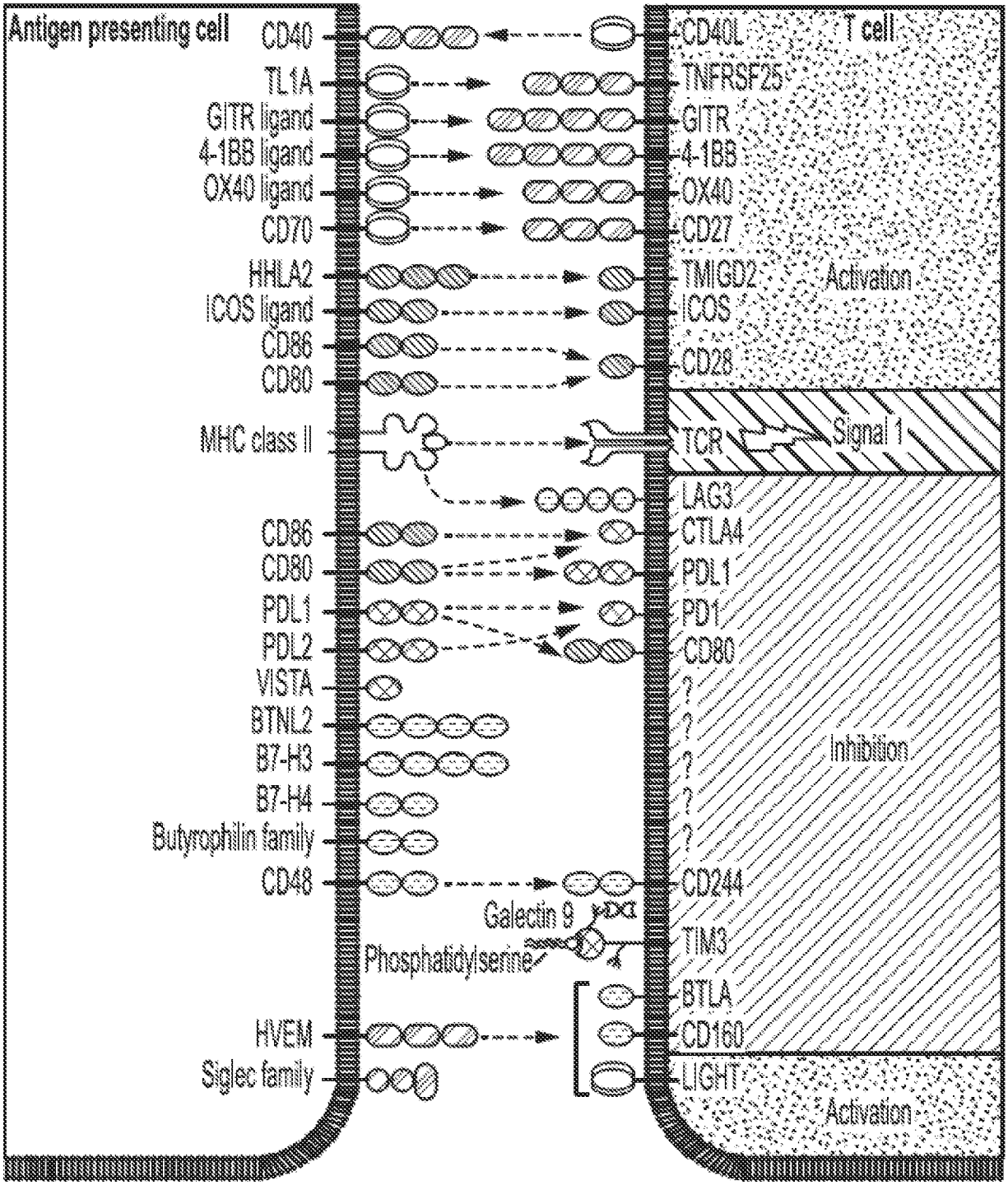

Antigen presenting cell
CD40
TL1A
GITR ligand
4-1BB ligand
OX40 ligand
CD70
HHLA2
ICOS ligand
CD86
CD80
MHC class II
CD86
CD80
PDL1
PDL2
VISTA
BTNL2
B7-H3
B7-H4
Butyrophilin family
CD48
HVEM
Siglec family
T cell
CD40L
TNFRSF25
GITR
4-1BB
OX40
CD27
TMIGD2
ICOS
CD28
Activation
TCR
Signal 1
LAG3
CTLA4
PDL1
PD1
CD80
?
?
?
?
?
?
Inhibition
CD244
Galectin 9
Phosphatidylserine
TIM3
BTLA
CD160
LIGHT
Activation

FIG. 3A

Reagent 1	Dose 1	Schedule 1	Reagent 2	Dose 2	Schedule 2	N=	# Rejection	% Survival @ Day 27
Vehicle	IP (PBS)	Days 8,11,13				8	**0**	**0**
Vehicle	IT (PBS)	Day 8				8	**0**	**0**
anti-CTLA4 (9D9)	100 µg	Days 8,11,13				8	**1**	**37.5**
DMXAA	100µg	Day 8				8	**3**	**75**
			anti-PD1 (RMP1-14)	100µg	Days 11,13,15	8	**0**	**12.5**
			anti-OX40 (OX86)	100µg	Days 11,13,15	8	**1**	**37.5**
PD1-Fc-GITRL	300µg	Days 8,11,13				8	**1**	**75**
anti-CTLA4 (9D9)	100 µg	Days 8,11,13	anti-PD1	100µg	Days 13,15,17	8	**4**	**62.5**
anti-CTLA4 (9D9)	100 µg	Days 8,11,13	anti-OX40	100µg	Days 13,15,17	8	**1**	**62.5**
anti-CTLA4 (9D9)	100 µg	Days 8,11,13	PD1-Fc-GITRL	300µg	Days 13,15,17	8	**3**	**75**
PD1-Fc-GITRL	300µg	Days 8,11,13	anti-CTLA4 (9D9)	100 µg	Days 13,15,17	7	**1**	**100**
PD1-Fc-GITRL	300µg	Days 8,11,13	DMXAA	100µg	Day 13	8	**3**	**100**
DMXAA	100µg	Day 8	anti-PD1	100µg	Days 11,13,15	8	**4**	**75**
DMXAA	100µg	Day 8	anti-OX40	100µg	Days 11,13,15	8	**3**	**75**
DMXAA	100µg	Day 8	PD1-Fc-GITRL	300µg	Days 11,13,15	8	**1**	**100**

FIG. 3B

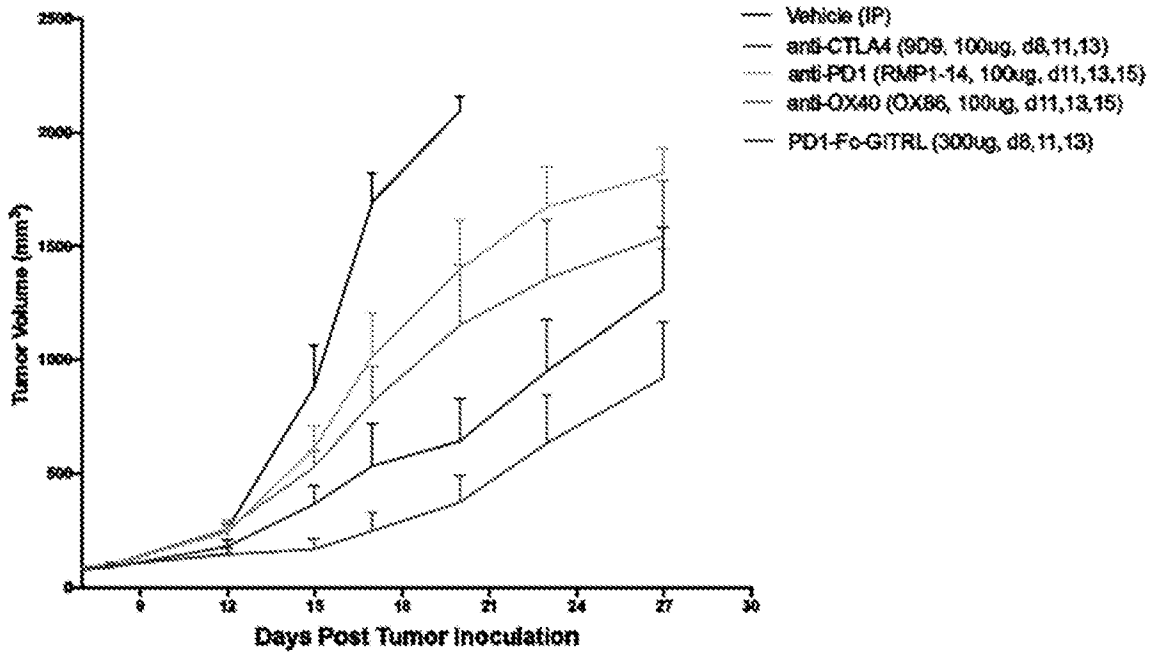

Vehicle (IP)
anti-CTLA4 (9D9, 100ug, d8,11,13)
anti-PD1 (RMP1-14, 100ug, d11,13,15)
anti-OX40 (OX86, 100ug, d11,13,15)
PD1-Fc-GITRL (300ug, d8,11,13)
Tumor Volume (mm³)
Days Post Tumor Inoculation

FIG. 3C

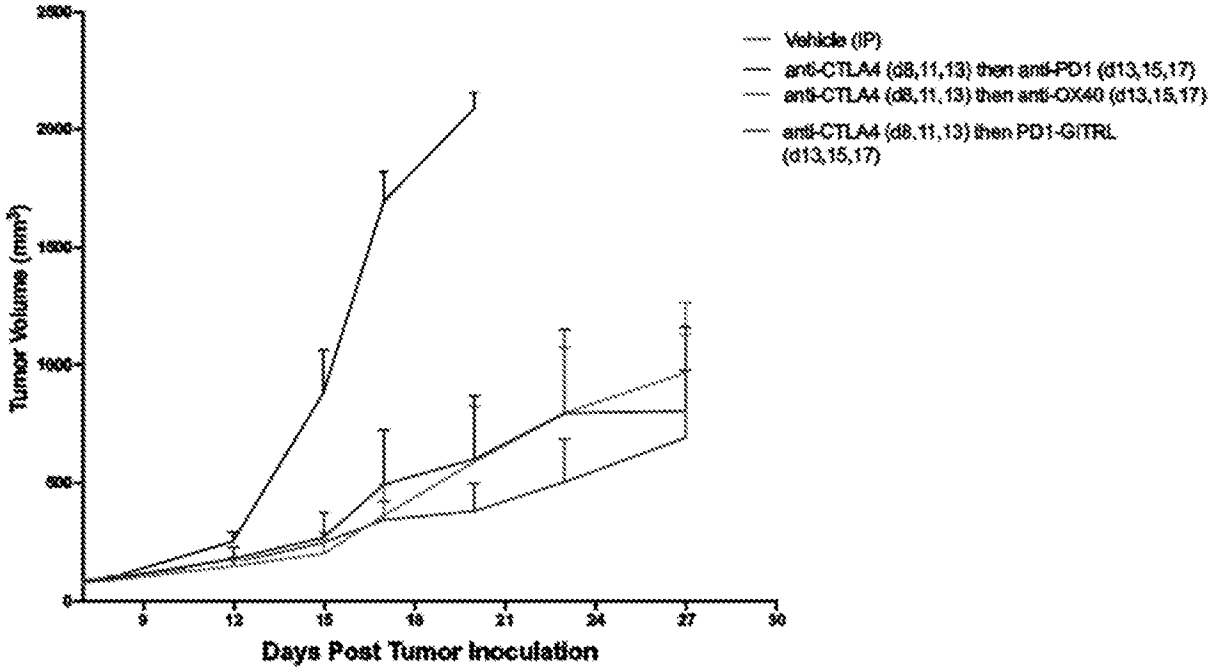

Vehicle (IP)
anti-CTLA4 (d8,11,13) then anti-PD1 (d13,15,17)
anti-CTLA4 (d8,11,13) then anti-OX40 (d13,15,17)
anti-CTLA4 (d8,11,13) then PD1-GITRL (d13,15,17)
Tumor Volume (mm³)
Days Post Tumor Inoculation

FIG. 3D

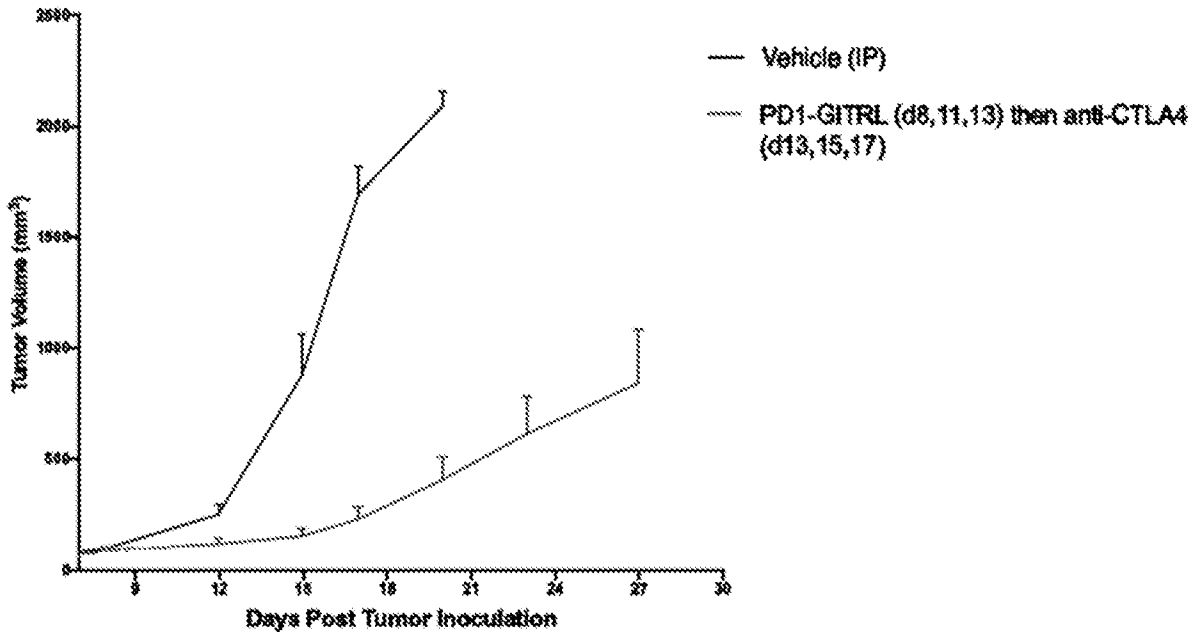

Vehicle (IP)
PD1-GITRL (d8,11,13) then anti-CTLA4 (d13,15,17)
Tumor Volume (mm³)
2500
2000
1500
1000
500
0
9
12
15
18
21
24
27
30
Days Post Tumor Inoculation

FIG. 4A

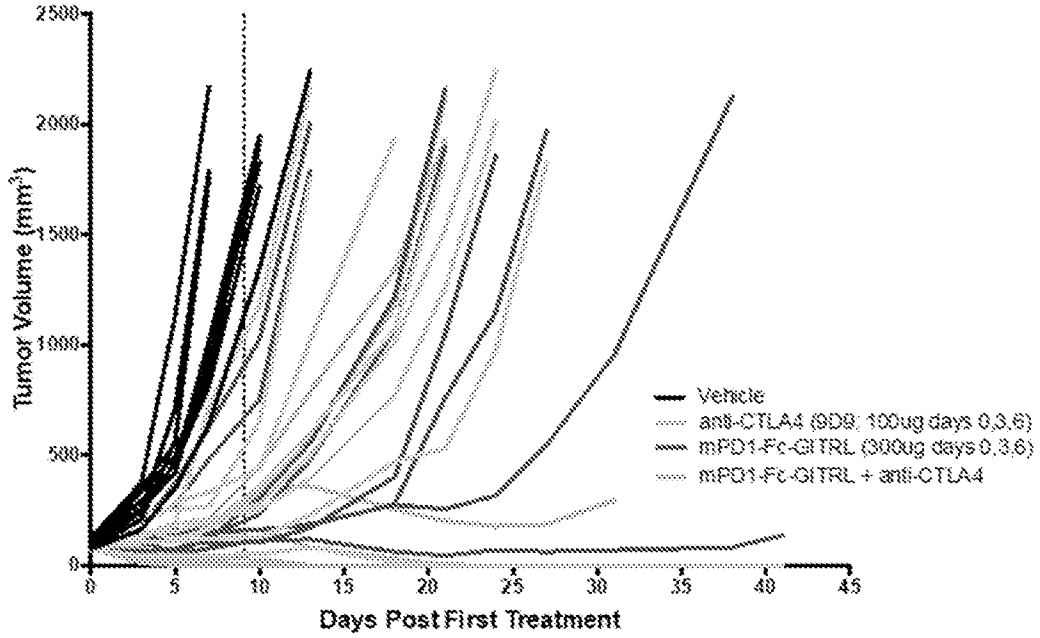

Tumor Volume (mm3)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
45
Days Post First Treatment
Vehicle
anti-CTLA4 (9D9; 100ug days 0,3,6)
mPD1-Fc-GITRL (300ug days 0,3,6)
mPD1-Fc-GITRL + anti-CTLA4

FIG. 4B

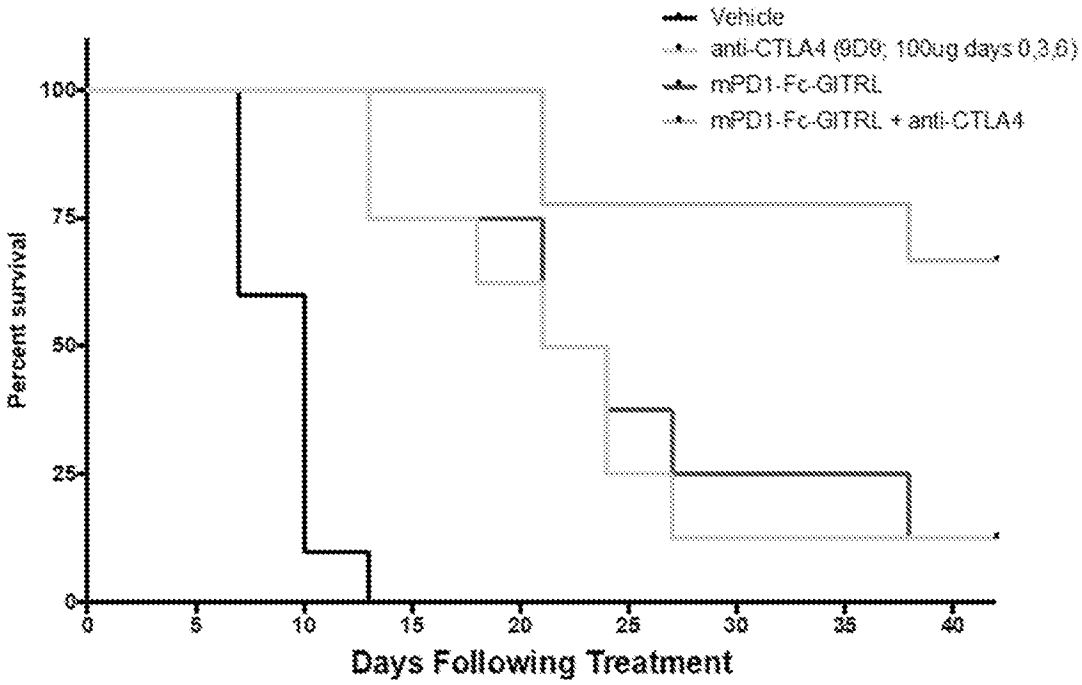

Percent survival
100
75
50
25
0
0
5
10
15
20
25
30
35
40
Days Following Treatment
Vehicle
anti-CTLA4 (9D9; 100ug days 0,3,6)
mPD1-Fc-GITRL
mPD1-Fc-GITRL + anti-CTLA4

FIG. 4C

	Dose	Treatment (IP) Schedule	Group Size	Survival Alive at Day 24	Rejection Primary Tumor		Rejection Secondary Tumor		Tumor Vol. Day 0 to 10 Treated vs. Vehicle
Group	(µg)	(days)	(n=)	%	#	%	#	%	Fold Change
Vehicle	PBS	0, 3, 6	10	0	0/10	0	N/A	N/A	N/A
anti-CTLA4 (clone 9D9)	100	0, 3, 6	8	50	1/8	12.5	1/1	100	-4.43
PD1-Fc-GITRL	300	0, 3, 6	8	50	0/8	0	N/A	N/A	-5.16
PD1-Fc-GITRL + anti-CTLA4	300/100	0, 3, 6	9	77.8	6/9	66.7	6/6	100	-19.29

FIG. 5A

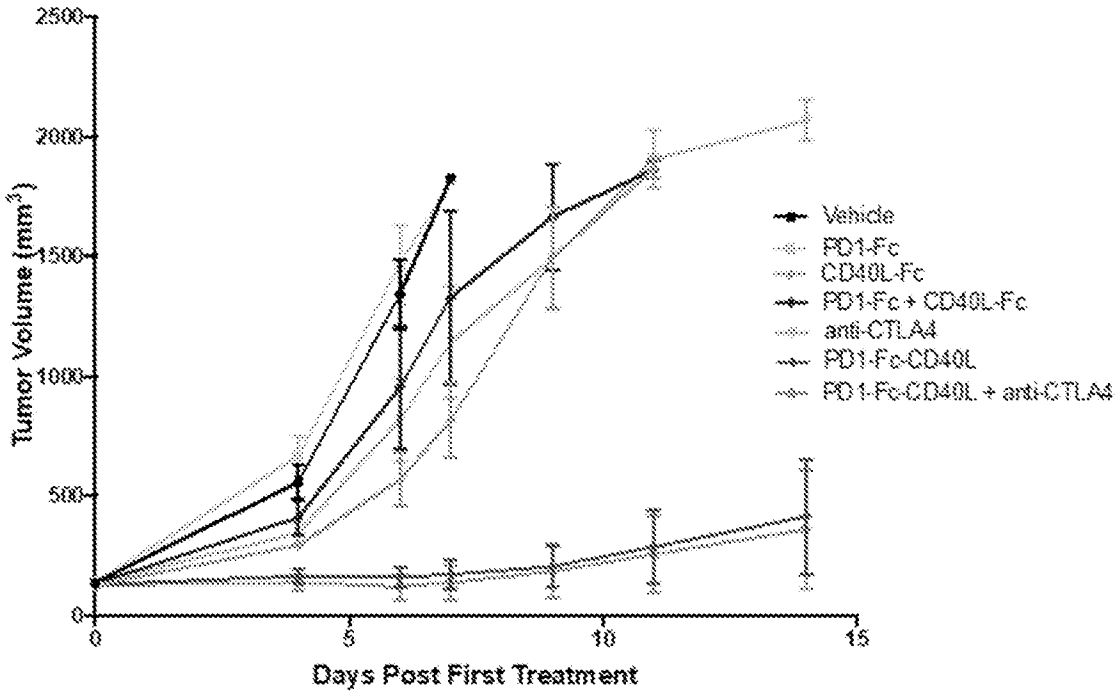


FIG. 5B

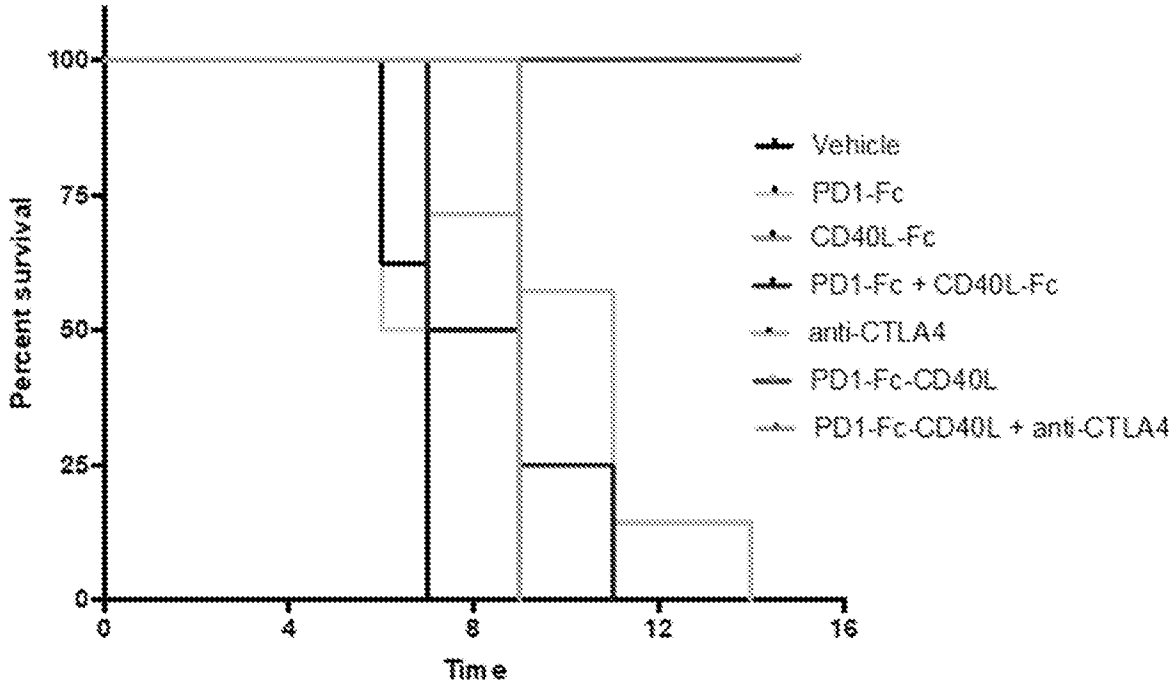
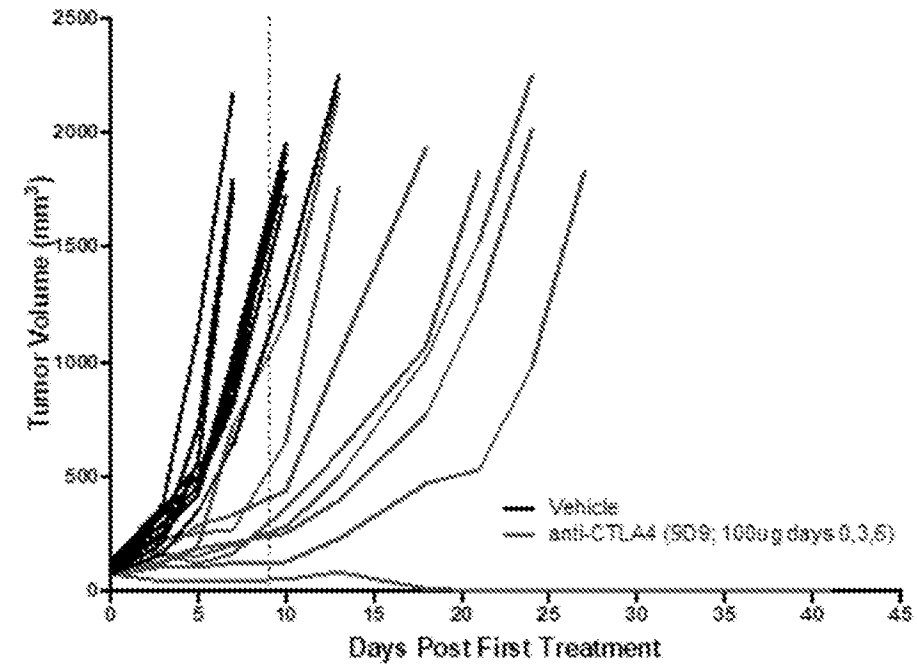


FIG. 5C

		Schedule	Group Size	Primary Tumor Rejection		Survival %
Group	Dose (µg)	(days)	(n=)	#	%	at day 14
Vechicle			8	0/8	0	0
PD1-Fc	150	0,2,4	6	0/6	0	0
Fc-CD40L	150	0,2,4	4	0/4	0	0
PD1-Fc + Fc-CD40L	150/150	0,2,4	4	0/4	0	0
anti-CTLA4 (9D9)	100	0,2,4	7	0/7	0	0
PD1-Fc-CD40L	300	0,2,4	5	1/5	20	100
PD1-Fc-CD40L + anti-CTLA4	300/100	0,2,4	5	2/5	40	100

FIG. 6A


Tumor Volume (mm³)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
45
Days Post First Treatment
Vehicle
anti-CTLA4 (SO9; 100ug days 0,3,6)

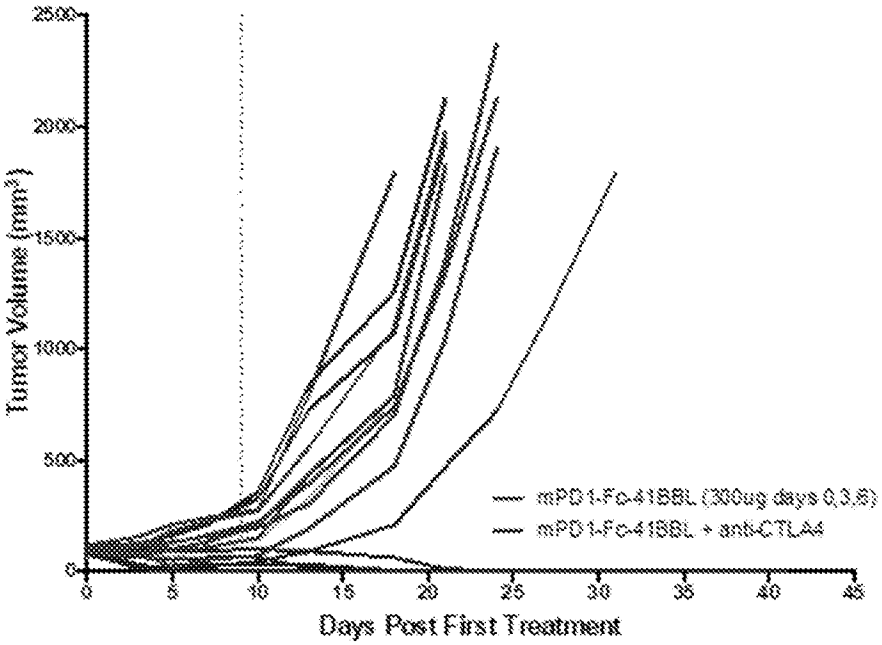

Tumor Volume (mm³)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
45
Days Post First Treatment
mPD1-Fc-41BBL (300ug days 0,3,6)
mPD1-Fc-41BBL + anti-CTLA4

FIG. 6B

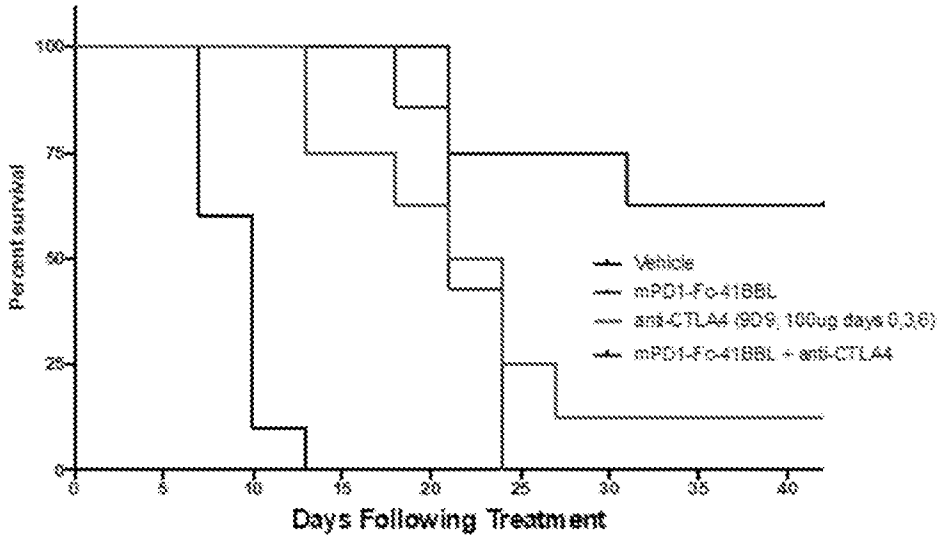


FIG. 6C

	Dose	Treatment (IP) Schedule	Group Size	Survival Alive at Day 24	Rejection Primary Tumor		Rejection Secondary Tumor		Tumor Vol. Day 0 to 10 Treated vs. Vehicle	Survival Mantel-Cox p-value
Group	(µg)	(days)	(n=)	%	#	%	#	%	Fold Change	vs. vehicle
Vehicle	PBS	0, 3, 6	10	0	0/10	0	N/A	N/A	N/A	N/A
anti-CTLA4 (clone 9D9)	100	0, 3, 6	8	50	1/8	12.5	1/1	100	-4.43	<.0001
PD1-Fc-41BBL	300	0, 3, 6	7	42.9	0/7	0	N/A	N/A	-9.14	<.0001
PD1-Fc-41BBL + anti-CTLA4	300/100	0, 3, 6	8	75	5/8	62.5	4/5	80	-44.91	<.0001

FIG. 6D

Group		Comparator	Survival Mantel-Cox p-value	Tumor Vol. Day 0 to 10 Fold Change
PD1-Fc-41BBL (ARC)	vs.	anti-CTLA4	0.6398	-2.06
ARC + anti-CTLA4 combo	vs.	PD1-Fc-41BBL (ARC)	0.0073	-4.91
ARC + anti-CTLA4 combo	vs.	anti-CTLA4	0.024	-10.15

FIG. 7A

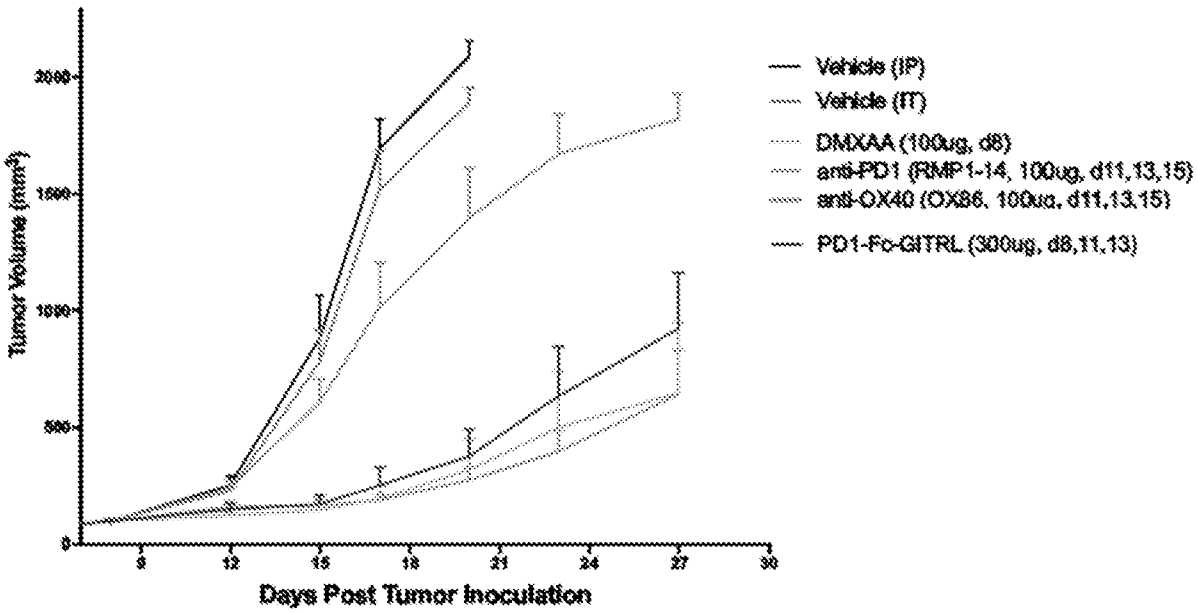
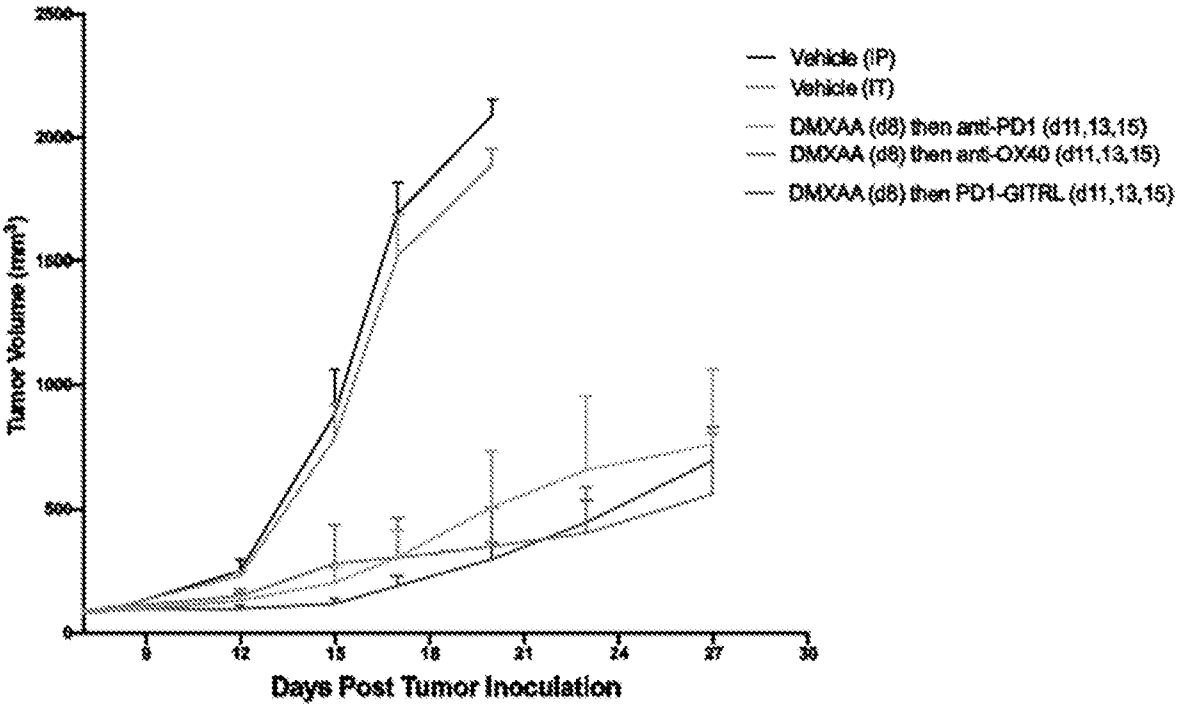

Vehicle (IP)
Vehicle (IT)
DMXAA (100ug, d8)
anti-PD1 (RMP1-14, 100ug, d11,13,15)
anti-OX40 (OX86, 100ug, d11,13,15)
PD1-Fc-GITRL (300ug, d8,11,13)
Tumor Volume (mm3)
Days Post Tumor Inoculation

FIG. 7B


Vehicle (IP)
Vehicle (IT)
DMXAA (d8) then anti-PD1 (d11,13,15)
DMXAA (d8) then anti-OX40 (d11,13,15)
DMXAA (d8) then PD1-GITRL (d11,13,15)
Tumor Volume (mm3)
Days Post Tumor Inoculation

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        35                  40                  45


Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
    50                  55                  60


Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
65                  70                  75                  80


Gln Asp Trp Leu Ser Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys
                85                  90                  95


Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln
            100                 105                 110
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        115                 120                 125

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro
    130                 135                 140

Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
145                 150                 155                 160

Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu
                165                 170                 175

Tyr Ser Arg Leu Thr Val Asp Lys Ser Ser Trp Gln Glu Gly Asn Val
            180                 185                 190

Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln
        195                 200                 205

Lys Ser Leu Ser Leu Ser Leu Gly Lys
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Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
        35                  40                  45
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Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
    50                  55                  60

Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Thr Pro His
65                  70                  75                  80

Ser Asp Trp Leu Ser Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys
                85                  90                  95

Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln
            100                 105                 110

Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met
        115                 120                 125

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro
    130                 135                 140

Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
145                 150                 155                 160

Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu
                165                 170                 175

Tyr Ser Arg Leu Thr Val Asp Lys Ser Ser Trp Gln Glu Gly Asn Val
            180                 185                 190

Phe Ser Cys Ser Val Leu His Glu Ala Leu His Asn His Tyr Thr Gln
        195                 200                 205

Lys Ser Leu Ser Leu Ser Leu Gly Lys
    210                 215
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<210> 3
<211> 217
<212> PRT
<213> Artificial Sequence

<220>

<223> Synthetic Polypeptide

<400> 3

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Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
1               5                  10                  15


Pro Lys Asp Gln Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
            20                  25                  30


Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
        35                  40                  45


Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
    50                  55                  60


Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
65                  70                  75                  80


Gln Asp Trp Leu Ser Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys
                85                  90                  95


Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln
            100                 105                 110


Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met
        115                 120                 125


Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro
    130                 135                 140


Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
145                 150                 155                 160


Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu
                165                 170                 175


Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val
            180                 185                 190
```

Phe Ser Cys Ser Val Leu His Glu Ala Leu His Asn His Tyr Thr Gln
195 200 205

Lys Ser Leu Ser Leu Ser Leu Gly Lys
210 215

<210> 4
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 4

Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro
1 5 10

<210> 5
<211> 11
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 5

Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro
1 5 10

<210> 6
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 6

Ser Lys Tyr Gly Pro Pro
1 5

<210> 7
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 7

Ile Glu Gly Arg Met Asp
1 5

<210> 8
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 8

Gly Gly Gly Val Pro Arg Asp Cys Gly
1 5

<210> 9
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 9

Ile Glu Gly Arg Met Asp Gly Gly Gly Gly Ala Gly Gly Gly Gly
1 5 10 15

<210> 10
<211> 8
<212> PRT
<213> Artificial Sequence

<220>

<223> Synthetic Polypeptide

<400> 10

Gly Gly Gly Ser Gly Gly Gly Ser
1 5

<210> 11
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 11

Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly
1 5 10

<210> 12
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 12

Glu Gly Lys Ser Ser Gly Ser Gly Ser Glu Ser Lys Ser Thr
1 5 10

<210> 13
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 13

Gly Gly Ser Gly
1

<210> 14
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 14

Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly
1 5 10

<210> 15
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 15

Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1 5 10 15

<210> 16
<211> 20
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 16

Glu Ala Ala Ala Arg Glu Ala Ala Ala Arg Glu Ala Ala Ala Arg Glu
1 5 10 15

Ala Ala Ala Arg
20

<210> 17
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 17

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Ala
1   5   10   15

Ser

<210> 18
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 18

Gly Gly Gly Gly Ala Gly Gly Gly Gly
1   5

<210> 19

<400> 19
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<210> 20
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 20

Gly Ser Gly Ser Gly Ser
1   5

<210> 21
<211> 10
<212> PRT

<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 21

Gly Ser Gly Ser Gly Ser Gly Ser Gly Ser
1 5 10

<210> 22
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 22

Gly Gly Gly Gly Ser Ala Ser
1 5

<210> 23
<211> 20
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 23

Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro
1 5 10 15

Ala Pro Ala Pro
20

<210> 24
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 24

Cys Pro Pro Cys
1

<210> 25
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 25

Gly Gly Gly Gly Ser
1 5

<210> 26
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 26

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10

<210> 27
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 27

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

<210> 28

<211> 20
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 28

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Gly Ser
20

<210> 29
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 29

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Gly Ser Gly Gly Gly Gly Ser
20 25

<210> 30
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 30

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser

20 25 30

<210> 31
<211> 35
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 31

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
20 25 30

Gly Gly Ser
35

<210> 32
<211> 40
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 32

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly
1 5 10 15

Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
20 25 30

Gly Gly Ser Gly Gly Gly Gly Ser
35 40

<210> 33
<211> 16
<212> PRT

<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 33

Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

<210> 34
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 34

Gly Gly Gly Gly Gly Gly Gly Gly
1 5

<210> 35
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 35

Gly Gly Gly Gly Gly Gly
1 5

<210> 36
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 36

Glu Ala Ala Ala Lys

1 5

<210> 37
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 37

Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1 5 10

<210> 38
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 38

Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1 5 10 15

<210> 39
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 39

Ala Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Ala
1 5 10

<210> 40
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 40

Ala Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1   5   10   15

Ala

<210> 41
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 41

Ala Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1   5   10   15

Glu Ala Ala Ala Lys Ala
            20

<210> 42
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 42

Ala Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1   5   10   15

Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Ala
            20   25

<210> 43

<211> 46
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 43

Ala Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys
1 5 10 15

Glu Ala Ala Ala Lys Ala Leu Glu Ala Glu Ala Ala Ala Lys Glu Ala
20 25 30

Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Ala
35 40 45

<210> 44
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 44

Pro Ala Pro Ala Pro
1 5

<210> 45
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 45

Lys Glu Ser Gly Ser Val Ser Ser Glu Gln Leu Ala Gln Phe Arg Ser
1 5 10 15

Leu Asp

<210> 46
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 46

Gly Ser Ala Gly Ser Ala Ala Gly Ser Gly Glu Phe
1 5 10

<210> 47
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 47

Gly Gly Gly Ser Glu
1 5

<210> 48
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 48

Gly Ser Glu Ser Gly
1 5

<210> 49
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 49

Gly Ser Glu Gly Ser
1   5

<210> 50
<211> 35
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 50

Gly Glu Gly Gly Ser Gly Glu Gly Ser Ser Gly Glu Gly Ser Ser Ser
1   5   10   15

Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu
            20   25   30

Gly Gly Ser
        35

<210> 51
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 51

Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro Ala Pro Glu Phe Leu
1   5   10   15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
            20   25   30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser

```
        35                  40                  45

Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
    50                  55                  60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
65                  70                  75                  80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser
                85                  90                  95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser
            100                 105                 110

Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
            180                 185                 190

Val Asp Lys Ser Ser Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Leu Gly Lys Ile Glu Gly Arg Met Asp
225                 230
```

<210> 52
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 52

```
Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro Ala Pro Glu Phe Leu
1               5                  10                  15


Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu
            20                  25                  30


Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
        35                  40                  45


Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
    50                  55                  60


Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
65                  70                  75                  80


Tyr Arg Val Val Ser Val Leu Thr Thr Pro His Ser Asp Trp Leu Ser
                85                  90                  95


Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser
            100                 105                 110


Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125


Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
    130                 135                 140


Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160
```

```
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
            180                 185                 190

Val Asp Lys Ser Ser Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Leu His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Leu Gly Lys Ile Glu Gly Arg Met Asp
225                 230
```

<210> 53
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 53

```
Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro Ala Pro Glu Phe Leu
1               5                   10                  15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu
            20                  25                  30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
        35                  40                  45

Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
    50                  55                  60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
65                  70                  75                  80
```

```
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser
                85                  90                  95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser
            100                 105                 110

Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
            180                 185                 190

Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Leu His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Leu Gly Lys Ile Glu Gly Arg Met Asp
225                 230
```

<210> 54
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 54

```
Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu
1               5                  10                  15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
            20                  25                  30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
        35                  40                  45

Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
    50                  55                  60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
65                  70                  75                  80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser
                85                  90                  95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser
            100                 105                 110

Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
            180                 185                 190

Val Asp Lys Ser Ser Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
```

195 200 205

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
210 215 220

Ser Leu Gly Lys Ile Glu Gly Arg Met Asp
225 230

<210> 55
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 55

Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu
1 5 10 15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu
20 25 30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
35 40 45

Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
50 55 60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
65 70 75 80

Tyr Arg Val Val Ser Val Leu Thr Thr Pro His Ser Asp Trp Leu Ser
85 90 95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser
100 105 110

Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln

```
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
            180                 185                 190

Val Asp Lys Ser Ser Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Leu His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Leu Gly Lys Ile Glu Gly Arg Met Asp
225                 230
```

<210> 56
<211> 234
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 56

```
Ser Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu
1               5                   10                  15

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu
            20                  25                  30

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
```

```
        35                  40                  45

Gln Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu
    50                  55                  60

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr
65                  70                  75                  80

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser
                85                  90                  95

Gly Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser
            100                 105                 110

Ile Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln
        115                 120                 125

Val Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val
    130                 135                 140

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
145                 150                 155                 160

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
                165                 170                 175

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr
            180                 185                 190

Val Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val
        195                 200                 205

Leu His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
    210                 215                 220

Ser Leu Gly Lys Ile Glu Gly Arg Met Asp
225                 230
```

<210> 57
<211> 143
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Sequence

<400> 57

Leu Asp Ser Pro Asp Arg Pro Trp Asn Pro Pro Thr Phe Ser Pro Ala
1 5 10 15

Leu Leu Val Val Thr Glu Gly Asp Asn Ala Thr Phe Thr Cys Ser Phe
20 25 30

Ser Asn Thr Ser Glu Ser Phe Val Leu Asn Trp Tyr Arg Met Ser Pro
35 40 45

Ser Asn Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln
50 55 60

Pro Gly Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg
65 70 75 80

Asp Phe His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr
85 90 95

Tyr Leu Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu
100 105 110

Ser Leu Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro
115 120 125

Thr Ala His Pro Ser Pro Ser Pro Arg Pro Ala Gly Gln Phe Gln
130 135 140

<210> 58
<211> 126
<212> PRT

<213> Artificial Sequence

<220>
<223> Synthetic Sequence

<400> 58

Glu Thr Ala Lys Glu Pro Cys Met Ala Lys Phe Gly Pro Leu Pro Ser
1 5 10 15

Lys Trp Gln Met Ala Ser Ser Glu Pro Pro Cys Val Asn Lys Val Ser
20 25 30

Asp Trp Lys Leu Glu Ile Leu Gln Asn Gly Leu Tyr Leu Ile Tyr Gly
35 40 45

Gln Val Ala Pro Asn Ala Asn Tyr Asn Asp Val Ala Pro Phe Glu Val
50 55 60

Arg Leu Tyr Lys Asn Lys Asp Met Ile Gln Thr Leu Thr Asn Lys Ser
65 70 75 80

Lys Ile Gln Asn Val Gly Gly Thr Tyr Glu Leu His Val Gly Asp Thr
85 90 95

Ile Asp Leu Ile Phe Asn Ser Glu His Gln Val Leu Lys Asn Asn Thr
100 105 110

Tyr Trp Gly Ile Ile Leu Leu Ala Asn Pro Gln Phe Ile Ser
115 120 125

<210> 59

<400> 59
000

<210> 60
<211> 215
<212> PRT
<213> Artificial Sequence

<220>

<223> Synthetic Sequence

<400> 60

```
His Arg Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp
1               5                  10                  15

Phe Val Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser
            20                  25                  30

Leu Ser Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe
        35                  40                  45

Val Lys Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser
    50                  55                  60

Phe Glu Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val
65                  70                  75                  80

Ile Ser Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu
                85                  90                  95

Lys Gly Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly
            100                 105                 110

Lys Gln Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln
        115                 120                 125

Val Thr Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile
    130                 135                 140

Ala Ser Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu
145                 150                 155                 160

Arg Ala Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser
                165                 170                 175

Ile His Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe
            180                 185                 190
```

Val Asn Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr
        195   200   205

Ser Phe Gly Leu Leu Lys Leu
    210   215

<210> 61
<211> 205
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Sequence

<400> 61

Ala Cys Pro Trp Ala Val Ser Gly Ala Arg Ala Ser Pro Gly Ser Ala
1   5   10   15

Ala Ser Pro Arg Leu Arg Glu Gly Pro Glu Leu Ser Pro Asp Asp Pro
            20   25   30

Ala Gly Leu Leu Asp Leu Arg Gln Gly Met Phe Ala Gln Leu Val Ala
        35   40   45

Gln Asn Val Leu Leu Ile Asp Gly Pro Leu Ser Trp Tyr Ser Asp Pro
    50   55   60

Gly Leu Ala Gly Val Ser Leu Thr Gly Gly Leu Ser Tyr Lys Glu Asp
65   70   75   80

Thr Lys Glu Leu Val Val Ala Lys Ala Gly Val Tyr Tyr Val Phe Phe
                85   90   95

Gln Leu Glu Leu Arg Arg Val Val Ala Gly Glu Gly Ser Gly Ser Val
            100   105   110

Ser Leu Ala Leu His Leu Gln Pro Leu Arg Ser Ala Ala Gly Ala Ala
        115   120   125

```
Ala Leu Ala Leu Thr Val Asp Leu Pro Pro Ala Ser Ser Glu Ala Arg
    130                 135                 140

Asn Ser Ala Phe Gly Phe Gln Gly Arg Leu Leu His Leu Ser Ala Gly
145                 150                 155                 160

Gln Arg Leu Gly Val His Leu His Thr Glu Ala Arg Ala Arg His Ala
                165                 170                 175

Trp Gln Leu Thr Gln Gly Ala Thr Val Leu Gly Leu Phe Arg Val Thr
            180                 185                 190

Pro Glu Ile Pro Ala Gly Leu Pro Ser Pro Arg Ser Glu
        195                 200                 205
```

<210> 62
<211> 505
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Sequence

<400> 62

```
Leu Asp Ser Pro Asp Arg Pro Trp Asn Pro Pro Thr Phe Ser Pro Ala
1               5                   10                  15

Leu Leu Val Val Thr Glu Gly Asp Asn Ala Thr Phe Thr Cys Ser Phe
            20                  25                  30

Ser Asn Thr Ser Glu Ser Phe Val Leu Asn Trp Tyr Arg Met Ser Pro
        35                  40                  45

Ser Asn Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln
    50                  55                  60

Pro Gly Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg
65                  70                  75                  80
```

```
Asp Phe His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr
                85                  90                  95

Tyr Leu Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu
            100                 105                 110

Ser Leu Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro
        115                 120                 125

Thr Ala His Pro Ser Pro Ser Pro Arg Pro Ala Gly Gln Phe Gln Ser
    130                 135                 140

Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly
145                 150                 155                 160

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu Met
                165                 170                 175

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln
            180                 185                 190

Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val
        195                 200                 205

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr
    210                 215                 220

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser Gly
225                 230                 235                 240

Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser Ile
                245                 250                 255

Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln Val
            260                 265                 270
```

```
Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser
        275                 280                 285

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
    290                 295                 300

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
305                 310                 315                 320

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val
                325                 330                 335

Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Leu
            340                 345                 350

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
        355                 360                 365

Leu Gly Lys Ile Glu Gly Arg Met Asp Gln Leu Glu Thr Ala Lys Glu
    370                 375                 380

Pro Cys Met Ala Lys Phe Gly Pro Leu Pro Ser Lys Trp Gln Met Ala
385                 390                 395                 400

Ser Ser Glu Pro Pro Cys Val Asn Lys Val Ser Asp Trp Lys Leu Glu
                405                 410                 415

Ile Leu Gln Asn Gly Leu Tyr Leu Ile Tyr Gly Gln Val Ala Pro Asn
            420                 425                 430

Ala Asn Tyr Asn Asp Val Ala Pro Phe Glu Val Arg Leu Tyr Lys Asn
        435                 440                 445

Lys Asp Met Ile Gln Thr Leu Thr Asn Lys Ser Lys Ile Gln Asn Val
    450                 455                 460

Gly Gly Thr Tyr Glu Leu His Val Gly Asp Thr Ile Asp Leu Ile Phe
465                 470                 475                 480
```

```
Asn Ser Glu His Gln Val Leu Lys Asn Asn Thr Tyr Trp Gly Ile Ile
                485                 490                 495


Leu Leu Ala Asn Pro Gln Phe Ile Ser
            500                 505


<210>  63
<211>  592
<212>  PRT
<213>  Artificial Sequence

<220>
<223>  Synthetic Sequence

<400>  63

Leu Asp Ser Pro Asp Arg Pro Trp Asn Pro Pro Thr Phe Ser Pro Ala
1               5                   10                  15


Leu Leu Val Val Thr Glu Gly Asp Asn Ala Thr Phe Thr Cys Ser Phe
            20                  25                  30


Ser Asn Thr Ser Glu Ser Phe Val Leu Asn Trp Tyr Arg Met Ser Pro
        35                  40                  45


Ser Asn Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln
    50                  55                  60


Pro Gly Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg
65                  70                  75                  80


Asp Phe His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr
                85                  90                  95


Tyr Leu Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu
            100                 105                 110


Ser Leu Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro
        115                 120                 125
```

```
Thr Ala His Pro Ser Pro Ser Pro Arg Pro Ala Gly Gln Phe Gln Ser
    130                 135                 140

Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly
145                 150                 155                 160

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu Met
                165                 170                 175

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln
            180                 185                 190

Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val
        195                 200                 205

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr
    210                 215                 220

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser Gly
225                 230                 235                 240

Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser Ile
                245                 250                 255

Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln Val
            260                 265                 270

Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser
        275                 280                 285

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
    290                 295                 300

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
305                 310                 315                 320
```

```
Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val
                325                 330                 335

Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Leu
            340                 345                 350

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
        355                 360                 365

Leu Gly Lys Ile Glu Gly Arg Met Asp His Arg Arg Leu Asp Lys Ile
    370                 375                 380

Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val Phe Met Lys Thr Ile
385                 390                 395                 400

Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser Leu Leu Asn Cys Glu
                405                 410                 415

Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys Asp Ile Met Leu Asn
            420                 425                 430

Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu Met Gln Lys Gly Asp
        435                 440                 445

Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser Glu Ala Ser Ser Lys
    450                 455                 460

Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly Tyr Tyr Thr Met Ser
465                 470                 475                 480

Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln Leu Thr Val Lys Arg
                485                 490                 495

Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr Phe Cys Ser Asn Arg
            500                 505                 510

Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser Leu Cys Leu Lys Ser
        515                 520                 525
```

Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala Ala Asn Thr His Ser
530 535 540

Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His Leu Gly Gly Val Phe
545 550 555 560

Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn Val Thr Asp Pro Ser
565 570 575

Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe Gly Leu Leu Lys Leu
580 585 590

<210> 64
<211> 582
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Sequence

<400> 64

Leu Asp Ser Pro Asp Arg Pro Trp Asn Pro Pro Thr Phe Ser Pro Ala
1 5 10 15

Leu Leu Val Val Thr Glu Gly Asp Asn Ala Thr Phe Thr Cys Ser Phe
20 25 30

Ser Asn Thr Ser Glu Ser Phe Val Leu Asn Trp Tyr Arg Met Ser Pro
35 40 45

Ser Asn Gln Thr Asp Lys Leu Ala Ala Phe Pro Glu Asp Arg Ser Gln
50 55 60

Pro Gly Gln Asp Cys Arg Phe Arg Val Thr Gln Leu Pro Asn Gly Arg
65 70 75 80

Asp Phe His Met Ser Val Val Arg Ala Arg Arg Asn Asp Ser Gly Thr
85 90 95

```
Tyr Leu Cys Gly Ala Ile Ser Leu Ala Pro Lys Ala Gln Ile Lys Glu
            100                 105                 110

Ser Leu Arg Ala Glu Leu Arg Val Thr Glu Arg Arg Ala Glu Val Pro
        115                 120                 125

Thr Ala His Pro Ser Pro Ser Pro Arg Pro Ala Gly Gln Phe Gln Ser
    130                 135                 140

Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly
145                 150                 155                 160

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Gln Leu Met
                165                 170                 175

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln
            180                 185                 190

Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val
        195                 200                 205

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr
    210                 215                 220

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Ser Gly
225                 230                 235                 240

Lys Glu Tyr Lys Cys Lys Val Ser Ser Lys Gly Leu Pro Ser Ser Ile
                245                 250                 255

Glu Lys Thr Ile Ser Asn Ala Thr Gly Gln Pro Arg Glu Pro Gln Val
            260                 265                 270

Tyr Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser
        275                 280                 285
```

```
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
    290                 295                 300

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
305                 310                 315                 320

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val
                325                 330                 335

Asp Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Leu
            340                 345                 350

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
        355                 360                 365

Leu Gly Lys Ile Glu Gly Arg Met Asp Ala Cys Pro Trp Ala Val Ser
    370                 375                 380

Gly Ala Arg Ala Ser Pro Gly Ser Ala Ala Ser Pro Arg Leu Arg Glu
385                 390                 395                 400

Gly Pro Glu Leu Ser Pro Asp Asp Pro Ala Gly Leu Leu Asp Leu Arg
                405                 410                 415

Gln Gly Met Phe Ala Gln Leu Val Ala Gln Asn Val Leu Leu Ile Asp
            420                 425                 430

Gly Pro Leu Ser Trp Tyr Ser Asp Pro Gly Leu Ala Gly Val Ser Leu
        435                 440                 445

Thr Gly Gly Leu Ser Tyr Lys Glu Asp Thr Lys Glu Leu Val Val Ala
    450                 455                 460

Lys Ala Gly Val Tyr Tyr Val Phe Phe Gln Leu Glu Leu Arg Arg Val
465                 470                 475                 480

Val Ala Gly Glu Gly Ser Gly Ser Val Ser Leu Ala Leu His Leu Gln
                485                 490                 495
```

```
Pro Leu Arg Ser Ala Ala Gly Ala Ala Ala Leu Ala Leu Thr Val Asp
            500                 505                 510

Leu Pro Pro Ala Ser Ser Glu Ala Arg Asn Ser Ala Phe Gly Phe Gln
        515                 520                 525

Gly Arg Leu Leu His Leu Ser Ala Gly Gln Arg Leu Gly Val His Leu
    530                 535                 540

His Thr Glu Ala Arg Ala Arg His Ala Trp Gln Leu Thr Gln Gly Ala
545                 550                 555                 560

Thr Val Leu Gly Leu Phe Arg Val Thr Pro Glu Ile Pro Ala Gly Leu
                565                 570                 575

Pro Ser Pro Arg Ser Glu
            580
```