

BISPECIFIC IL-2- AND ANTI-PD-1-BASED THERAPEUTICS AND METHODS OF USE THEREOF

FIELD

[0001] The present disclosure relates generally to IL2-based therapeutics and methods of use thereof and more specifically to a fusion protein comprising an IL2 moiety and an antigen-binding moiety that binds specifically to human PD-1 as well as methods of use thereof.

BACKGROUND

[0002] Interleukin 2 (IL-2 or IL2) is a pluripotent cytokine produced primarily by activated T cells. It stimulates the proliferation and differentiation of T cells, induces the generation of cytotoxic T lymphocytes (CTLs) and the differentiation of peripheral blood lymphocytes to cytotoxic cells and lymphokine-activated killer (LAK) cells, promotes cytokine and cytolytic molecule expression by T cells, facilitates the proliferation and differentiation of B-cells and the synthesis of immunoglobulin by B-cells, and stimulates the generation, proliferation, and activation of natural killer (NK) cells (see Waldmann, 2006, *Nat Rev Immunol* 6:595-601 and Malek, 2008, *Annu Rev Immunol* 26:453-79). IL2 is involved in the maintenance of peripheral CD4⁺ CD25⁺ regulatory T (Treg) cells (see, e.g., Fontenot *et al.*, 2005, *Nature Immunol* 6:1142-51), which are also known as suppressor T cells. They suppress effector T cells from destroying their (self-)target, either through cell-cell contact by inhibiting T cell help and activation or through release of immunosuppressive cytokines such as IL-10 or TGF β . Depletion of Treg cells was shown to enhance IL2-induced anti-tumor immunity (Imai *et al.*, 2007, *Cancer Sci* 98:416-23).

[0003] However, IL2 is not optimal for inhibiting tumor growth due to its pleiotropic effects. The use of IL2 as an antineoplastic agent has been limited by the serious toxicities that accompany the doses necessary for a tumor response. Proleukin® (marketed by Prometheus Laboratories, San Diego, Calif.) is a recombinant form of IL2 that is approved for the treatment of metastatic melanoma and metastatic renal cancer, but its side effects are so severe that its use is only recommended in a hospital setting with access to intensive care. The major side effect of IL2 therapy is vascular leak syndrome (VLS), which leads to the accumulation of interstitial fluid in the lungs and liver, resulting in pulmonary edema and liver damage. There is no treatment for VLS other than withdrawal of IL2. It has been shown that IL2-induced pulmonary edema resulted from direct binding of IL2 to lung endothelial cells, which express low to intermediate levels of functional high-affinity IL2 receptors (Krieg *et al.*, 2010, *Proc Nat Acad*

Sci USA 107:11906-11). Despite the general acceptance in the field of CD122 directed IL2 therapies being developed for cancer therapy, it has been surprisingly discovered that such molecules have poor therapeutic indices for cancer therapy, with high, toxic doses required to confer modest anti-cancer effects.

[0004] Thus, there is a pressing need in the art for novel IL2 therapies with improved therapeutic efficacy and safety profiles.

SUMMARY

[0005] This disclosure addresses one or more of the needs mentioned above. The fusion proteins of the present disclosure comprise an antigen-binding moiety that specifically binds to human PD-1, which targets tumor-reactive T cells. The antigen-binding moiety is helpful in selectively reconstituting activity on tumor-reactive T cells. The fusion proteins further comprise an IL2 moiety wherein the IL2 moiety comprises IL2 bound to IL2R α . In certain embodiments, the fusion proteins comprise an unmodified IL2 sequence. In certain embodiments, the fusion proteins retain an ability to engage endogenous IL2R α . The fusion protein comprises the IL2 moiety in a trans-sequestered conformation. This maintains engagement with activated CD8⁺ T cells; however, the bound configuration of the IL2 moiety helps in masking of the IL2 and attenuating its activity, thus leading to reduced systemic toxicity. For example, the fusion proteins of the present disclosure do not induce acute pulmonary edema (vascular leakage) in mice as compared to wild-type IL2. The fusion proteins of the present disclosure provide enhanced anti-tumor efficacy and improved therapeutic index as compared to IL2 alone or in combination with a PD-1 inhibitor (*e.g.*, an anti-PD-1 antibody or antigen-binding fragment thereof).

[0006] In one aspect, the disclosed technology relates to a fusion protein including: (i) an antigen-binding moiety that binds specifically to human programmed cell death protein 1 (PD-1) and (ii) an interleukin 2 (IL2) moiety. In some embodiments, the antigen-binding moiety includes an antibody or antigen-binding fragment thereof that binds specifically to human PD-1. In some embodiments, the antibody or antigen-binding fragment thereof that binds to human PD-1 is a human monoclonal antibody. In some embodiments, the antigen-binding moiety includes three heavy chain complementarity determining regions (HCDRs) (HCDR1, HCDR2, and HCDR3) and three light chain CDRs (LCDR1, LCDR2, and LCDR3), wherein: HCDR1 includes an amino acid sequence of SEQ ID NO: 43, 4, or 24; HCDR2 includes an amino acid sequence of SEQ ID NO: 45, 6, or 26; HCDR3 includes an amino acid sequence of SEQ ID NO: 47, 8, or 28; LCDR1 includes an amino acid sequence of SEQ ID NO: 12 or 32; LCDR2 includes

an amino acid sequence of SEQ ID NO: 14; and LCDR3 includes an amino acid sequence of SEQ ID NO: 16 or 35.

[0007] In some embodiments, the antigen-binding moiety includes HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 including respective amino acid sequences of (i) SEQ ID NOs: 43, 45, 47, 12, 14, and 16; (ii) SEQ ID NOs: 4, 6, 8, 12, 14, and 16; or (iii) SEQ ID NOs: 24, 26, 28, 32, 14, and 35. In some embodiments, the antigen-binding moiety includes HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 including respective amino acid sequences of SEQ ID NOs: 43, 45, 47, 12, 14, and 16. In some embodiments, the antigen-binding moiety includes a heavy chain variable region (HCVR) including an amino acid sequence of SEQ ID NO: 41, 2, or 22; and a light chain variable region (LCVR) including an amino acid sequence of SEQ ID NO: 10 or 30. In some embodiments, the HCVR includes the amino acid sequence of SEQ ID NO: 2 and the LCVR includes the amino acid sequence of SEQ ID NO: 10. In some embodiments, the HCVR includes the amino acid sequence of SEQ ID NO: 22 and the LCVR includes the amino acid sequence of SEQ ID NO: 30. In some embodiments, the HCVR includes the amino acid sequence of SEQ ID NO: 41 and the LCVR includes the amino acid sequence of SEQ ID NO: 10. In some embodiments, the antigen-binding moiety includes a heavy chain constant region of SEQ ID NO: 55 and a light chain constant region of SEQ ID NO: 56. In some embodiments, the antigen-binding moiety includes a heavy chain and a light chain wherein the heavy chain includes an amino acid sequence of SEQ ID NO: 61, 57, or 59 and the light chain includes an amino acid sequence of SEQ ID NO: 62, 58, or 60. In some embodiments, the antigen-binding moiety includes a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62, 57/58, or 59/60.

[0008] In some embodiments, the antigen-binding moiety includes a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62. In some embodiments, the IL2 moiety includes (i) IL2 or a fragment thereof; and (ii) IL2 receptor alpha (IL2R α) or a fragment thereof. In some embodiments, the IL2 or fragment thereof is human IL2 (hIL2) or a fragment thereof. In some embodiments, the IL2R α or fragment thereof is human IL2R α (hIL2R α) or a fragment thereof. In some embodiments, the IL2 or fragment thereof includes the amino acid sequence of SEQ ID NO: 53. In some embodiments, the IL2R α or fragment thereof includes the amino acid sequence of SEQ ID NO: 51. In some embodiments, the IL2 or fragment thereof is connected to the C-terminal of the IL2R α or fragment thereof via a linker. In some embodiments, the IL2 moiety is connected to the C-terminal of the heavy chain constant region of the antigen-binding moiety via a linker. In some embodiments, the linker includes an amino acid sequence of one or more repeats of GGGGS (SEQ ID NO: 67). In some embodiments, the linker includes an amino

acid sequence of SEQ ID NO: 50 or 52. In some embodiments, the IL2 moiety includes the amino acid sequence of SEQ ID NO: 54.

[0009] In another aspect, the disclosed technology relates to a fusion protein including: (i) a first polypeptide including a light chain variable region (LCVR) of a human antibody that binds specifically to human PD-1; and (ii) a second polypeptide that includes (a) a heavy chain variable region (HCVR) of the antibody that binds specifically to human PD-1 and (b) an IL2 moiety. In some embodiments, the HCVR includes three heavy chain complementarity determining regions (HCDRs) (HCDR1, HCDR2, and HCDR3) and the LCVR includes three light chain CDRs (LCDR1, LCDR2, and LCDR3), wherein: HCDR1 includes an amino acid sequence of SEQ ID NO: 43, 4, or 24; HCDR2 includes an amino acid sequence of SEQ ID NO: 45, 6, or 26; HCDR3 includes an amino acid sequence of SEQ ID NO: 47, 8, or 28; LCDR1 includes an amino acid sequence of SEQ ID NO: 12 or 32; LCDR2 includes an amino acid sequence of SEQ ID NO: 14; and LCDR3 includes an amino acid sequence of SEQ ID NO: 16 or 35. In some embodiments, the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 including amino acid sequences of (i) SEQ ID NOs: 43, 45, 47, 12, 14, and 16; (ii) SEQ ID NOs: 4, 6, 8, 12, 14, and 16; or (iii) SEQ ID NOs: 24, 26, 28, 32, 14 and 35. In some embodiments, the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 include the amino acid sequences of SEQ ID NOs: 43, 45, 47, 12, 14, and 16, respectively.

[0010] In some embodiments, the HCVR and the LCVR include amino acid sequences of (i) SEQ NOs: 41 and 10; (ii) SEQ ID NOs: 2 and 10; or (iii) SEQ ID NOs: 22 and 30. In some embodiments, the first polypeptide includes a light chain constant region linked to the LCVR. In some embodiments, the light chain constant region includes an amino acid sequence of SEQ ID NO: 56. In some embodiments, the second polypeptide includes a heavy chain constant region linked to the HCVR and wherein the IL2 moiety is linked to the C-terminal of the heavy chain constant region. In some embodiments, the heavy chain constant region includes an amino acid sequence of SEQ ID NO: 55. In some embodiments, the first polypeptide includes a light chain sequence of SEQ ID NO: 62, 58, or 60. In some embodiments, the second polypeptide includes a heavy chain sequence of SEQ ID NO: 61, 57, or 59.

[0011] In some embodiments, the fusion protein includes a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62, 57/58, or 59/60. In some embodiments, the fusion protein includes a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62. In some embodiments, the IL2 moiety includes (i) IL2 or a fragment thereof; and (ii) IL2R α or a fragment thereof. In some embodiments, the IL2 moiety is connected to the C-terminal of the heavy chain constant

region via a linker. In some embodiments, the IL2 or fragment thereof is connected to the C-terminal of IL2R α or fragment thereof via a linker. In some embodiments, the IL2 or fragment thereof is human IL2 (hIL2) or a fragment thereof. In some embodiments, the IL2R α or fragment thereof is human IL2R α (hIL2R α) or a fragment thereof. In some embodiments, the IL2 or fragment thereof includes the amino acid sequence of SEQ ID NO: 53. In some embodiments, the IL2R α or fragment thereof includes the amino acid sequence of SEQ ID NO: 51. In some embodiments, the linker includes an amino acid sequence of one or more repeats of GGGGS (SEQ ID NO: 67). In some embodiments, the linker includes an amino acid sequence of SEQ ID NO: 50 or 52. In some embodiments, the IL2 moiety includes the amino acid sequence of SEQ ID NO: 54. In some embodiments, the first polypeptide includes the amino acid sequence of SEQ ID NO: 62, 58, or 60.

[0012] In some embodiments, the second polypeptide includes the amino acid sequence of SEQ ID NO: 63, 64, or 65. In some embodiments, the first polypeptide includes the amino acid sequence of SEQ ID NO: 58 and the second polypeptide includes the amino acid sequence of SEQ ID NO: 64. In some embodiments, the first polypeptide includes the amino acid sequence of SEQ ID NO: 60 and the second polypeptide includes the amino acid sequence of SEQ ID NO: 63. In some embodiments, the first polypeptide includes the amino acid sequence of SEQ ID NO: 62 and the second polypeptide includes the amino acid sequence of SEQ ID NO: 65. In some embodiments, the fusion protein forms a dimeric fusion protein. In some embodiments, the fusion protein dimerizes through their respective heavy chain constant regions.

[0013] In some embodiments, the fusion protein does not cross-compete with REGN2810, pembrolizumab or nivolumab for binding to PD-1. In some embodiments, the fusion protein exhibits reduced activity in activating human IL2R α / β / γ and IL2R β / γ complexes as compared to IL2. In some embodiments, the fusion protein exhibits increased activity in activating human IL2R α as compared to a non-targeted IL2R α -IL2. In some embodiments, the fusion protein exhibits increased activity in stimulating T cells as measured by a level of IFN- γ release as compared to a wild-type human IL2. In some embodiments, the fusion protein exhibits attenuated binding to IL2R α , IL2R β and IL2R γ .

[0014] In another aspect, the disclosed technology relates to a nucleic acid or plurality of nucleic acids including a polynucleotide sequence encoding a fusion protein disclosed herein. In another aspect, the disclosed technology relates to vector including a nucleic acid or plurality of nucleic acids disclosed herein. In another aspect, the disclosed technology relates to a host cell including a nucleic acid or plurality of nucleic acids or a vector

disclosed herein. In some embodiments, the host cell expresses a first vector including a polynucleotide sequence that encodes the first polypeptide of a fusion protein disclosed herein and a second vector including a polynucleotide sequence that encodes the second polypeptide of a fusion protein disclosed herein.

[0015] In another aspect, the disclosed technology relates to a method of producing a fusion protein disclosed herein, including culturing a host cell disclosed herein under conditions permitting production of the fusion protein or fragment, and recovering the fusion protein or fragment thereof so produced.

[0016] In another aspect, the disclosed technology relates to a pharmaceutical composition including a fusion protein disclosed herein. In some embodiments, the pharmaceutical composition further includes an anti-PD-1 antibody or antigen-binding fragment thereof. In some embodiments, the anti-PD-1 antibody or antigen-binding fragment thereof does not cross-compete with the fusion protein for binding to PD-1.

[0017] In another aspect, the disclosed technology relates to a method of treating cancer, including administering to a subject in need thereof a therapeutically effective amount of a fusion protein or pharmaceutical composition disclosed herein. In some embodiments, the method includes administering to the subject the fusion protein in an amount of 0.005 mg/kg to 10 mg/kg of the subject's body weight. In some embodiments, the method further includes administering a second therapeutic agent or therapy to the subject.

BRIEF DESCRIPTION OF THE FIGURES

[0018] FIGS. 1A, 1B, 1C, and 1D are graphs showing the results of an *in vivo* study as described in Example 6. FIGS. 1A and 1B show tumor volume (mm³) (FIG. 1A) and percent survival (FIG. 1B) of isotype control Ab, isotype control-hIL2R α -IL2 + anti-PD-1, REGN10486 + Isotype control Ab treated mice. FIGS. 1C and 1D show tumor volume (mm³) (FIG. 1C) and percent survival (FIG. 1D) of Isotype control, REGN10486, REGN10595, and REGN10597 treated mice.

[0019] FIGS. 2A and 2B are a set of graphs showing the results of a FACS binding assay that compares binding of anti-alpha-hPD1 antibody ("ahPD1" or "ahPD1") parental antibodies to PD1+ cells (FIG. 2A) with binding of anti-ahPD1 antibody-IL2R α -IL2 fusion constructs to PD1+ cells (FIG. 2B), as described in Example 8.

[0020] FIGS. 3A and 3B show the experimental design (FIG. 3A) and resulting native mass spectra (FIG. 3B) of a mixture of anti-PD-1-IL2R α -IL2 + an hIgG4 antibody compared to specified antibody mixtures, as described in Example 9.

[0021] FIGS. 4A and 4B are a set of graphs showing IFN γ release and proliferation of T-cells in response to the antibody or cytokine titration as described in Example 10. Allogeneic PBMCs mixed with T-cells were treated with a dose titration of recombinant IL2, anti-PD-1-IL2R α -IL2 (REGN10597, REGN10595, and REGN10486) or Isotype-IL2R α -IL2 control (REGN9903) and IFN γ release (FIG. 4A) and proliferation (FIG. 4B) were evaluated.

[0022] FIGS. 5A and 5B are a set of graphs showing IFN γ release of T-cells in response to antibody or cytokine titration in the absence (FIG. 5A) or presence (FIG. 5B) of REGN2810 as described in Example 11. RAJI/CD80 KO/CD86 KO/PDL1 cells were incubated with T-cells and treated with a fixed dose of anti-CD3 $\times$ CD20 (REGN1979) and a dose titration of isotype control (REGN7540), isotype-IL2R α -IL2 antibody (REGN9903), anti-PD1 antibody with variable regions of mAb9048 (REGN15187), anti-PD1-IL2R α -IL2 (REGN10597), or a combination of REGN9903 + REGN15187 in the absence (FIG. 5A) or presence (FIG. 5B) of REGN2810. IFN γ release was measured.

[0023] FIGS. 6A, 6B, 6C, 6D, 6E, 6F, 6G, and 6H are graphs showing *in vivo* activity assessment of anti-PD1-IL2R α -IL2 fusion construct REGN10597 in PD1 $\times$ LAG3 humanized mice, primarily in comparison to: the combination of a non-targeted control REGN9904 and a PD-1 blocking antibody REGN2810 (FIGS. 6A and 6B), the comparator anti-PD1-targeted “non-alpha” (3m) molecule REGN13233 (FIGS. 6C, 6D, 6E, and 6F), and a construct containing all the same components as REGN10597 but with IL2 and IL2R α fused to each other in the reverse order (FIGS. 6G and 6H), as described in Example 12.

[0024] FIGS. 7A and 7B show graphs for the IL2 binding assay described in Example 13. YT/STAT5-Luc/IL2Ra KO (FIG. 7A) or YT/STAT5-Luc/IL2Ra OE (FIG. 7B) were incubated with a titration of an antibody in-line fused to IL2 (REGN8512; gray open circle with dashed line) or an antibody in-line fused to IL2Ra and IL2 (REGN9904; black filled circle with solid line). Cells were washed, stained with a secondary AF647 anti-human IgG, washed, fixed, and acquired on an iQue Plus flow cytometer.

[0025] FIGS. 8A and 8B are graphs showing IL2 reporter assay comparison of Ab-IL2Ra-IL2 and Ab-IL2-IL2Ra, as described in Example 14. YT/STAT5-Luc/IL2Ra KO/hPD1 (FIG. 8A) or YT/STAT5-Luc/hIL2Ra/hPD1 (FIG. 8B) were incubated with a titration of a non-targeted IL2Ra-IL2 (black open round symbol with black dashed line, REGN9903), a non-targeted IL2-IL2Ra (gray square open symbol with gray dashed line), anti-PD1-IL2Ra-IL2 (black filled round symbol with black solid line, REGN10597), or an anti-PD1-IL2-IL2Ra (gray square filled symbol with gray solid line). 4h and 30minutes later, STAT5 activity was assessed by luminescent readout.

[0026] FIGS. 9A and 9B are graphs showing IL2 reporter assay comparison of Ab-IL2Ra-IL2 and Ab-IL2(3m), as described in Example 14. YT/STAT5-Luc/IL2Ra KO/hPD1 (FIG. 9A) or YT/STAT5-Luc/hIL2Ra/hPD1 (FIG. 9B) were incubated with a titration of a non-targeted IL2Ra-IL2 (black open round symbol with black dashed line, REGN9904), a non-targeted IL2(3m) (gray diamond open symbol with gray dashed line), anti-PD1-IL2Ra-IL2 (black filled round symbol with black solid line, REGN10597), or an anti-PD1-IL2(3m) (gray diamond filled symbol with gray solid line). 4h later, STAT5 activity was assessed by luminescent readout.

DETAILED DESCRIPTION

[0027] It is to be understood that the present disclosure is not limited to the particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, and that the scope of the present disclosure will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, preferred methods and materials are now described. All publications mentioned herein are hereby incorporated by reference in their entirety unless otherwise stated.

Fusion Proteins

[0028] In one aspect, this disclosure provides a fusion protein comprising: (i) an antigen-binding moiety that binds specifically to human PD-1, and (ii) an IL2 moiety. The disclosed fusion protein is surprisingly effective in stimulating T cells and inhibiting tumor growth as compared to an anti-hPD1 antibody (such as REGN2810). In certain embodiments, the fusion protein exhibits a better safety profile as compared to an anti-PD-1-targeted IL2 molecule (such as REGN13233).

[0029] The disclosed fusion protein can be monomers or multimers, e.g., dimers (homodimers or heterodimers) or high order complexes. For simplicity, the fusion proteins that are homodimers (or higher order multimers of the same polypeptide) are described by their constituent monomers; however, upon recombinant expression of the component monomers in a suitable cell line, a homodimeric (or higher order multimer) molecule can be produced.

[0030] In some embodiments, the antigen-binding moiety comprises an antibody or antigen-binding fragment thereof that binds specifically to human PD-1. In some embodiments, the antigen-binding moiety that binds to human PD-1 is a human monoclonal antibody.

[0031] As used herein, the term “antibody” refers to an immunoglobulin molecule comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds (*i.e.*, “full antibody molecules”), as well as a multimer thereof (*e.g.*, IgM) or antigen-binding fragments thereof. Each heavy chain is comprised of a heavy chain variable region (“HCVR” or “VH”) and a heavy chain constant region (comprised of domains CH1, CH2, and CH3). Each light chain is comprised of a light chain variable region (“LCVR” or “VL”) and a light chain constant region (CL). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. In some embodiments, the FRs of the antibody (or antigen-binding fragment thereof) may be identical to the human germline sequences or may be naturally or artificially modified. An amino acid consensus sequence may be defined based on a side-by-side analysis of two or more CDRs. The term “antibody,” as used herein, also includes antigen-binding fragments of full antibody molecules.

[0032] As used herein, the terms “antigen-binding fragment” of an antibody, “antigen-binding portion” of an antibody, and the like, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen to form a complex. Antigen-binding fragments of an antibody may be derived, *e.g.*, from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, *e.g.*, commercial sources, DNA libraries (including, *e.g.*, phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, etc.

[0033] Non-limiting examples of antigen-binding fragments include: (i) Fab fragments; (ii) F(ab')₂ fragments; (iii) Fd fragments; (iv) Fv fragments; (v) single-chain Fv (scFv) molecules; (vi) dAb fragments; and (vii) minimal recognition units consisting of the amino acid residues that

mimic the hypervariable region of an antibody (e.g., an isolated complementarity determining region (CDR) such as a CDR3 peptide), or a constrained FR3-CDR3-FR4 peptide. Other engineered molecules, such as domain-specific antibodies, single domain antibodies, domain-deleted antibodies, chimeric antibodies, CDR-grafted antibodies, diabodies, triabodies, tetrabodies, minibodies, nanobodies (e.g., monovalent nanobodies, bivalent nanobodies, etc.), small modular immunopharmaceuticals (SMIPs), and shark variable IgNAR domains, are also encompassed within the expression “antigen-binding fragment,” as used herein.

[0034] An antigen-binding fragment of an antibody will typically comprise at least one variable domain. The variable domain may be of any size or amino acid composition and will generally comprise at least one CDR adjacent to or in frame with one or more framework sequences. In antigen-binding fragments having a V_H domain associated with a V_L domain, the V_H and V_L domains may be situated relative to one another in any suitable arrangement. For example, the variable region may be dimeric and contain V_H - V_H , V_H - V_L or V_L - V_L dimers. Alternatively, the antigen-binding fragment of an antibody may contain a monomeric V_H or V_L domain.

[0035] In some embodiments, an antigen-binding fragment of an antibody may contain at least one variable domain covalently linked to at least one constant domain. Non-limiting, exemplary configurations of variable and constant domains that may be found within an antigen-binding fragment of an antibody of the present disclosure include: (i) V_H - C_H1 ; (ii) V_H - C_H2 ; (iii) V_H - C_H3 ; (iv) V_H - C_H1 - C_H2 ; (v) V_H - C_H1 - C_H2 - C_H3 ; (vi) V_H - C_H2 - C_H3 ; (vii) V_H - C_L ; (viii) V_L - C_H1 ; (ix) V_L - C_H2 ; (x) V_L - C_H3 ; (xi) V_L - C_H1 - C_H2 ; (xii) V_L - C_H1 - C_H2 - C_H3 ; (xiii) V_L - C_H2 - C_H3 ; and (xiv) V_L - C_L . In any configuration of variable and constant domains, including any of the exemplary configurations listed above, the variable and constant domains may be either directly linked to one another or may be linked by a full or partial hinge or linker region. A hinge region may consist of at least 2 (e.g., 5, 10, 15, 20, 40, 60 or more) amino acids which result in a flexible or semi-flexible linkage between adjacent variable and/or constant domains in a single polypeptide molecule. Moreover, an antigen-binding fragment of an antibody of the present disclosure may comprise a homo-dimer or hetero-dimer (or other multimer) of any of the variable and constant domain configurations listed above in non-covalent association with one another and/or with one or more monomeric V_H or V_L domain (e.g., by disulfide bond(s)).

[0036] The antibodies or antigen-binding fragments thereof comprised in the fusion proteins disclosed herein may be human antibodies or antigen-binding fragments thereof. As used herein, the term “human antibody” refers to antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies or

antigen-binding fragments thereof that are comprised in the fusion proteins of the present disclosure may nonetheless include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example, in the CDRs and, in particular, CDR3. However, the term “human antibody,” as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0037] The antibodies comprised in the fusion proteins disclosed herein may be recombinant human antibodies. As used herein, the term “recombinant human antibody” includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see, e.g., Taylor *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In some embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis), and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[0038] In some embodiments, the antibodies or antigen-binding fragments thereof specifically bind human PD-1. As used herein, the term “specifically binds” or the like means that an antibody or antigen-binding fragment thereof forms a complex with an antigen that is relatively stable under physiologic conditions. Methods for determining whether an antibody specifically binds to an antigen are well known in the art and include, for example, equilibrium dialysis, surface plasmon resonance, and the like. For example, an antibody that “specifically binds” human PD-1, as used in the context of the present disclosure, includes antibodies that bind human PD-1 or a portion thereof with a K_D of less than about 500 nM, less than about 300 nM, less than about 200 nM, less than about 100 nM, less than about 90 nM, less than about 80 nM, less than about 70 nM, less than about 60 nM, less than about 50 nM, less than about 40 nM, less than about 30 nM, less than about 20 nM, less than about 10 nM, less than about 5

nM, less than about 4 nM, less than about 3 nM, less than about 2 nM, less than about 1 nM or less than about 0.5 nM, as measured in a surface plasmon resonance assay. An isolated antibody that specifically binds human PD-1 may, however, have cross-reactivity to other antigens, such as PD-1 molecules from other (non-human) species.

[0039] In some embodiments, the antibody or antigen-binding fragment thereof comprises three heavy chain complementarity determining regions (HCDRs) (HCDR1, HCDR2, and HCDR3) and three light chain CDRs (LCDR1, LCDR2, and LCDR3), wherein: HCDR1 comprises an amino acid sequence of SEQ ID NO: 4, 24, or 43; HCDR2 comprises an amino acid sequence of SEQ ID NO: 6, 26, or 45; HCDR3 comprises an amino acid sequence of SEQ ID NO: 8, 28, or 47; LCDR1 comprises an amino acid sequence of SEQ ID NO: 12 or 32; LCDR2 comprises an amino acid sequence of SEQ ID NO: 14; and LCDR3 comprises an amino acid sequence of SEQ ID NO: 16 or 35.

[0040] In some embodiments, the antibody or antigen-binding fragment thereof comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising respective amino acid sequences of (i) SEQ ID NOs: 4, 6, 8, 12, 14, and 16; (ii) SEQ ID NOs: 24, 26, 28, 32, 14, and 35; or (iii) SEQ ID NOs: 43, 45, 47, 12, 14, and 16.

[0041] In some embodiments, the antibody or antigen-binding fragment thereof comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising amino acid sequences of SEQ ID NOs: 43, 45, 47, 12, 14, and 16, respectively.

[0042] In some embodiments, the antibody or antigen-binding fragment thereof comprises a heavy chain variable region (HCVR) comprising an amino acid sequence of SEQ ID NO: 2, 22, or 41 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 2, 22, or 41; and a light chain variable region (LCVR) comprising an amino acid sequence of SEQ ID NO: 10 or 30 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 10 or 30.

[0043] In some embodiments, the antibody or antigen-binding fragment thereof comprises a HCVR comprising the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 2 and a LCVR comprising the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 10.

[0044] In some embodiments, the antibody or antigen-binding fragment thereof comprises a HCVR comprising the amino acid sequence of SEQ ID NO: 22 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 22

and a LCVR comprising the amino acid sequence of SEQ ID NO: 30 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 30.

[0045] In some embodiments, the antibody or antigen-binding fragment thereof comprises a HCVR comprising the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 41 and a LCVR comprising the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 10.

[0046] In some embodiments, the fusion protein further comprises a heavy chain constant region of SEQ ID NO: 55 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 55 and a light chain constant region of SEQ ID NO: 56 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 56.

[0047] In some embodiments, the fusion protein comprises a heavy chain and a light chain wherein the heavy chain comprises an amino acid sequence of SEQ ID NO: 57, 59, or 61 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 57, 59, or 61 and the light chain has an amino acid sequence of SEQ ID NO: 58, 60, or 62 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 58, 60, or 62.

[0048] In some embodiments, the fusion protein comprises a heavy chain and a light chain wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 57 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 57 and the light chain comprises the amino acid sequence of SEQ ID NO: 58 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 58.

[0049] In some embodiments, the fusion protein comprises a heavy chain and a light chain wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 59 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 59 and the light chain comprises the amino acid sequence of SEQ ID NO: 61 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 61.

[0050] In some embodiments, the fusion protein comprises a heavy chain and a light chain wherein the heavy chain comprises the amino acid sequence of SEQ ID NO: 61 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 61 and the light chain comprises the amino acid sequence of SEQ ID NO: 62 or an

amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 62.

[0051] In some embodiments, the fusion protein comprises a heavy chain/light chain sequence pair of SEQ ID NOs: 57/58, 59/60, or 61/62. In some embodiments, the fusion protein comprises a heavy chain/light chain amino acid sequence pair of SEQ ID NOs: 61 / 62.

[0052] In some embodiments, the IL2 moiety comprises (i) IL2 or a fragment thereof; and (ii) IL2 receptor alpha (IL2R α) or a fragment thereof.

[0053] In some embodiments, the IL2 moiety may include a wild type or variant IL2 domain, which is fused to an IL2 binding domain of IL-2R α , optionally via a linker. The IL2 binding domain of IL-2R α can be N-terminal or C-terminal to the wild type or variant IL2 domain. In some embodiments, the IL2 binding domain of IL-2R α is N-terminal to the wild type or variant IL2 domain.

[0054] In some embodiments, the IL2 moiety comprises the amino acid sequence of SEQ ID NO: 54 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 54.

[0055] In eukaryotic cells, human IL2 is synthesized as a precursor polypeptide of 153 amino acids, from which 20 amino acids are removed to generate mature secreted IL2 (Taniguchi *et al.*, 1983, Nature 302(5906):305-10). Mature human IL2 has the following amino acid sequence:

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLCLE
EELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRW
ITFCQSIISTLT (SEQ ID NO: 75)

[0056] In some embodiments, the IL2 moieties can be IL2R α directed, e.g., they have one or more amino acid substitutions in the IL2 domain that make them preferentially bind to IL-2R α as compared to IL-2R β . In some embodiments, the IL2 moieties are not CD122 directed, e.g., they do not have amino acid substitutions in the IL2 domain that make them preferentially bind to IL-2R β as compared to IL-2R α .

[0057] In some embodiments, the fusion protein of the disclosure has one or more amino acid substitutions in the IL2 domain that reduce binding to IL-2R β . For example, in some embodiments, the IL2 moiety can have up to 50-fold (and in some embodiments up to 100-fold) to 1,000-fold attenuated binding to human IL-2R β as compared to wild-type human IL2. In some embodiments, the IL2 moiety with reduced binding to IL-2R β may retain its affinity to IL-2R α or have reduced binding to IL-2R α . For example, in some embodiments, the IL2 moiety may have up to 50-fold attenuated binding to human IL2-R α as compared to wild-type human IL2. In one

embodiment, the IL2 domain comprises one or more amino acid substitutions that reduce affinity to IL-2R β and preserve affinity to IL-2R α . An exemplary amino acid substitution is N88D. Other amino acid substitutions that reduce or abolish the affinity of IL2 to IL-2R β are D20T, N88R, N88D or Q126D (see, *e.g.*, US Patent Publication No. US 2007/0036752).

[0058] In some embodiments, the IL2 domain comprises one or more amino acid substitutions that reduce affinity to IL-2R α and preserve or reduce affinity to a lesser degree, to IL-2R β , resulting in CD122 directed IL2 moieties. Exemplary CD122 directed IL2 domains are those comprising both H16A and F42A substitutions. Accordingly, in some embodiments, the IL2 moiety comprises the amino acid sequence of human IL2 with H16A and F42A substitutions.

[0059] In some embodiments, the IL2 moiety comprises an amino acid substitution that eliminates the O-glycosylation site of IL2 at a position corresponding to residue 3 of human IL2. Exemplary amino acid substitutions at T3 are T3A, T3G, T3Q, T3E, T3N, T3D, T3R, T3K, and T3P. In a specific embodiment, the substitution is T3A.

[0060] In some embodiments, the IL2 domain may include a substitution at C125. C125 can be substituted with S, V, or A to reduce protein aggregation, as described in U.S. Patent No. 4,518,584. In some embodiments, the IL2 domain may include a substitution of methionine 104 with a neutral amino acid such as alanine, as described in U.S. Patent No. 5,206,344. In some embodiments, the IL2 domain may be devoid of the N-terminal alanine residue of IL2, resulting in des-A1 IL2.

[0061] In some embodiments, the IL2 domain can have amino acid deletions and/or substitutions selected from des-A1 M104A IL2, des-A1 M104A C125S IL2, M104A IL2, M104A C125A IL2, des-A1 M104A C125A IL2, and M104A C125S IL2, in addition to other variations alter the binding of IL2 to its receptor. These and other mutants may be found in U.S. Patent No. 5,116,943 and in Weiger *et al.*, 1989, Eur J Biochem 180:295-300.

[0062] In some embodiments, the IL2 domain may include an amino acid sequence having at least about 90%, at least about 91%, at least about 92%, about at least 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or 100% sequence identity to mature human IL2.

[0063] . In some embodiments, the IL2 or fragment thereof comprises the amino acid sequence of SEQ ID NO: 53 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 53.

[0064] In some embodiments, the IL2 moiety further includes an IL2 binding domain of IL-2R α (referred to as "IL2-R α domain"), *e.g.*, the extracellular domain of an IL-2R α , fused at the C-terminus or the N-terminus of IL2, optionally via a linker. In some embodiments, the IL2R α

or fragment thereof is human IL2R α (hIL2R α) or a fragment thereof. In some embodiments, the IL2-R α domain may include the mature human IL-2R α extracellular domain (corresponding to amino acids 22-272 of human IL-R α). In some embodiments, the IL2-R α domain may include an IL2 binding portion of the human IL-2R α extracellular domain (comprising the two “sushi” domains, which corresponds to amino acids 22-186 of human IL-2R α). In some embodiments, the IL2-R α domain may include an alternative IL2 binding portion of the human IL-2R α extracellular domain, which corresponds to amino acids 22-240 of human IL-2R α .

[0065] In some embodiments, the IL2-R α domain or the IL2 binding portion of the IL-2R α extracellular domain has an amino acid sequence with at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or 100% sequence identity to any of the sequences above, *i.e.*, any one of amino acids 22-186 of IL-2R α , amino acids 22-240 of IL-2R α , or amino acids 22-272 of IL-2R α , or any IL2 binding portion thereof.

[0066] In some embodiments, the IL2-R α domain or the IL2 binding portion can comprise or consist of an amino acid sequence having at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or 100% sequence identity to an IL2 binding portion of human IL-2R α , optionally wherein the binding portion has an amino acid sequence of (a) at least 160 amino acids, at least 161 amino acids, at least 162 amino acids, at least 164 amino acids or at least 165 amino acids and/or (b) up to 251, up to 240, up to 230, up to 220, up to 210, up to 200, up to 190, up to 180 or up to 170 amino acids of the extracellular domain of human IL2-R α . In particular embodiments, the portion of human IL-2R α is bounded by any one of (a) and (b) in the preceding sentence, *e.g.*, at least 160 and up to 180 amino acids from human IL-2R α , at least 162 and up to 200 amino acids from human IL-2R α , at least 160 and up to 220 amino acids from human IL-2R α , at least 164 and up to 190 amino acids from human IL-2R α , and so on and so forth.

[0067] In some embodiments, the IL2-R α domain or the IL2 binding portion comprises or consists of an amino acid sequence having at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% or 100% sequence identity to amino acids 22-186, with or without an additional up to 5 amino acids, up to 10 amino acids, up to 15 amino acids, up to 20 amino acids, up to 30 amino acids, or up to 40 amino acids C-terminal to amino acid residue 186, of IL2-R α .

[0068] In some embodiments, the IL2-R α domain or the IL-2R α extracellular domain has at least one fewer O-glycosylation and/or N-glycosylation compared to the extracellular domain of native IL-2R α , for example, by a substitution at one or more of amino acid N49,

amino acid N68, amino acid T74, amino acid T85, amino acid T197, amino acid T203, amino acid T208, and amino acid T216. In some embodiments, the one or more substitutions are from asparagine to an amino acid selected from alanine, threonine, serine, arginine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tryptophan, tyrosine, and valine. In some embodiments, the one or more substitutions are from threonine to an amino acid selected from alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan, tyrosine, and valine. In some embodiments, the one or more substitutions are at amino acid S50 (e.g., S50P), amino acid S51 (e.g., S51R, S51N, S51D, S51C, S51Q, S51E, S51G, S51H, S51I, S51L, S51K, S51M, S51F, S51P, S51W, S51Y, or S51V), amino acid T69 (e.g., T69P), amino acid T70 (e.g., T70R, T70N, T70D, T70C, T70Q, T70E, T70G, T70H, T70I, T70L, T70K, T70M, T70F, T70P, T70W, T70Y, or T70V), amino acid C192 (e.g., C192R, C192N, C192D, C192Q, C192E, C192G, C192H, C192I, C192L, C192K, C192M, C192F, C192P, C192W, C192Y, or C192V), or any combination thereof.

[0069] In some embodiments, the IL2R α or fragment thereof comprises the amino acid sequence of SEQ ID NO: 51 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 51.

[0070] The fusion protein can include one or more linkers (e.g., peptide linker or non-peptide linker) connecting the various components of the molecule. In some embodiments, two or more components of the fusion protein are connected to one another by a peptide linker. By way of example and not limitation, linkers can be used to connect (a) an IL2 moiety and an antigen-binding moiety; (b) different domains within an IL2 moiety (e.g., an IL2 domain and an IL-R α domain); or (c) different domains within an antigen-binding moiety (e.g., different components of anti-PD-1 antibody).

[0071] A peptide linker can range from 2 amino acids to 60 or more amino acids, and in some embodiments, a peptide linker ranges from 3 amino acids to 50 amino acids, from 4 to 30 amino acids, from 5 to 25 amino acids, from 10 to 25 amino acids, 10 amino acids to 60 amino acids, from 12 amino acids to 20 amino acids, from 20 amino acids to 50 amino acids, or from 25 amino acids to 35 amino acids in length.

[0072] In some embodiments, a peptide linker is at least 5 amino acids, at least 6 amino acids or at least 7 amino acids in length and optionally is up to 30 amino acids, up to 40 amino acids, up to 50 amino acids or up to 60 amino acids in length. In some embodiments, the linker ranges from 5 amino acids to 50 amino acids in length, e.g., ranges from 5 to 50, from 5

to 45, from 5 to 40, from 5 to 35, from 5 to 30, from 5 to 25, or from 5 to 20 amino acids in length. In other embodiments of the foregoing, the linker ranges from 6 amino acids to 50 amino acids in length, *e.g.*, ranges from 6 to 50, from 6 to 45, from 6 to 40, from 6 to 35, from 6 to 30, from 6 to 25, or from 6 to 20 amino acids in length. In yet other embodiments of the foregoing, the linker ranges from 7 amino acids to 50 amino acids in length, *e.g.*, ranges from 7 to 50, from 7 to 45, from 7 to 40, from 7 to 35, from 7 to 30, from 7 to 25, or from 7 to 20 amino acids in length.

[0073] In some embodiments, the linker comprises polar (*e.g.*, serine (S)) or charged (*e.g.*, lysine (K)) residues. In some embodiments, the linker is a flexible linker, *e.g.*, comprising one or more glycine (G) or serine (S) residues.

[0074] Examples of flexible linkers that can be used in the fusion protein of the disclosure include those disclosed by Chen *et al.*, 2013, *Adv Drug Deliv Rev.* 65(10): 1357-1369 and Klein *et al.*, 2014, *Protein Engineering, Design & Selection* 27(10): 325-330. Particularly useful flexible linkers are or comprise repeats of glycines and serines, *e.g.*, a monomer or multimer of GnS or SGn, where n is an integer from 1 to 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. In one embodiment, the linker is or comprises a monomer or multimer of repeat of G4S (GGGGS; SEQ ID NO: 67), *e.g.*, (GGGGS)*n*.

[0075] Polyglycine linkers can suitably be used in the fusion protein of the disclosure. In some embodiments, a peptide linker comprises two consecutive glycines (2Gly), three consecutive glycines (3Gly), four consecutive glycines (4Gly) (SEQ ID NO:68), five consecutive glycines (5Gly) (SEQ ID NO:69), six consecutive glycines (6Gly) (SEQ ID NO:70), seven consecutive glycines (7Gly) (SEQ ID NO:71), eight consecutive glycines (8Gly) (SEQ ID NO:72) or nine consecutive glycines (9Gly) (SEQ ID NO:73).

[0076] In some embodiments, the IL2 moiety and the antigen-binding moiety are connected via a linker. In some embodiments, the linker comprises an amino acid sequence of one or more repeats of GGGGS (SEQ ID NO: 67). In some embodiments, the linker comprises an amino acid sequence of SEQ ID NO: 50 or 52. In some embodiments, the IL2 moiety is linked to the C-terminal of the antigen-binding moiety via a peptide linker. In some embodiments, the linker comprises an amino acid sequence of SEQ ID NO: 50.

[0077] In another aspect, this disclosure provides a fusion protein comprising: (i) a first polypeptide comprising a light chain variable region (LCVR) of a human antibody that binds specifically to human PD-1; and (ii) a second polypeptide that comprises (a) a heavy chain variable region (HCVR) of the antibody that binds specifically to human PD-1; and (b) an IL2 moiety.

[0078] In some embodiments, the HCVR comprises three heavy chain complementarity determining regions (HCDRs) (HCDR1, HCDR2, and HCDR3) and the LCVR comprises three light chain CDRs (LCDR1, LCDR2, and LCDR3), wherein: HCDR1 comprises an amino acid sequence of SEQ ID NO: 4, 24, or 43; HCDR2 comprises an amino acid sequence of SEQ ID NO: 6, 26, or 45; HCDR3 comprises an amino acid sequence of SEQ ID NO: 8, 28, or 47; LCDR1 comprises an amino acid sequence of SEQ ID NO: 12 or 32; LCDR2 comprises an amino acid sequence of SEQ ID NO: 14; and LCDR3 comprises an amino acid sequence of SEQ ID NO: 16 or 35.

[0079] In some embodiments, the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprise respective amino acid sequences of (i) SEQ ID NOs: 4, 6, 8, 12, 14, and 16; (ii) SEQ ID NOs: 24, 26, 28, 32, 14, and 35; or (iii) SEQ ID NOs: 43, 45, 47, 12, 14, and 16.

[0080] In some embodiments, the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprise respective amino acid sequences of SEQ ID NOs: 43, 45, 47, 12, 14, and 16.

[0081] In some embodiments, the HCVR comprises an amino acid sequence of SEQ ID NO: 2, 22, or 41 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 2, 22, or 41. In some embodiments, the LCVR comprises an amino acid sequence of SEQ ID NO: 10 or 30 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 10 or 30.

[0082] In some embodiments, the HCVR comprises the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 2 and the LCVR comprises the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 10.

[0083] In some embodiments, the HCVR comprises the amino acid sequence of SEQ ID NO: 22 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 22 and the LCVR comprises the amino acid sequence of SEQ ID NO: 30 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 30.

[0084] In some embodiments, the HCVR comprises the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 41 and the LCVR comprises the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 10.

[0085] In some embodiments, the HCVR and the LCVR comprise respective amino acid sequences of (i) SEQ ID NOs: 2 and 10; (ii) SEQ ID NOs: 22 and 30; or (iii) SEQ ID NOs: 41 and 10. In some embodiments, the HCVR and the LCVR comprise respective amino acid sequences of SEQ ID NOs: 41 and 10.

[0086] In some embodiments, the first polypeptide further comprises a light chain constant region linked to the LCVR. In some embodiments, the light chain constant region comprises the amino acid sequence of SEQ ID NO: 56 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 56.

[0087] In some embodiments, the second polypeptide further comprises a heavy chain constant region arranged between the HCVR and the IL2 moiety. In some embodiments, the heavy chain constant region comprises the amino acid sequence of SEQ ID NO: 55 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 55.

[0088] In some embodiments, the first polypeptide comprises a light chain amino acid sequence of SEQ ID NO: 58, 60, or 62 or a light chain amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 58, 60, or 62.

[0089] In some embodiments, the second polypeptide comprises a heavy chain amino acid sequence of SEQ ID NO: 57, 59, or 61 or a heavy chain amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 57, 59, or 61.

[0090] In some embodiments, the fusion protein comprises a heavy chain/light chain sequence pair of SEQ ID NOs: 57/58, 59/60, or 61/62. In some embodiments, the fusion protein comprises a heavy chain/light chain amino acid sequence pair of SEQ ID NOs: 61 / 62.

[0091] In some embodiments, the IL2 moiety is connected to the C-terminal of the heavy chain constant region via a linker. In some embodiments, the linker comprises an amino acid sequence of one or more repeats of GGGGS (SEQ ID NO: 67). In some embodiments, the linker comprises an amino acid sequence of SEQ ID NO: 50 or 52. In one embodiment, the linker comprises the amino acid sequence of SEQ ID NO: 50.

[0092] In some embodiments, the IL2 moiety comprises (i) IL2 or a fragment thereof; and (ii) IL2 receptor alpha (IL2R α) or a fragment thereof.

[0093] In some embodiments, the IL2 or fragment thereof is human IL2 (hIL2) or a fragment thereof. In some embodiments, the IL2 or fragment thereof comprises the amino acid sequence of SEQ ID NO: 53 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 53.

[0094] In some embodiments, the IL2R α or fragment thereof is human IL2R α (hIL2R α) or a fragment thereof. In some embodiments, the IL2R α or fragment thereof comprises the amino acid sequence of SEQ ID NO: 51 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 51.

[0095] In some embodiments, the IL2 moiety comprises the amino acid sequence of SEQ ID NO: 54 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 54.

[0096] In some embodiments, the IL2 or fragment thereof is linked to the C-terminal of the IL2R α or fragment thereof via a linker. In one embodiment, the linker comprises the amino acid sequence of SEQ ID NO: 52.

[0097] In some embodiments, the first polypeptide comprises the amino acid sequence of SEQ ID NO: 58, 60, or 62 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 58, 60, or 62. In some embodiments, the second polypeptide comprises the amino acid sequence of SEQ ID NO: 63, 64, or 65 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 63, 64, or 65.

[0098] In some embodiments, the first polypeptide comprises the amino acid sequence of SEQ ID NO: 58 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 58 and the second polypeptide comprises the amino acid sequence of SEQ ID NO: 64 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 64.

[0099] In some embodiments, the first polypeptide comprises the amino acid sequence of SEQ ID NO: 60 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 60 and the second polypeptide comprises the amino acid sequence of SEQ ID NO: 63 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 63.

[0100] In some embodiments, the first polypeptide comprises the amino acid sequence of SEQ ID NO: 62 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 62 and the second polypeptide comprises the amino acid sequence of SEQ ID NO: 65 or an amino acid sequence having 80%, 85%, 90%, 95%, 97%, 98% or 99% sequence identity to SEQ ID NO: 65.

[0101] Also provided in this disclosure is a dimeric fusion protein formed by a fusion protein described herein. In some embodiments, the dimeric fusion protein is a homodimeric fusion protein, wherein each constituent monomer comprises the fusion protein described

herein. In some embodiments, each constituent monomer consists of the fusion protein described herein. In some embodiments, the monomers of the dimeric fusion protein dimerize through the heavy chain constant region of each monomer.

Biological Activity

[0102] The fusion proteins of the present disclosure exhibit attenuated binding to IL2R α , IL2R β and IL2R γ . In some embodiments, the fusion proteins do not compete with REGN2810, pembrolizumab or nivolumab. In some embodiments, the fusion proteins exhibit reduced activity in activating human IL2R α / β / γ trimeric and IL2R β / γ dimeric receptor complexes as compared to IL2 and increased activity in activating human IL2R α / β / γ trimeric and IL2R β / γ dimeric receptor complexes as compared to a non-targeted IL2R α -IL2 construct. In some embodiments, the fusion proteins exhibit increased activity in stimulating antigen-activated T cells as measured by a level of IFN- γ release as compared to a wild-type human IL2. In some embodiments, the fusion proteins showed enhanced anti-tumor efficacy as compared to IL2 alone or in combination with an anti-PD-1 antibody.

Nucleic Acids and Host Cells

[0103] In another aspect, the disclosure provides isolated nucleic acid or nucleic acids comprising one or more polynucleotide sequences encoding the fusion protein of the disclosure. In some embodiments, the fusion protein is encoded by a single nucleic acid. In other embodiments, for example, in the case of a heterodimeric molecule or a molecule comprising an anti-PD-1 antibody composed of more than one polypeptide chain, the fusion protein can be encoded by a plurality (e.g., two, three, four or more) nucleic acids.

[0104] A single nucleic acid can encode a fusion protein that comprises a single polypeptide chain, a fusion protein that comprises two or more polypeptide chains, or a portion of a fusion protein that comprises more than two polypeptide chains (for example, a single nucleic acid can encode two polypeptide chains of a fusion protein comprising three, four or more polypeptide chains, or three polypeptide chains of a fusion protein comprising four or more polypeptide chains). For separate control of expression, the open reading frames encoding two or more polypeptide chains can be under the control of separate transcriptional regulatory elements (e.g., promoters and/or enhancers). The open reading frames encoding two or more polypeptides can also be controlled by the same transcriptional regulatory elements and separated by internal ribosome entry site (IRES) sequences allowing for translation into separate polypeptides.

[0105] In some embodiments, a fusion protein comprising two or more polypeptide chains is encoded by two or more nucleic acids. The number of nucleic acids encoding a fusion protein can be equal to or less than the number of polypeptide chains in the fusion protein (for example, when more than one polypeptide chains are encoded by a single nucleic acid).

[0106] The nucleic acids of the disclosure can be DNA or RNA (e.g., mRNA).

[0107] In another aspect, the disclosure provides host cells and vectors containing the nucleic acids of the disclosure. The nucleic acids may be present in a single vector or separate vectors present in the same host cell or separate host cell, as described in more detail hereinbelow.

[0108] In some embodiments, the host cell expresses a first vector comprising a polynucleotide sequence that encodes a HCVR of an antibody as set forth herein and a second vector comprising a polynucleotide sequence that encodes a LCVR of an antibody as set forth herein.

[0109] In some embodiments, the host cell expresses a first vector comprising a polynucleotide sequence that encodes the first polypeptide of the fusion protein set forth herein and a second vector comprising a polynucleotide sequence that encodes the second polypeptide of the fusion protein set forth herein.

[0110] This disclosure provides vectors comprising nucleotide sequences encoding a fusion protein or a fusion protein component described herein, for example, one or two of the polypeptide chains of a half antibody. The vectors include, but are not limited to, a virus, plasmid, cosmid, lambda phage or a yeast artificial chromosome (YAC).

[0111] Numerous vector systems can be employed. For example, one class of vectors utilizes DNA elements that are derived from animal viruses such as, for example, bovine papillomavirus, polyomavirus, adenovirus, vaccinia virus, baculovirus, retroviruses (Rous Sarcoma Virus, MMTV or MOMLV) or SV40 virus. Another class of vectors utilizes RNA elements derived from RNA viruses such as Semliki Forest virus, Eastern Equine Encephalitis virus, and Flaviviruses.

[0112] Additionally, cells that have stably integrated the DNA into their chromosomes can be selected by introducing one or more markers which allow for the selection of transfected host cells. The marker may provide, for example, prototrophy to an auxotrophic host, biocide resistance (e.g., antibiotics), or resistance to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed or introduced into the same cell by co-transformation. Additional elements may also be needed for

optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals.

[0113] Once the expression vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors can be transfected or introduced into an appropriate host cell. Various techniques may be employed to achieve this, such as, for example, protoplast fusion, calcium phosphate precipitation, electroporation, retroviral transduction, viral transfection, gene gun, lipid-based transfection or other conventional techniques. Methods and conditions for culturing the resulting transfected cells and for recovering the expressed polypeptides are known to those skilled in the art and may be varied or optimized depending upon the specific expression vector and mammalian host cell employed, based upon the present description.

[0114] The disclosure also provides host cells comprising a nucleic acid of the disclosure. In one embodiment, the host cells are genetically engineered to comprise one or more nucleic acids described herein. In one embodiment, the host cells are genetically engineered by using an expression cassette. The phrase “expression cassette” refers to nucleotide sequences, which are capable of affecting expression of a gene in hosts compatible with such sequences. Such cassettes may include a promoter, an open reading frame with or without introns, and a termination signal. Additional factors necessary or helpful in effecting expression may also be used, such as, for example, an inducible promoter.

[0115] The disclosure also provides host cells comprising the vectors described herein.

[0116] The cell can be, but is not limited to, a eukaryotic cell, a bacterial cell, an insect cell, or a human cell. Suitable eukaryotic cells include, but are not limited to, Vero cells, HeLa cells, COS cells, CHO cells, HEK293 cells, BHK cells, and MDCKII cells. Suitable insect cells include, but are not limited to, Sf9 cells.

Pharmaceutical Compositions

[0117] The fusion protein or the dimeric fusion protein as disclosed herein may be in the form of compositions comprising the fusion protein (e.g., dimeric fusion protein) and one or more carriers, excipients and/or diluents.

[0118] The compositions may be formulated for specific uses, such as for veterinary uses or pharmaceutical uses in humans. The form of the composition (e.g., dry powder, liquid formulation, etc.) and the excipients, diluents and/or carriers used will depend upon the intended

uses of the fusion protein or the dimeric fusion protein and, for therapeutic uses, the mode of administration.

[0119] For therapeutic uses, the compositions may be supplied as part of a sterile, pharmaceutical composition that includes a pharmaceutically acceptable carrier. This composition can be in any suitable form (depending upon the desired method of administering it to a patient). The pharmaceutical composition can be administered to a patient by a variety of routes such as orally, transdermally, subcutaneously, intranasally, intravenously, intramuscularly, intratumorally, intrathecally, topically or locally. The most suitable route for administration in any given case will depend on the particular antibody, the subject, the nature and severity of the disease, and the physical condition of the subject. In some embodiments, the pharmaceutical composition will be administered intravenously or subcutaneously.

[0120] Pharmaceutical compositions can be conveniently presented in unit dosage forms containing a predetermined amount of a fusion protein of the disclosure per dose. The quantity of the fusion protein included in a unit dose will depend on the disease being treated, as well as other factors that are well known in the art. Such unit dosages may be in the form of a lyophilized dry powder containing an amount of fusion protein suitable for a single administration or in the form of a liquid. Dry powder unit dosage forms may be packaged in a kit with a syringe, a suitable quantity of diluent and/or other components useful for administration. Unit dosages in liquid form may be conveniently supplied in the form of a syringe pre-filled with a quantity of fusion protein suitable for a single administration. The pharmaceutical compositions may also be supplied in bulk from containing quantities of fusion protein suitable for multiple administrations.

[0121] Pharmaceutical compositions may be prepared for storage as lyophilized formulations or aqueous solutions by mixing a fusion protein having the desired degree of purity with optional pharmaceutically-acceptable carriers, excipients or stabilizers typically employed in the art (all of which are referred to herein as “carriers”), *i.e.*, buffering agents, stabilizing agents, preservatives, isotonicifiers, non-ionic detergents, antioxidants, and other miscellaneous additives (see, Remington’s Pharmaceutical Sciences, 16th edition (Osol, ed. 1980)). Such additives should be nontoxic to the recipients at the dosages and concentrations employed.

Methods of Use

[0122] The fusion proteins of the disclosure are useful in treating disease states where stimulation of the immune system of the host is beneficial, in particular conditions where an enhanced cellular immune response is desirable. These may include disease states where the host immune response is insufficient or deficient. Disease states for which the fusion protein

of the disclosure can be administered comprise, for example, a tumor or infection where a cellular immune response would be a critical mechanism for specific immunity. Specific disease states for which fusion protein of the present disclosure can be employed include cancer, for example, renal cell carcinoma or melanoma; immune deficiency, specifically in HIV-positive patients, immunosuppressed patients, chronic infection, and the like. The fusion protein of the disclosure may be administered per se or in any suitable pharmaceutical composition.

[0123] In one aspect, a fusion protein of the disclosure for use as a medicament is provided. In further aspects, a fusion protein of the disclosure for use in treating a disease is provided. In some embodiments, a fusion protein of the disclosure for use in a method of treatment is provided. In some embodiments, the disclosure provides a fusion protein as described herein for use in the treatment of a disease in a subject in need thereof. In some embodiments, the disclosure provides a fusion protein for use in a method of treating a subject having a disease comprising administering to the subject a therapeutically effective amount of the fusion protein. In some embodiments, the disease to be treated is a proliferative disorder. In some embodiments, the disease is cancer.

[0124] In some embodiments, the method further comprises administering to the subject a therapeutically effective amount of at least one additional therapeutic agent, e.g., an anti-cancer agent if the disease to be treated is cancer. In further embodiments, the disclosure provides a fusion protein for use in stimulating the immune system. In some embodiments, the disclosure provides a fusion protein for use in a method of stimulating the immune system in a subject comprising administering to the subject an effective amount of the fusion protein to stimulate the immune system.

[0125] "Stimulation of the immune system" according to any of the embodiments herein may include any one or more of a general increase in immune function, an increase in T cell function, an increase in B cell function, a restoration of lymphocyte function, an increase in the expression of IL-2 receptors, an increase in T cell responsiveness, an increase in natural killer cell activity or lymphokine-activated killer (LAK) cell activity, and the like.

[0126] In a further aspect, the disclosure provides for the use of a fusion protein of the disclosure in the manufacture or preparation of a medicament for the treatment of a disease in a subject in need thereof. In one embodiment, the medicament is for use in a method of treating a disease comprising administering to a subject having the disease a therapeutically effective amount of the medicament. In some embodiments, the disease to be treated is a proliferative disorder. In some embodiments, the disease is cancer. In one such embodiment, the method further comprises administering to the subject a therapeutically effective amount of at least one

additional therapeutic agent, e.g., an anti-cancer agent if the disease to be treated is cancer. In a further embodiment, the medicament is for stimulating the immune system. In a further embodiment, the medicament is for use in a method of stimulating the immune system in a subject comprising administering to the subject an amount effective of the medicament to stimulate the immune system.

[0127] In some embodiments, the disease to be treated is a proliferative disorder, preferably cancer. Non-limiting examples of cancers include bladder cancer, brain cancer, head and neck cancer, pancreatic cancer, lung cancer, breast cancer, ovarian cancer, uterine cancer, cervical cancer, endometrial cancer, esophageal cancer, colon cancer, colorectal cancer, rectal cancer, gastric cancer, prostate cancer, blood cancer, skin cancer, squamous cell carcinoma, bone cancer, and kidney cancer. Other cell proliferation disorders that can be treated using a fusion protein of the present disclosure include, but are not limited to, neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid), eye, head and neck, nervous system (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic region, and urogenital system. Also included are pre-cancerous conditions or lesions and cancer metastases. In some embodiments, the cancer is chosen from the group consisting of renal cell cancer, skin cancer, lung cancer, colorectal cancer, breast cancer, brain cancer, head and neck cancer. Similarly, other cell proliferation disorders can also be treated by the fusion protein of the present disclosure. Examples of such cell proliferation disorders include, but are not limited to: hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenstrom's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other cell proliferation disease, besides neoplasia, located in an organ system listed above. In another embodiment, the disease is related to autoimmunity, transplantation rejection, post-traumatic immune responses, and infectious diseases (e.g., HIV). More specifically, the fusion protein may be used in eliminating cells involved in immune cell-mediated disorders, including lymphoma; autoimmunity, transplantation rejection, graft-versus-host disease, ischemia, and stroke. A skilled artisan readily recognizes that in many cases, the fusion protein may not provide a cure but may only provide partial benefit. In some embodiments, a physiological change having some benefit is also considered therapeutically beneficial. Thus, in some embodiments, an amount of fusion protein that provides a physiological change is considered an "effective amount" or a "therapeutically effective amount." The subject, or patient, in need of treatment is typically a mammal, more specifically a human.

[0128] Various dosing schedules, including but not limited to, single or multiple administrations over various time-points, bolus administration, and pulse infusion, are contemplated herein.

[0129] The fusion protein of the disclosure will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent a disease condition, the fusion protein of the disclosure, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount.

[0130] One typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the fusion protein would be in the range from about 0.005 mg/kg to about 10 mg/kg. In other non-limiting examples, a dose may also comprise from about 1 µg/kg/body weight, about 5 µg/kg/body weight, about 10 µg/kg/body weight, about 50 µg/kg/body weight, about 100 µg/kg body weight, about 200 µg/kg/body weight, about 350 µg/kg body weight, about 500 µg/kg body weight, about 1 mg/kg body weight, about 5 mg/kg body weight, about 10 mg/kg body weight, about 50 mg/kg body weight, or about 100 mg/kg body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg body weight to about 50 mg/kg body weight, about 5 µg/kg body weight to about 100 mg/kg body weight, etc., can be administered, based on the numbers described above. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g., every week or every three weeks (e.g., such that the patient receives from about two to about twenty, or, e.g., about six doses of the fusion protein). An initial higher loading dose, followed by one or more lower doses, may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

[0131] A therapeutically effective dose of the fusion protein described herein will generally provide therapeutic benefit without causing substantial toxicity. Toxicity and therapeutic efficacy of a fusion protein can be determined by standard pharmaceutical procedures in cell culture or experimental animals. Cell culture assays and animal studies can be used to determine the LD50 (the dose lethal to 50% of a population) and the ED50 (the dose therapeutically effective in 50% of a population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which can be expressed as the ratio LD50/ED50. A fusion protein that exhibits large therapeutic indices is preferred. In one embodiment, the fusion

protein, according to the present disclosure, exhibits a high therapeutic index. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosages suitable for use in humans. The dosage lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon a variety of factors, *e.g.*, the dosage form employed, the route of administration utilized, the condition of the subject, and the like. (See, *e.g.*, Fingl *et al.*, 1975, In: The Pharmacological Basis of Therapeutics, Ch. 1, p. 1, incorporated herein by reference in its entirety).

[0132] Due to lower toxicity, the fusion protein of the disclosure can have higher maximum therapeutic doses than wild type IL2.

[0133] The fusion protein, according to the disclosure, may be administered in combination with one or more other agents or therapies. For instance, a fusion protein of the disclosure may be co-administered with at least one additional therapeutic agent or therapy. The term “therapeutic agent” encompasses any agent administered to treat a symptom or disease in a subject in need of such treatment. Such additional therapeutic agent may comprise any active ingredients suitable for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. In some embodiments, an additional therapeutic agent is an immunomodulatory agent, a cytostatic agent, an inhibitor of cell adhesion, a cytotoxic agent, an activator of cell apoptosis, or an agent that increases the sensitivity of cells to apoptotic inducers. In a particular embodiment, the additional therapeutic agent is an anti-cancer agent, for example, a microtubule disruptor, an antimetabolite, a topoisomerase inhibitor, a DNA intercalator, an alkylating agent, a hormonal therapy, a kinase inhibitor, a receptor antagonist, an activator of tumor cell apoptosis, or an antiangiogenic agent.

[0134] In certain embodiments, the second therapeutic agent or therapy is selected from: radiation, surgery, a chemotherapeutic agent, an oncolytic virus, a cancer vaccine, a PD-1 inhibitor, a B7-H3 inhibitor, a B7-H4 inhibitor, a lymphocyte activation gene 3 (LAG3) inhibitor, a T cell membrane protein 3 (TIM3) inhibitor, a galectin 9 (GAL9) inhibitor, a V-domain immunoglobulin (Ig)-containing suppressor of T-cell activation (VISTA) inhibitor, a Killer-Cell Immunoglobulin-Like Receptor (KIR) inhibitor, a B and T lymphocyte attenuator (BTLA) inhibitor, a T cell immunoreceptor with Ig and ITIM domains (TIGIT) inhibitor, a CTLA4 inhibitor, a CD38 inhibitor, a CD47 inhibitor, a CD28 activator, a 4-1BB activator, a GITR agonist, a CD40 agonist, an OX40 modulator, an indoleamine-2,3-dioxygenase (IDO) inhibitor, a vascular endothelial growth factor (VEGF) antagonist, an angiopoietin-2 (Ang2) inhibitor, a transforming growth factor beta (TGF β) inhibitor, an epidermal growth factor receptor (EGFR) inhibitor, an antibody to a

tumor-specific antigen, Bacillus Calmette-Guerin vaccine, granulocyte-macrophage colony-stimulating factor, a cytotoxin, an interleukin 6 receptor (IL-6R) inhibitor, an interleukin 4 receptor (IL-4R) inhibitor, an IL-10 inhibitor, IL-7, IL-12, IL-21, IL-15, IL-18, type-I interferons, an antibody-drug conjugate, an anti-inflammatory drug, and combinations thereof.

[0135] In certain embodiments, a fusion protein of the present disclosure may be used in combination with an anti-PD-1 antibody wherein the antibody does not cross-compete with the fusion protein for binding to human PD-1.

[0136] Such other agents are suitably present in combination in amounts that are effective for the purpose intended. The effective amount of such other agents depends on the amount of fusion protein used, the type of disorder or treatment, and other factors discussed above. The fusion protein is generally used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

[0137] Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate compositions) and separate administration, in which case, administration of the fusion protein of the disclosure can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. The fusion protein of the disclosure can also be used in combination with radiation therapy and/or surgery.

Additional Definitions

[0138] To aid in understanding the detailed description of the compositions and methods according to the disclosure, a few express definitions (in addition to those disclosed elsewhere herein) are provided to facilitate an unambiguous disclosure of the various aspects of the disclosure. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

[0139] As used herein, the term “fusion protein” or “fusion polypeptide” means a protein comprising two or more polypeptide sequences which are joined together covalently or non-covalently. For example, the fusion polypeptides encompassed by the present disclosure may include translation products of a chimeric gene construct that joins the nucleic acid sequences encoding a first polypeptide with the nucleic acid sequence encoding a second polypeptide to form a single open reading frame. Alternatively, the fusion protein may be encoded by two or more gene constructs on separate vectors that may be co-expressed in a host cell. In other

words, a “fusion polypeptide” or “fusion protein” is a recombinant protein of two or more proteins which are joined by a peptide bond or via several peptides. In some embodiments, the fusion protein may also comprise a peptide linker between the two domains.

[0140] As used herein, the terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, pegylation, or any other manipulation, such as conjugation with a labeling component. As used herein, the term “amino acid” includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs, and peptidomimetics.

[0141] As used herein, a “wild type” form of IL-2 is a form of IL-2 that is otherwise the same as the mutant IL-2 polypeptide except that the wild-type form has a wild-type amino acid at each amino acid position of the mutant IL-2 polypeptide. For example, if the IL-2 mutant is the full-length IL-2 (*i.e.*, IL-2 not fused or conjugated to any other molecule), the wild-type form of this mutant is full-length native IL-2. If the IL-2 mutant is a fusion between IL-2 and another polypeptide encoded downstream of IL-2 (*e.g.*, an antibody chain), the wild-type form of this IL-2 mutant is IL-2 with a wild-type amino acid sequence fused to the same downstream polypeptide. Furthermore, if the IL-2 mutant is a truncated form of IL-2 (the mutated or modified sequence within the non-truncated portion of IL-2), then the wild-type form of this IL-2 mutant is a similarly truncated IL-2 that has a wild-type sequence.

[0142] The fusion protein as disclosed herein may include one or more conservative modifications. The fusion protein with one or more conservative modifications may retain the desired functional properties, which can be tested using the functional assays known in the art. As used herein, the term “conservative sequence modifications” refers to amino acid modifications that do not significantly affect or alter the binding characteristics of the protein containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions, and deletions. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include: amino acids with basic side chains (*e.g.*, lysine, arginine, histidine); acidic side chains (*e.g.*, aspartic acid, glutamic

acid); uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan); nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine); beta-branched side chains (e.g., threonine, valine, isoleucine); and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). includes one or more conservative modifications. The Cas protein with one or more conservative modifications may retain the desired functional properties, which can be tested using the functional assays known in the art. As used herein, the term “conservative sequence modifications” refers to amino acid modifications that do not significantly affect or alter the binding characteristics of the protein containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions, and deletions. Modifications can be introduced by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include: amino acids with basic side chains (e.g., lysine, arginine, histidine); acidic side chains (e.g., aspartic acid, glutamic acid); uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan); nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine); beta-branched side chains (e.g., threonine, valine, isoleucine); and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[0143] As used herein, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, $\% \text{ homology} = \# \text{ of identical positions} / \text{total } \# \text{ of positions} \times 100$), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

[0144] The percent identity between two amino acid sequences can be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) algorithm which has been incorporated into the GAP program in

the GCG software package (available at www.gcg.com), using either a Blossum62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

[0145] Additionally or alternatively, the protein sequences of the present disclosure can further be used as a “query sequence” to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the XBLAST program (version 2.0) of Altschul, *et al.* (1990) J. Mol. Biol. 215:403-10. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the antibody molecules of the present disclosure. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used (see www.ncbi.nlm.nih.gov).

[0146] As used herein, the term “substantial identity” or “substantially identical,” when referring to a nucleic acid or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 90%, and more preferably at least about 95%, 96%, 97%, 98% or 99% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, BLAST or GAP, as discussed below. A nucleic acid molecule having substantial identity to a reference nucleic acid molecule may, in certain instances, encode a polypeptide having the same or substantially similar amino acid sequence as the polypeptide encoded by the reference nucleic acid molecule. As applied to polypeptides, the term “substantial similarity” or “substantially similar” means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 90% sequence identity, even more preferably at least 95%, 98% or 99% sequence identity. Preferably, residue positions, which are not identical, differ by conservative amino acid substitutions. A “conservative amino acid substitution” is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art.

See, *e.g.*, Pearson (1994) *Methods Mol. Biol.* 24: 307-331, which is herein incorporated by reference.

[0147] As used herein, the term “recombinant,” as used herein, refers to proteins or fragments thereof of the present disclosure created, expressed, isolated, or obtained by technologies or methods known in the art as recombinant DNA technology, which include, *e.g.*, DNA splicing and transgenic expression. The term refers to fusion proteins expressed in a non-human mammal (including transgenic non-human mammals, *e.g.*, transgenic mice), or a cell (*e.g.*, CHO cells) expression system or isolated from a recombinant combinatorial human antibody library.

[0148] As used herein, the term “associated” in the context of a fusion protein or a component thereof (*e.g.*, a targeting moiety such as an antibody) refers to a functional relationship between two or more polypeptide chains. In particular, the term “associated” means that two or more polypeptides are associated with one another, *e.g.*, non-covalently through molecular interactions or covalently through one or more disulfide bridges or chemical cross-linkages, so as to produce a functional Fusion protein. Examples of associations that might be present in a fusion protein of the disclosure include (but are not limited to) associations between homodimeric or heterodimeric Fc domains in an Fc region, associations between VH and VL regions in a Fab or scFv, associations between CH1 and CL in a Fab, and associations between CH3 and CH3 in a domain substituted Fab.

[0149] As used herein, the term “monovalent” as used herein in reference to an IL2 moiety and/or a targeting moiety in a fusion protein means a fusion protein that has only a single IL2 moiety and/or targeting moiety (*e.g.*, anti-PD-1 antibody or antigen-binding portion thereof), respectively.

[0150] As used herein, the term “bivalent” as used herein in reference to an IL2 moiety and/or a targeting moiety in a fusion protein means a fusion protein that has two IL2 moieties and/or targeting moieties (*e.g.*, anti-PD-1 antibody or antigen-binding portion thereof), respectively. Typically, a fusion protein that is bivalent for an IL2 moiety and/or a targeting moiety is dimeric (either homodimeric or heterodimeric).

[0151] As used herein, the terms “complementarity determining region” or “CDR,” as used herein, refer to the sequences of amino acids within antibody variable regions which confer antigen specificity and binding affinity. In general, there are three CDRs in each heavy chain variable region (CDR-H1, CDR-H2, HCDR-H3) and three CDRs in each light chain variable region (CDR-L1, CDR-L2, CDR-L3). Exemplary conventions that can be used to identify the boundaries of CDRs include, *e.g.*, the Kabat definition, the Chothia definition, the

ABM definition, and the IMGT definition. See, e.g., Kabat, 1991, "Sequences of Proteins of Immunological Interest," National Institutes of Health, Bethesda, Md. (Kabat numbering scheme); Al-Lazikani *et al.*, 1997, J. Mol. Biol. 273:927-948 (Chothia numbering scheme); Martin *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:9268-9272 (ABM numbering scheme); and Lefranc *et al.*, 2003, Dev. Comp. Immunol. 27:55-77 (IMGT numbering scheme). Public databases are also available for identifying CDR sequences within an antibody.

[0152] As used herein, the term "Fc domain" refers to a portion of the heavy chain that pairs with the corresponding portion of another heavy chain. The term "Fc region" refers to the region of antibody-based binding molecules formed by association of two heavy chain Fc domains. The two Fc domains within the Fc region may be the same or different from one another. In a native antibody the Fc domains are typically identical, but one or both Fc domains might advantageously be modified to allow for heterodimerization, e.g., via a knob-in-hole interaction.

[0153] As used herein, the term "EC50" refers to the half maximal effective concentration of a molecule (such as a fusion protein) which induces a response halfway between the baseline and maximum after a specified exposure time. The EC50 essentially represents the concentration of an antibody or fusion protein where 50% of its maximal effect is observed. In some embodiments, the EC50 value equals the concentration of a fusion protein that gives half-maximal STAT5 activation in an assay.

[0154] An epitope, or antigenic determinant, is a portion of an antigen (e.g., target molecule) recognized by an antibody or other antigen-binding moiety as described herein. An epitope can be linear or conformational.

[0155] As used herein, the term "subject" includes human and non-human animals. Non-human animals include all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, and reptiles. Except when noted, the terms "patient" or "subject" are used herein interchangeably.

[0156] As used herein, the term "target molecule" as used herein refers to any biological molecule (e.g., protein, carbohydrate, lipid or combination thereof) expressed on a cell surface or in the extracellular matrix that can be specifically bound by a targeting moiety in a fusion protein of the disclosure.

[0157] As used herein, the terms "treat," "treatment," and "treating" refer to the reduction or amelioration of the progression, severity and/or duration of a proliferative disorder, or the amelioration of one or more symptoms (preferably, one or more discernible symptoms) of a proliferative disorder resulting from the administration of one or more fusion protein of the

disclosure. In specific embodiments, the terms “treat,” “treatment,” and “treating” refer to the amelioration of at least one measurable physical parameter of a proliferative disorder, such as growth of a tumor, not necessarily discernible by the patient. In other embodiments, the terms “treat,” “treatment,” and “treating” refer to the inhibition of the progression of a proliferative disorder, either physically by, *e.g.*, stabilization of a discernible symptom, physiologically by, *e.g.*, stabilization of a physical parameter, or both. In other embodiments, the terms “treat,” “treatment,” and “treating” refer to the reduction or stabilization of tumor size or cancerous cell count.

[0158] As used herein, the term “cancer” refers to a disease characterized by the uncontrolled (and often rapid) growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers are described herein and include, but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, adrenal gland cancer, autonomic ganglial cancer, biliary tract cancer, bone cancer, endometrial cancer, eye cancer, fallopian tube cancer, genital tract cancers, large intestinal cancer, cancer of the meninges, oesophageal cancer, peritoneal cancer, pituitary cancer, penile cancer, placental cancer, pleura cancer, salivary gland cancer, small intestinal cancer, stomach cancer, testicular cancer, thymus cancer, thyroid cancer, upper aerodigestive cancers, urinary tract cancer, vaginal cancer, vulva cancer, lymphoma, leukemia, lung cancer and the like.

[0159] As used herein, the term “tumor” is used interchangeably with the term “cancer” herein, *e.g.*, both terms encompass solid and liquid, *e.g.*, diffuse or circulating, tumors. As used herein, the term “cancer” or “tumor” includes premalignant, as well as malignant cancers and tumors.

[0160] As used herein, the term “host cell,” as used herein, refers to cells into which a nucleic acid of the disclosure has been introduced. The terms “host cell” and “recombinant host cell” are used interchangeably herein. It is understood that such terms refer to the particular subject cell and to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. Typical host cells are eukaryotic host cells, such as mammalian host cells.

[0161] As used herein, “expression” refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the

process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene product(s).” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0162] As used herein, an “isolated” nucleic acid molecule or polynucleotide refers to a nucleic acid molecule, DNA or RNA that has been removed from its native environment. For example, a recombinant polynucleotide encoding a therapeutic polypeptide contained in a vector is considered isolated for the purposes of the present disclosure. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. An isolated polynucleotide includes a polynucleotide molecule contained in cells that ordinarily contain the polynucleotide molecule, but the polynucleotide molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the present disclosure, as well as positive and negative strand forms, and double-stranded forms. Isolated polynucleotides or nucleic acids, according to the present disclosure, further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

[0163] As used herein, the term “disease” as used herein is intended to be generally synonymous and is used interchangeably with, the terms “disorder” and “condition” (as in medical condition), in that all reflect an abnormal condition of the human or animal body or of one of its parts that impairs normal functioning, is typically manifested by distinguishing signs and symptoms, and causes the human or animal to have a reduced duration or quality of life.

[0164] As used herein, the term “composition” or “pharmaceutical composition” refers to a mixture of at least one component useful with the presently disclosed fusion protein in combination with other components, such as carriers, stabilizers, diluents, dispersing agents, suspending agents, thickening agents, and/or excipients. The pharmaceutical composition facilitates administration of one or more components of the disclosure to an organism.

[0165] As used herein, the term “pharmaceutically acceptable” refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the composition, and is relatively non-toxic, *i.e.*, the material may be administered to a subject without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

[0166] As used herein, the term “pharmaceutically acceptable carrier” includes a pharmaceutically acceptable salt, pharmaceutically acceptable material, composition or carrier, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting a compound(s) of the present disclosure within or to the subject such that it may perform its intended function. Typically, such compounds are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each salt or carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation, and not injurious to the subject. Some examples of materials that may serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose, and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer’s solution; ethyl alcohol; phosphate buffer solutions; diluent; granulating agent; lubricant; binder; disintegrating agent; wetting agent; emulsifier; coloring agent; release agent; coating agent; sweetening agent; flavoring agent; perfuming agent; preservative; antioxidant; plasticizer; gelling agent; thickener; hardener; setting agent; suspending agent; surfactant; humectant; carrier; stabilizer; and other non-toxic compatible substances employed in pharmaceutical formulations, or any combination thereof. As used herein, “pharmaceutically acceptable carrier” also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of one or more components of the present disclosure, and are physiologically acceptable to the subject. Supplementary active compounds may also be incorporated into the compositions.

[0167] As used herein, the term “modulate” is meant to refer to any change in biological state, *i.e.*, increasing, decreasing, and the like.

[0168] As used herein, the terms “increased,” “increase” or “enhance” or “activate” are all used herein to generally mean an increase by a statically significant amount; for the avoidance of any doubt, the terms “increased,” “increase” or “enhance” or “activate” means an increase of at least 10% as compared to a reference level, for example, an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including

a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

[0169] As used herein, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise.

[0170] As used herein, the terms “including,” “comprising,” “containing,” or “having” and variations thereof are meant to encompass the items listed thereafter and equivalents thereof as well as additional subject matter unless otherwise noted.

[0171] As used herein, the phrases “in one embodiment,” “in various embodiments,” “in some embodiments,” and the like are used repeatedly. Such phrases do not necessarily refer to the same embodiment, but they may unless the context dictates otherwise.

[0172] As used herein, the terms “and/or” or “/” means any one of the items, any combination of the items, or all of the items with which this term is associated.

[0173] As used herein, the word “substantially” does not exclude “completely,” e.g., a composition that is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition.

[0174] As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In some embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value). Unless indicated otherwise herein, the term “about” is intended to include values, e.g., weight percents, proximate to the recited range that are equivalent in terms of the functionality of the individual ingredient, the composition, or the embodiment.

[0175] As disclosed herein, a number of ranges of values are provided. It is understood that each intervening value, to the tenth of the unit of the lower limit, unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed by the present disclosure. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither, or both limits are

included in the smaller ranges is also encompassed by the present disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also encompassed by the present disclosure.

[0176] As used herein, the term “each,” when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection. Exceptions can occur if explicit disclosure or context clearly dictates otherwise.

[0177] The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the present disclosure and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the present disclosure.

[0178] All methods described herein are performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. In regard to any of the methods provided, the steps of the method may occur simultaneously or sequentially. When the steps of the method occur sequentially, the steps may occur in any order, unless noted otherwise. In cases in which a method comprises a combination of steps, each and every combination or sub-combination of the steps is encompassed within the scope of the disclosure, unless otherwise noted herein.

[0179] Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure. Publications disclosed herein are provided solely for their disclosure prior to the filing date of the present disclosure. Nothing herein is to be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure. Further, the dates of publication provided may be different from the actual publication dates, which may need to be independently confirmed.

[0180] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

EXAMPLES

[0181] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the present disclosure and are not intended to limit the scope of what the inventors regard as their invention. Likewise, the disclosure is not limited to any particular preferred embodiments described herein. Indeed, modifications and variations of the embodiments may be apparent to those skilled in the art upon reading this specification and can be made without departing from its spirit and scope. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, room temperature is about 25°C, and pressure is at or near atmospheric.

EXAMPLE 1: Generation of Human Antibodies to human PD-1

[0182] Human antibodies to PD-1 protein were generated in a VELOCIMMUNE® mouse comprising DNA encoding human immunoglobulin heavy and kappa light chain variable regions. The mice were immunized with a fragment of PD-1 that ranges from about amino acids 25 – 170 of GenBank Accession NP_005009.2 in the presence of an adjuvant. The antibody immune response was monitored by a PD-1-specific immunoassay. When a desired immune response was achieved, splenocytes were harvested and fused with mouse myeloma cells to preserve their viability and form hybridoma cell lines. The hybridoma cell lines were screened and selected to identify cell lines that produce PD-1-specific antibodies.

[0183] Anti-PD-1 antibodies were isolated directly from antigen-positive mouse B cells without fusion to myeloma cells, as described in U.S. Patent No. 7,582,298, herein specifically incorporated by reference in its entirety.

[0184] Using this method, several fully human anti-PD-1 antibodies (*i.e.*, antibodies possessing human variable domains and human constant domains) were obtained.

[0185] Exemplary antibodies generated as disclosed above were designated as mAb29512, mAb7798, and mAb9048, having HCVR, HCDR1, HCDR2, HCDR3, LCVR, LCDR1, LCDR2, and LCDR3 amino acid and nucleic acid sequences as identified in Tables 1 and 2.

[0186] Exemplary anti-PD-1 antibodies were used in the construction of IL2-based reagents using standard molecular biology techniques known in the art. Table 4 discloses the amino acid sequences of the reagents.

[0187] The biological properties of the exemplary proteins generated in accordance with the methods of this Example are described in detail in the Examples set forth below.

EXAMPLE 2: Heavy and Light Chain Variable Region Amino Acid and Nucleotide Sequences

[0188] Table 1 sets forth the amino acid sequence identifiers of the heavy and light chain variable region sequences, CDR sequences of exemplary anti-PD-1 antibodies.

Table 1: Amino Acid Identifiers

Antibody ID	SEQ ID NOs:							
	HCVR	HCDR1	HCDR2	HCDR3	LCVR	LCDR1	LCDR2	LCDR3
mAb29512	2	4	6	8	10	12	14	16
mAb7798	22	24	26	28	30	32	14	35
mAb9048	41	43	45	47	10	12	14	16

[0189] The corresponding nucleic acid sequence identifiers of the exemplary anti-PD-1 antibodies are set forth in Table 2.

Table 2: Nucleic Acid Identifiers

Antibody ID	SEQ ID NOs:							
	HCVR	HCDR1	HCDR2	HCDR3	LCVR	LCDR1	LCDR2	LCDR3
mAb29512	1	3	5	7	9	11	13	15
mAb7798	21	23	25	27	29	31	33	34
mAb9048	40	42	44	46	9	11	13	15

[0190] The antibodies may have a human or mouse Fc isotype. As will be appreciated by a person of ordinary skill in the art, an antibody having a particular Fc isotype can be converted to an antibody with a different Fc isotype (e.g., an antibody with a mouse IgG1 Fc can be converted to an antibody with a human IgG1 or a human IgG4, etc.), but in any event, the variable domains (including the CDRs) – which are indicated by the numerical identifiers shown in Table 1 – will remain the same, and the binding properties to the antigen are expected to be identical or substantially similar regardless of the nature of the Fc domain.

[0191] Exemplary antibodies of mAb29512, mAb7798, and mAb9048 comprising a human IgG4 Fc comprising a serine to proline mutation in the hinge region (S108P) were designated as H4H29512, REGN2810, and H4H9048, respectively. Table 3 sets forth the amino

acid sequence identifiers of full-length heavy chain and light chain sequences of these antibodies.

Table 3: Heavy chain (HC) and light chain (LC) sequences of anti-PD-1 antibodies

Antibody ID	Antibody Name	SEQ ID NOs:			
		HC		LC	
		DNA	PEP	DNA	PEP
mAb29512	H4H29512	17	18	19	20
mAb7798	REGN2810	36	37	38	39
mAb9048	H4H9048	48	49	19	20

[0192] REGN2810 (also known as cemiplimab; LIBTAYO®) was first disclosed in US 9,987,500 and has since been approved for the treatment of cutaneous squamous cell carcinoma, basal cell carcinoma, and non-small cell lung cancer.

[0193] Table 4 sets forth the amino acid sequence identifiers of the anti-PD-1-IL2R α -IL2 reagents.

Table 4: Amino acid identifiers of anti-PD-1-IL2R α -IL2

ID	SEQ ID NOs:										
	HCVR	HCDR1	HCDR2	HCDR3	LCVR	LCDR1	LCDR2	LCDR3	HC	LC	IL2 moiety
REGN10595	2	4	6	8	10	12	14	16	57	58	54
REGN10486	22	24	26	28	30	32	14	35	59	60	54
REGN10597	41	43	45	47	10	12	14	16	61	62	54

[0194] The IL2 moiety is connected to the C-terminal of the heavy chain constant region (SEQ ID NO: 55) via a linker comprising an amino acid sequence of SEQ ID NO: 50. For REGN10595, the heavy chain (HC) SEQ ID NO: 57 includes the amino acid sequences of the HCVR (SEQ ID NO: 2), the heavy chain constant region (SEQ ID NO: 55), the linker (SEQ ID NO: 50), and the IL2 moiety (SEQ ID NO: 54). For REGN10486, the heavy chain (HC) SEQ ID NO: 59 includes the amino acid sequences of the HCVR (SEQ ID NO: 22), the heavy chain constant region (SEQ ID NO: 55), the linker (SEQ ID NO: 50), and the IL2 moiety (SEQ ID NO: 54). For REGN10597, the heavy chain (HC) SEQ ID NO: 61 includes the amino acid sequences of the HCVR (SEQ ID NO: 41), the heavy chain constant region (SEQ ID NO: 55), the linker (SEQ ID NO: 50), and the IL2 moiety (SEQ ID NO: 54).

Control Constructs used in the following Examples:

[0195] The following control constructs were included in the following Examples for comparative purposes: “Comp 1”: a monoclonal anti-PD-1 antibody having V_H/V_L sequences of antibody “MK-3475” according to WO 2008/156712 (Merck Sharp & Dohme); “Comp 2”: a monoclonal anti-PD-1 antibody having V_H/V_L sequences of antibody “5C4” according to WO2006/121168 (Medarex, Inc/E R Squibb); and “REGN13233”: an anti-PD-1 antibody comprising V_H/V_L sequences of SEQ ID NOs: 41/10 and linked to an IL2 variant (IL2(3m)) having abolished CD25 binding (Klein et al., 2017, Oncoimmunology).

EXAMPLE 3: Binding kinetics of anti-PD1-IL2R α -IL2 fusion constructs and parental bivalent anti-PD1 antibodies

[0196] To evaluate binding kinetics of anti-PD1-IL2R α -IL2 fusion constructs and parental bivalent anti-PD1 antibodies, equilibrium dissociation constants (K_D values) for human PD-1 expressed with a C-terminal myc-myc-hexahistidine tag (hPD-1.mmH, SEQ ID NO: 66) binding to purified anti-PD-1 parental mAbs and anti-PD1-IL2R α -IL2 fusion constructs were determined using a real-time surface plasmon resonance biosensor using a Biacore 3000 or 4000 instrument. The CM5 Biacore sensor surface was derivatized by amine coupling with a monoclonal mouse anti-human Fc antibody (GE, # BR-1008-39). All Biacore binding studies were performed in a buffer composed of 0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.05% v/v Surfactant P20 (HBS-EP running buffer). Different concentrations of hPD-1.mmH prepared in HBS-EP running buffer (ranging from 100 to 3.7 nM in 3-fold dilutions or to or 90 nM to 3.33 nM in 3-fold dilutions) were injected over the anti-PD-1 antibody or anti-PD1-IL2R α -IL2 fusion construct captured surface at a flow rate of 30 or 50 μ L/minute. Antibody-reagent association was monitored for 4 or 5 minutes, while dissociation in HBS-EP running buffer was monitored for 10 minutes. At the end of each cycle, the anti-PD-1 mAb or anti-PD1-IL2R α -IL2 fusion construct capture surface was regenerated using a 12 sec injection of 20mM phosphoric acid. All binding kinetics experiments were performed at 25°C.

[0197] Kinetic association (k_a) and dissociation (k_d) rate constants were determined by fitting the real-time sensorgrams to a 1:1 binding model using Scrubber 2.0c curve fitting software. Binding dissociation equilibrium constants (K_D) and dissociative half-lives ($t_{1/2}$) were calculated from the kinetic rate constants as:

$$K_D \text{ (M)} = \frac{k_d}{k_a}, \quad \text{and} \quad t_{1/2} \text{ (min)} = \frac{\ln(2)}{60 \cdot k_d}$$

[0198] The binding kinetics of anti-PD1-IL2R α -IL2 fusion constructs and parental bivalent anti-PD1 antibodies are summarized in Table 5.

Table 5. hPD-1.mmH binding kinetics to anti-PD-1 mAbs and anti-PD1-IL2R α -IL2 fusion constructs at 25°C

REGN # / Ab PID #	mAb Capture (RU)	90nM or 100nM hPD-1.mmH Bind (RU)	ka (1/Ms)	kd (1/s)	KD (M)	t1/2 (min)
REGN10486	241.8 $\pm$ 6.4	46.9	1.07E+05	3.02E-04	2.83E-09	38.3
REGN2810	337.6 $\pm$ 3.9	88.7	9.88E+04	2.53E-04	2.56E-09	45.7
REGN10595	186.5 $\pm$ 1.5	44.7	1.04E+05	4.76E-04	4.59E-09	24.3
mAb29512	481.1 $\pm$ 1.3	123.8	1.38E+05	4.27E-04	3.10E-09	27.1
REGN10597	211.5 $\pm$ 2.6	41.8	1.48E+05	5.82E-04	3.92E-09	19.9
mAb9048	229.91	52.7	1.37E+05	1.23E-03	8.95E-09	9.4

EXAMPLE 4: Cross-competition of anti-PD-1 mAbs and anti-PD1-IL2R α -IL2 fusion constructs

[0199] To evaluate cross-competition of anti-PD-1 mAbs and anti-PD1-IL2R α -IL2 fusion constructs, binding competition between anti-PD1-IL2R α -IL2 and commercial anti-PD-1 mAbs was determined using a real-time, label-free bio-layer interferometry (BLI) assay on an Octet HTX biosensor (ForteBio Corp., A Division of Sartorius). The entire experiment was performed at 25°C in a buffer comprised of 0.01M HEPES pH7.4, 0.15M NaCl, 3mM EDTA, 0.05% v/v Surfactant P20, 0.1mg/mL BSA (Octet HBS-EP buffer) with the plate shaking at a speed of 1000rpm. To assess whether two antibodies are able to compete with one another for binding to their respective epitopes on the hPD-1.mmH (SEQ ID NO: 66), approximately ~0.34nm of hPD-1.mmH was first captured onto HIS1K antibody-coated Octet biosensors (ForteBio Inc, # 18-5120) by submerging the biosensors for 2 minutes into wells containing a 20 μ g/mL solution of hPD-1.mmH. The antigen-captured biosensors were then saturated with the first anti-PD-1 antibody or anti-PD1-IL2R α -IL2 fusion construct (subsequently referred to as mAb-1) by immersion into wells containing a 50 μ g/mL solution of mAb-1 for 5 minutes. The biosensors were then subsequently submerged into wells containing a 50 μ g/mL solution of a second anti-PD-1 antibody or anti-PD1-IL2R α -IL2 fusion construct (subsequently referred to as mAb-2) for 3 minutes. All the biosensors were washed in Octet HBS-EP buffer in between each step of the experiment. The real-time binding response was monitored during the course of the experiment, and the binding response at the end of every step was recorded—the response of mAb-2 binding to hPD-1.mmH pre-complexed with mAb-1 was compared, and competitive/non-competitive behaviors of different anti-PD-1 antibodies were determined using a 50% inhibition threshold.

[0200] Table 6 defines the relationships of antibodies competing in both directions, independent of the order of binding.

Table 6. Cross-competition of anti-PD-1 mAbs and anti-PD1-IL2R α -IL2 fusion constructs for binding to hPD-1.mmh

First mAb (mAb-1) Captured using HIS1K Octet Biosensors	mAb-2 Antibodies Shown to Compete with mAb-1
REGN10486	REGN2810
REGN2810	REGN10486
REGN10595	REGN10597
REGN10597	REGN10595

[0201] Table 6 shows that REGN10486 as well as its parental antibody REGN2810 did not cross-compete with either REGN10595 or REGN10597 for binding to hPD-1.

EXAMPLE 5: *In vitro* functional characterization of anti-PD1-IL2R α -IL2 constructs cell line engineering

[0202] Engineering of YT/reporter cells: The human T/NK cell leukemia YT cell line was electroporated with a Signal Transducer and Activator of Transcription 5 (STAT5) - luciferase reporter construct and maintained in Iscoves + 20% FBS + P/S/G + 200 μ g/mL hygromycin. A single cell clone, having high responsiveness to IL-2, was identified and renamed YT/Stat5-Luc cl.4. IL2R α (CD25) was knocked out in this clone using CRISPR-Cas9 technology, and the resulting cell line, YT/STAT5-Luc/IL2R α KO, was validated by flow cytometry. Human IL2R α was then stably reintroduced into the YT/STAT5-Luc/IL2R α KO cell line (amino acids M1-I272 of accession number NP_000408.1), and the resulting cell line, YT/STAT5-Luc/hIL2R α , was validated by flow cytometry and maintained in Iscoves + 20% FBS + P/S/G + 200 μ g/mL hygromycin + 15 μ g/mL blasticidin. YT/Stat5-Luc cl.4, YT/Stat5-Luc/IL2R α KO, and YT/Stat5-Luc/hIL2R α cells were engineered to stably express human PD1 (amino acids M1-L288 of accession number NP_005009.2, with a 2Q=>E mutation) and cells selected in media supplemented with 1mg/mL G418. Cells were validated by flow cytometry and renamed YT/STAT5-Luc/hPD1, YT/STAT5-Luc/IL2R α KO/hPD1, and YT/STAT5-Luc/hIL2R α /hPD1.

[0203] Engineering of PD1 reporter cells: Jurkat E6-1 cells were engineered to stably express an Activation Protein 1 (AP1) luciferase reporter construct, and antibiotic-resistant cells were selected and maintained in RPMI + 10% FBS + P/S/G + 1 μ g/mL puromycin. The resulting

pool of reporter cells was engineered to express human PD1 (amino acids M1-L288 of accession number NP_005009.2 with a 2Q=>E mutation), and a high PD-1 expressing clonal cell line was isolated by fluorescence-activated cell sorting and maintained in RPMI + 10% FBS + P/S/G + 1 µg/mL puromycin. The resulting clone was renamed Jurkat/AP1-Luc/hPD1 cl. 4E5.

[0204] Engineering of HEK293/anti-CD3/PD-L1 cells: HEK293 were transduced with a lentiviral vector encoding human CD79a (hlga: M1-P226 of accession number NP_001774.1), human CD79b (hlgb: M1-E229 of accession number NP_000617.1), anti-CD3 variable domains (anti-CD3 mlgE heavy chain, anti-CD3 kappa light chain; clone 2706N, IgE genbank# AAB59424.1, CemX-migis-Cyto/TM PIR# PIH1215) and human PD-L1 (amino acids M1-T290 of accession number NP_054862.1) then single cell sorted and maintained in DMEM + 10% FBS + P/S/G + 500ug/mL G418 + 100ug/mL hygromycin + 1ug/mL puromycin. Anti-CD3 and PD-L1 expression was confirmed by flow cytometry and the resulting clonal line was renamed 293/aCD3/hPD-L1 cl. A3.

[0205] Engineering of Raji/CD80 KO/CD86 KO and Raji/CD80 KO/CD86 KO/PD-L1 cells: CRISPR-Cas9 technology was used to eliminate CD80 and CD86 expression in Raji cells. Raji/CD80 KO/CD86 KO cells were single-cell cloned, and a resulting clone (Raji/CD80 KO/CD86 KO cl.1D6) was engineered to express human PD-L1 (amino acids M1-T290 of accession number NP_054862.1). The resulting cell line Raji/CD80 KO/CD86 KO/hPD-L1 was validated by flow cytometry and maintained in RPMI-1640 + 10% FBS + HEPES + NaPyr + P/S/G + 0.5ug/mL puromycin.

PD1 Antagonistic assay

[0206] HEK293 cells expressing membrane-bound anti-hCD3 epsilon and human PD-L1 were incubated with Jurkat/AP1-Luc/hPD-1 reporter T cells. Clustering of CD3 in complex with T cell receptor on reporter cells via anti-CD3 on HEK293 cells leads to activation of the transcription factor AP1, which drives luciferase reporter gene expression. However, luciferase expression in reporter T cells can be suppressed by the interaction of the inhibitory receptor PD-1 with PD-L1 on HEK293 cells, which can be overcome by the addition of antibodies blocking the PD1/PD-L1 axis.

[0207] RPMI1640 supplemented with 10% FBS and P/S/G was used as an assay medium to prepare cell suspensions and antibody dilutions. A day prior to screening, engineered Jurkat/AP1-Luc/hPD1 reporter cells were diluted to 3×10^5 cells/mL. On the day of the assay, cells were spun down, resuspended in the assay medium, and plated at 2.5×10^4 293/aCD3/hPD-L1 cells/well in 96 well white flat-bottom plates. Serially diluted (1:4) non-

targeted IL2R α -IL2 [anti-PSMA-IL2R α -IL2 (IL2R α -IL2 fused to an unrelated (PSMA) antibody), anti-PD1 (REGN2810, H4H29512 or H4H9048), anti-PD1-IL2R α -IL2 (REGN10486, REGN10595, REGN10597) or isotype controls (isotype control 1 or isotype control 2) over an 11-point titration range (500nM to 477 fM), with a 12th point containing no recombinant protein, were then added to the plated cells, followed by the addition of 2.5×10^4 Jurkat/AP1-Luc/hPD1 reporter cells/well. Plates were incubated for 5 hours at 37°C / 5% CO₂, and then 100 μ L ONE-Glo™ (Promega) reagent was added to the wells to lyse the cells and detect luciferase activity. The emitted light was measured in relative light units (RLU) on a multilabel plate reader Envision (PerkinElmer). EC₅₀ values of the antibodies were determined using GraphPad Prism™ software from a four-parameter logistic equation over a 12-point dose-response curve. Fold induction was calculated using the following equation:

$$\text{Fold induction} = \frac{\text{Highest mean RLU value within tested dose range}}{\text{Mean RLU values of no protein control}}$$

IL2 Reporter assay

[0208] In this experiment, engineered YT reporter cells are stimulated via either recombinant IL2-Fc, non-targeted IL2R α -IL2, or anti-PD1-IL2R α -IL2 fusion constructs. Functional IL2 receptors are formed by the differential assembly of IL2R subunits (IL2R α , IL2R β , and IL2R γ), with assembly of the IL2R β /IL2R γ subunits comprising a low affinity receptor and receptors containing all three subunits (IL2R α /IL2R β /IL2R γ) forming a high affinity receptor. Binding of cytokine by IL2R leads to activation of STAT5, which drives luciferase production in the engineered cell lines. To assess the relative potency of anti-PD1-IL2R α -IL2 in the presence of the low affinity (IL2R β / γ) or high affinity (IL2R α / β / γ) IL2 receptor, YT/STAT5-Luc reporter cells that expressed endogenous IL2R α were engineered to lack or overexpress IL2R α . While the reporter cells express endogenous PD1, derivatives in which PD1 was over-expressed were generated to assess the impact of PD1 expression levels on anti-PD1-IL2R α -IL2 potency.

[0209] RPMI1640 supplemented with 10% FBS and P/S/G was used as assay medium to prepare cell suspensions and antibody dilutions. A day prior to screening, engineered reporter YT/Stat5-Luc cells over-expressing or devoid of IL2R α and endogenously expressing or over-expressing PD1 were diluted at 3×10^5 cells/mL. On the day of the assay, 2.5×10^4 reporter cells/well were plated, in fresh assay media, in 96 well white flat-bottom plates, and were incubated with IL2-Fc (SEQ ID NO: 74), non-targeted IL2R α -IL2, REGN10486, REGN10595, REGN10597, or an isotype control, serially diluted (1:4) over an 11-point titration

range (200nM to 191 fM), and a 12th point containing no recombinant protein. Plates were incubated for 5 hours at 37°C / 5% CO₂, and then 100 µL ONE-Glo™ (Promega) reagent was added to the wells to lyse the cells and detect luciferase activity. The emitted light was measured in RLU on a multilabel plate reader Envision (PerkinElmer). EC₅₀ values of the antibodies were determined using GraphPad Prism™ software from a four-parameter logistic equation over a 12-point dose-response curve. Fold induction was calculated using the following equation:

$$\text{Fold induction} = \frac{\text{Highest mean RLU value within tested dose range}}{\text{Mean RLU values of no protein control}}$$

PD1 Competition assay

[0210] Anti-PD1 bivalent antibody or an isotype control are added to engineered YT reporter cells prior to their stimulation via either a non-targeted IL2Rα-IL2 or anti-PD1-IL2Rα-IL2 fusion constructs. Competition of the bivalent anti-PD1, with anti-PD1-IL2Rα-IL2, would blunt the ability of anti-PD1-IL2Rα-IL2 to target, and therefore activate, PD-1 expressing STAT5-Luc reporter cells. PD1 over-expressing reporter cells are used to maximize targeting efficiency and allow the best detection of anti-PD1 bivalent competition with anti-PD1-IL2Rα-IL2.

[0211] RPMI1640 supplemented with 10% FBS and P/S/G was used as assay medium to prepare cell suspensions and antibody dilutions. A day prior to screening, YT/Stat5-Luc/hPD1 reporter cells, knocked out or overexpressing IL2Rα, were diluted at 3 x 10⁵ cells/mL. On the day of the assay, 2.5 x 10⁴ reporter cells/well, were plated in assay medium, into 96 well white flat-bottom plates, and a titration of anti-PD1 bivalent or isotype control antibodies (5 point, 1:10 dilution ranging from 500nM to 0.05nM with the 6th point containing no recombinant protein) were added, followed by the addition of a titration of non-targeted IL2Rα-IL2, REGN10486, REGN10595, or REGN10597 (62.5nM to 59.6 fM; 1:4 serial dilution over an 11-point dilution range with a 12th point containing no recombinant protein). Plates were incubated for 4 hours at 37°C / 5% CO₂, and then 100 µL ONE-Glo™ (Promega) reagent was added to the wells to lyse cells and detect luciferase activity. The emitted light was measured in RLU on a multilabel plate reader Envision (PerkinElmer). EC₅₀ values of the antibodies were determined using the GraphPad Prism™ software from a four-parameter logistic equation over a 12-point dose-response curve. Fold induction was calculated using the following equation:

$$\text{Fold induction} = \frac{\text{Highest mean RLU value within tested dose range}}{\text{Mean RLU values of no protein control}}$$

Primary T cell stimulation assay

[0212] Primary T-cells are stimulated via a 4-day coculture with mitomycin-treated Raji/CD80 KO/CD86 KO cells. As Raji cells endogenously express CD20, a CD20xCD3 bispecific antibody was added to activate primary T-cells. A titration of an isotype control, IL2, anti-PD1, a non-targeted IL2R α -IL2 antibody (REGN9904) or anti-PD1-IL2R α -IL2 (REGN10486, REGN10595 or REGN10597) were added during the coculture, and their impact on T-cell activity was determined by measuring the release of IFN- γ in the cell culture supernatant using homogenous, no wash, AlphaLISA kits (Perkin Elmer).

[0213] Human peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor leukopak using an EasySep Direct Human PBMC Isolation kit (Stemcell) following the manufacturer's recommended protocol. T cells were isolated from PBMCs using an EasySep Human T cell isolation kit (Stemcell) in agreement with the manufacturer's protocol. Cells were spun down, resuspended in stimulation media (X-VIVO 15 cell culture media supplemented with 10% FBS, HEPES, NaPyr, NEAA, and 0.01 mM BME), and 1×10^5 cells/well plated out into 96-well round-bottom plates. Raji/CD80 KO/CD86 KO and Raji/CD80 KO/CD86 KO/hPD-L1 cells were treated with 20 μ g/mL mitomycin C, in primary stimulation media at a concentration of 10×10^6 cells/mL for 1 hour at 37°C, in order to arrest cell growth. Cells were then washed 3 times with D-PBS containing 2% FBS, and 5×10^4 cells were added per well. Subsequently, 0.5nM of an anti-CD3xanti-CD20 bispecific antibody was added, in conjunction with a titration of anti-PD1, IL2, a combination of REGN2810 + IL2, a non-targeted IL2R α -IL2 antibody, anti-PD1-IL2R α -IL2 (REGN10486, REGN10595 or REGN10597) or isotype control antibody (isotype control 1 or isotype control 2) in a 9-point, 1:6 serial dilution ranging from 500nM to 0.3pM, with the 10th dilution point containing only 0.5nM constant of the bispecific antibody. The plates were incubated for 96 hours at 37°C / 5% CO₂. IFN- γ was quantified in the cell culture supernatant using an AlphaLISA assay according to the manufacturer's protocols using samples with known IFN- γ concentrations in order to extrapolate the pg/mL of IFN- γ in each sample well. The measurements were acquired on the multilabel plate reader Envision (Perkin Elmer). The EC₅₀ values of the cytokines were determined using the GraphPad PrismTM software from a four-parameter logistic equation over a 10-point dose-response curve, where the 10th dilution point contained only 0.5 nM constant of the bispecific antibody.

Results summary and conclusions

PD1 Antagonistic assay:

[0214] The ability of anti-PD1-IL2R α -IL2 fusion constructs to block PD1/PD-L1-mediated suppression of TCR signaling was assessed using an AP1-reporter cell-based bioassay. Incubation of 293/aCD3/hPD-L1 cells with Jurkat/AP1-Luc/hPD1 reporter cells leads to reduced reporter activity due to PD1 activation by PD-L1. The ability of bivalent anti-PD1 antibodies or antibody fused IL2R α -IL2 proteins to block PD1-PD-L1 interaction and rescue reporter activity was evaluated. EC₅₀ and fold induction values are summarized in Table 7 for engineered reporter cells.

[0215] When reporter cells are treated with control proteins (isotype control Ab 1, isotype control Ab 2, or a non-targeted IL2R α -IL2), no increase in luciferase activity was detected. In contrast, incubation of the reporter cells with anti-PD1 antibody, REGN2810, or the corresponding anti-PD1-IL2R α -IL2, REGN10486, enhanced luciferase activity (19.210 and 17.740-fold, respectively) with similar potency (3.668E-09 and 5.351E-09, respectively). Bivalent PD1 antibodies, H4H29512 and H4H9048 on the other hand, did not enhance luciferase activity, and corresponding anti-PD1-IL2R-IL2 proteins, REGN10595 and REGN10597, induced low luciferase activity (2.725 and 3.038-fold, respectively) with weak potency (1.266E-08 and 1.264E-08, respectively).

IL2 Reporter assay:

[0216] The ability of anti-PD1-IL2R α -IL2 fusion constructs to induce PD1 targeted IL-2 receptor signaling was assessed using a STAT5-reporter assay. YT/STAT5-Luc reporter cells that expressed endogenous IL2R α , lacked IL2R α expression (KO cells), or overexpressed IL2R α , as well as expressed endogenous or overexpressed PD1, were co-incubated with an isotype control, IL2-Fc, a non-targeted IL2R α -IL2 or anti-PD1-IL2R α -IL2 (REGN10486, REGN10595, or REGN10597) and STAT5 reporter activity was assessed. Fold induction values and EC₅₀ are summarized in Tables 8 and 9, respectively.

[0217] In comparison to IL2, non-targeted IL2R α -IL2 exhibited reduced induction of STAT5 reporter activity, regardless of IL2R α expression. PD1 targeted molecules displayed increased potency compared to non-targeted IL2R α -IL2, irrespective of IL2R α expression. Furthermore, when PD1 was overexpressed on reporter cells, anti-PD1-IL2R α -IL2 potency further increased toward a potency similar to IL2.

PD-1 Competition assay:

[0218] The ability of anti-PD1 bivalent mAbs to interfere with anti-PD1-IL2R α -IL2 fusion constructs was assessed using a STAT5-reporter bioassay. YT/STAT5-Luc reporter cells that over-expressed IL2R α or lacked IL2R α expression, and overexpressed PD1, were incubated with 500nM of either isotype control or anti-PD1 bivalent mAbs (REGN2810, Comp 1 or Comp 2) prior to incubation with a titration of non-targeted IL2R α -IL2 or PD1 targeted anti-PD1-IL2R α -IL2 constructs (REGN10486, REGN10595, or REGN10597). Following incubation, STAT5 reporter activity was assessed. Competition of anti-PD1 bivalent mAbs with anti-PD1-IL2R α -IL2 would result in loss of PD1 targeting by anti-PD1-IL2R α -IL2, leading to reduced reporter activity. EC₅₀ values are summarized in Table 10.

[0219] In the absence of bivalent PD1 mAbs, PD1 targeted IL2R α -IL2 (REGN10486, REGN10595, REGN10597) had greater potency compared to non-targeted IL2R α -IL2 (REGN9904). While REGN10486 potency was strongly reduced by incubation of reporter cells with PD1 antibody (REGN2810, Comp 1 or Comp 2), the impact on REGN10595 and REGN10597 potency was minimal, regardless of IL2R α expression. The addition of anti-PD1 (REGN2810, Comp 1 or Comp 2) had no effect on the potency of the non-targeted IL2R α -IL2.

Primary T cell stimulation assay:

[0220] The ability of anti-PD1-IL2R α -IL-2 to enhance T cell stimulation was assessed in a functional primary T-cell assay measuring IFN- γ cytokine production. T cells incubated with mitomycin C treated Raji/CD80 KO/CD86 KO or Raji/CD80 KO/CD86 KO/hPD-L1 cells were treated with a constant amount of 0.5nM of an anti-CD3xanti-CD20 bispecific antibody and a titration of isotype control, IL2, an anti-PD1 bivalent antibody (REGN2810) alone or in combination with IL2, a non-targeted IL2R α -IL2, or anti-PD1-IL2R α -IL2 (REGN10486, REGN10595 or REGN10597). Co-incubation of the T cells with the Raji cells for 4 days led to measurable IFN- γ release. In the presence of Raji/CD80 KO/CD86 KO cells, treatment of T cells with either anti-PD1-IL2R α -IL2, IL2, or IL2 in combination with REGN2810, leads to similar levels of IFN γ release, whereas non-targeted IL2R α -IL2, Isotype Control antibodies, and REGN2810, lead to lower levels of IFN γ release. In the presence of Raji/CD80 KO/CD86 KO cells expressing hPD-L1, overall IFN γ release is reduced in isotype control treated samples, compared to RAJI cells not expressing PD-L1. Treatment with non-targeted IL2R α -IL2 and REGN2810 lead to a similar increase in IFN γ release, compared to isotype controls, while treatment with IL2, REGN10597 and REGN10595 lead to an even greater response. The greatest response is observed for REGN10486 or the combination of IL2 with REGN2810. EC₅₀

and maximal interferon release values across the antibody dose range are summarized in Table 11.

Table 7. EC₅₀ and fold induction values for anti-PD1 and anti-PD1-IL2R α -IL2 constructs determined by the PD1 antagonistic assay

	Fold induction*	EC ₅₀ (M)
Isotype control 1	1.126	ND
isotype control 2	1.202	ND
Non-targeted IL2R α -IL2	1.076	ND
REGN2810	19.210	3.668E-09
REGN10486	17.740	5.351E-09
H4H29512	1.286	ND
REGN10595	2.725	1.266E-08
H4H9048	1.237	ND
REGN10597	3.038	1.264E-08

ND: Not determined because a concentration-dependent response was not observed.

*Fold induction is the highest mean RLU value within the tested dose range relative to the mean RLU in the absence of antibody.

Table 8. Fold induction for IL2-Fc, non-targeted IL2R α -IL2, and anti-PD1-IL2R α -IL2 constructs determined by the IL2 reporter assay

	Isotype control	Non-targeted IL2R α -IL2	IL2-Fc	REGN10486	REGN10595	REGN10597
YT/Stat5-Luc	1.072	3.206	4.955	3.426	3.254	3.295
YT/Stat5-Luc/IL2R α KO	1.118	2.622	5.397	3.093	2.834	2.851
YT/Stat5-Luc/hIL2R α	1.074	4.868	5.485	5.080	5.016	5.187
YT/Stat5-Luc/hPD1	1.082	4.521	7.967	5.605	6.638	6.393
YT/Stat5-Luc/IL2R α KO/hPD1	1.061	2.586	3.870	3.347	3.612	3.538
YT/Stat5-Luc/hIL2R α /hPD1	1.000	2.770	3.060	2.714	3.013	2.879

*Fold induction is the highest mean RLU value within the tested dose range relative to the mean RLU in the absence of antibody.

Table 9. EC₅₀ for IL2-Fc, non-targeted IL2R α -IL2, and anti-PD1-IL2R α -IL2 constructs determined by the IL2 reporter assay

	Isotype control	Non-targeted IL2R α -IL2	IL2-Fc	REGN10486	REGN10595	REGN10597
YT/Stat5-Luc	ND	NC	4.023E-10	NC	NC	NC
YT/Stat5-Luc/IL2R α KO	ND	NC	1.327E-09	NC	NC	NC
YT/Stat5-Luc/hIL2R α	ND	1.631E-09	2.415E-11	7.524E-10	8.87E-10	8.482E-10
YT/Stat5-Luc/hPD1	ND	NC	3.918E-10	1.702E-10	2.193E-10	1.749E-10
YT/Stat5-Luc/IL2R α KO/hPD1	ND	NC	4.423E-10	1.948E-10	1.865E-10	1.913E-10
YT/Stat5-Luc/hIL2R α /hPD1	ND	9.714E-10	2.316E-11	1.668E-11	5.777E-11	3.533E-11

NC: Not calculated because the data did not fit a 4-parameter logistic equation.

ND: Not determined because a concentration-dependent response was not observed.

Table 10. EC₅₀ values for non-targeted IL2R α -IL2 and anti-PD1-IL2R α -IL2 constructs in presence of anti-PD1 antibody or isotype control

		-	Isotype control	REGN2810	Comp 1	Comp 2
Non-targeted IL2R α -IL2	YT/STAT5-Luc/IL2R α KO/hPD1	NC	NC	NC	NC	NC
	YT/STAT5-Luc/hIL2R α /hPD1	7.033E-10	7.906E-10	6.506E-10	9.182E-10	9.376E-10
REG10486	YT/STAT5-Luc/IL2R α KO/hPD1	9.388E-11	1.295E-10	NC	NC	NC
	YT/STAT5-Luc/hIL2R α /hPD1	1.499E-11	2.018E-11	5.860E-10	6.927E-10	4.531E-10
REGN10595	YT/STAT5-Luc/IL2R α KO/hPD1	2.499E-10	3.623E-10	4.550E-10	3.480E-10	2.941E-10
	YT/STAT5-Luc/hIL2R α /hPD1	5.300E-11	6.223E-11	1.375E-10	1.335E-10	1.531E-10
REGN10597	YT/STAT5-Luc/IL2R α KO/hPD1	1.285E-10	1.317E-10	2.216E-10	3.058E-10	2.807E-10
	YT/STAT5-Luc/hIL2R α /hPD1	5.448E-11	7.094E-11	1.172E-10	7.649E-11	8.046E-11

NC: Not calculated because the data did not fit a 4-parameter logistic equation.

Table 11. EC₅₀ and Max IFN γ release values from primary T-cells treated with IL2, anti-PD1 or anti-PD1-IL2R α -IL2 constructs

	Max IFN γ (pg/mL)		EC ₅₀ (M)	
	Raji/ CD80 KO/ CD86 KO	Raji/ CD80 KO/ CD86 KO/ hPD-L1	Raji /CD80 KO/ CD86 KO	Raji/CD80 KO/ CD86 KO/ hPD-L1
Isotype control 1	1512	386	ND	ND
Isotype control 2	1563	343	ND	ND
Non-targeted IL2R α -IL2	1687	859	7.325E-09	NC
REGN10486	2306	1687	NC	1.457E-09
REGN10595	2108	1157	1.096E-09	2.047E-09
REGN10597	2484	1261	4.798E-10	1.839E-09
IL2	2544	1081	4.914E-10	2.660E-10
REGN2810	1172	858	ND	1.833E-09
IL2 + REGN2810	2500	1868	3.148E-10	2.016E-10

NC: Not calculated because the data did not fit a 4-parameter logistic equation.

ND: Not determined because a concentration-dependent response was not observed.

EXAMPLE 6: *In vivo* anti-tumor efficacy assessment in PD1xLAG3 humanized mice

Study #1:

[0221] Human PD1 x LAG3 knockin mice (described in Burova E. *et al.*, Mol Cancer Ther 2019 (18) (11) 2051-2062) were inoculated s.c. with 3×10^5 MC38 tumor cells on day 0 and were randomized on day 7 when the average tumor size reached 95mm³. Mice were then treated intraperitoneally with isotype control Ab (0.33mg/kg), isotype-IL2R α -IL2 (0.5mg/kg) + anti-hPD1 antibody (0.33mg/kg) or anti hPD1-IL2R α -IL2 (0.5mg/kg) + isotype control Ab (0.33mg/kg) semi-weekly for four total injections. Average tumor volumes (mm³ + SEM) in each treatment group were plotted (FIG. 1A). Tumor sizes were calculated as $v = ab^2/2$, where a represents the longest tumor diameter and b is the perpendicular tumor diameter. Arrows in FIG. 1A indicate the days of treatment.

[0222] Kaplan-Meier survival curves in each treatment group were also plotted (FIG. 1B). Loss of survival was defined as euthanasia when tumors showed profound ulceration or when tumors reached 20mm in any dimension or 2250mm³ in total volume.

Study #2:

[0223] Human PD1 x LAG3 knockin mice were inoculated s.c. with 3×10^5 MC38 tumor cells on day 0 and were randomized on day 7 when the average tumor size reached 105mm³. Mice were then treated intraperitoneally with isotype (0.5mg/kg) or three different

clones of anti-hPD1-IL2R α -IL2 (0.5mg/kg) semi-weekly for four total injections. Average tumor volumes (mm³ + SEM) in each treatment group were shown (FIG. 1C). Tumor sizes were calculated as $v=ab^2/2$, where a represents the longest tumor diameter and b is the perpendicular tumor diameter. Arrows in the figure indicate the days of treatment.

[0224] Kaplan-Meier survival curves in each treatment group were also plotted (FIG. 1D). Loss of survival was defined as euthanasia when tumors showed profound ulceration or when tumors reached 20mm in any dimension or 2250mm³ in total volume.

Results summary and conclusions

[0225] In Study #1, anti-tumor efficacy of anti-hPD1-IL2R α -IL2 was evaluated in comparison with the combination of its two individual components, Isotype-IL2R α -IL2 and equal molar of parental anti-hPD1 blocking antibody.

[0226] While isotype-IL2R α -IL2 + anti-hPD1 was not able to confer effective tumor control at the dose tested, the same molar dose of anti-hPD1-IL2R α -IL2 + isotype treatment was able to regress established tumors, leading to long-term tumor-free survival in the majority of treated mice (FIGS. 1A-B). This result demonstrated the superior anti-tumor efficacy of anti-hPD1-targeted IL2R α -IL2 to the combination of untargeted-IL2R α -IL2 and parental anti hPD1 antibody.

[0227] In Study #2, antitumor activities of three different clones of anti-hPD1-IL2R α -IL2 were compared. One of the anti-hPD1 clones is a strong blocker, whereas the other two minimally block hPD1 signaling. All three clones of the anti-hPD1-IL2R α -IL2 molecule showed comparably potent anti-tumor efficacies and resulted in similar long-term tumor-free survival rates (FIGS. 1C-D). This result indicated anti-hPD1-targeted delivery of IL2R α -IL2, more than its blocking activity, is primarily responsible for the robust anti-tumor effect of anti-hPD1-IL2R α -IL2 therapy. It also indicated the potential to combine an anti-hPD1-IL2R α -IL2 molecule with a non-competing PD1 blocking reagent to further boost its therapeutic efficacy.

EXAMPLE 7: Ability of anti-PD1-IL2R α -IL2 fusion constructs to bind recombinant monomeric IL2R α , IL2R β , or IL2R γ protein in solution

Human IL-2 Receptor Alpha Binding

[0228] Binding of human IL2R α expressed with a C-terminal hexahistidine tag (hIL2R α .6H, R&D, Catalog # 10305-RL-050) to purified anti-PD1 antibodies with C-terminal IL2R α -IL2 fusions was determined using a real-time surface plasmon resonance biosensor technology with Biacore S-200 instrument. To prepare hIgG capturing surfaces, CM5 Biacore

sensor surfaces were derivatized by amine coupling with a mixture of monoclonal anti-human Fab antibodies (Cytiva, catalog # 28-9583-25). All Biacore binding studies were performed at 25°C in a buffer composed of 0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, and 0.05% v/v Surfactant P20 (HBS-EP running buffer). Anti-PD1-IL2R α -IL2 constructs and controls were captured onto this anti-hFab surface by injecting 3 μ g/mL solutions for 1 minute at 8 μ L / min. The solution of hIL2R α .6H at 1 μ M prepared in the HBS-EP running buffer was then injected over the captured antibody IL2 fusion construct captured surfaces at a flow rate of 50 μ L/minute. IL2 fusion antibody association was monitored for 1 minute followed by a 1 minute dissociation phase in HBS-EP running buffer. Binding responses were measured at the end of injection and are presented in Table 12. At the end of each cycle, the anti-PD1-IL2R α -IL2 fusion construct capture surface was regenerated using a 30 sec injection of 10mM glycine, pH 1.5.

Human IL-2 Receptor Beta / Gamma Binding

[0229] Binding of human IL2 receptor beta (hIL2R β) expressed with a C-terminal myc-myc-hexahistidine tag (hIL2R β .mmH, REGN9169) or human IL2 receptor gamma (hIL2R γ) expressed with a C-terminal myc-myc-hexahistidine tag (hIL2R γ .mmH, REGN1183) binding to anti-PD1-IL2R α -IL2 fusion constructs were determined using a real-time surface plasmon resonance biosensor technology on a Biacore S-200 instrument. The CM5 Biacore sensor surface was derivatized by amine coupling with a mixture of monoclonal anti-human Fab antibodies (Cytiva, catalog # 28-9583-25). All Biacore binding studies were performed at 25°C in a buffer composed of 0.01M HEPES pH 7.4, 0.15M NaCl, 3mM EDTA, 0.05% v/v Surfactant P20 (HBS-EP running buffer). All IL2 receptor components were prepared in the same buffer. Anti-PD1-IL2R α -IL2 constructs and controls were captured onto this anti-hFab surface by injecting 3 μ g/mL solutions for 1 minute at 8 μ L / min.

[0230] It is reported in the literature (Liparoto, *et al. Biochemistry*. 2002) that IL2R γ possesses an IL2R β or IL2R α / β dependent affinity for IL2. Addition of IL2R β , therefore, allows the formation of a detectable ternary complex. A sequential binding experiment was designed to explore IL2R γ binding in the presence of pre-bound IL2R β . To the anti-PD1-IL2R α -IL2 surfaces, a 30 sec injection of 1 μ M IL2R β .mmH at 50 μ L/min was immediately followed by 1 minute injection of a mixture solution of 1 μ M IL2R β .mmh and 1 μ M IL2R γ .mmH. Binding signals at the end of each injection were recorded. Results are presented in Table 13. At the end of each cycle, the anti-PD1-IL2R α -IL2 fusion construct capture surface was regenerated using a 30 sec injection of 10mM glycine, pH 1.5.

[0231] To calculate the activity of anti-PD1-IL2R α -IL2 fusion constructs, the theoretical maximum signal (TRmax) was first calculated based on the amount of anti-PD1-IL2-IL2R α fusion captured, the stoichiometry of IL2 receptor interaction, and the molecular weights of the interacting proteins. Then the activity of anti-PD1-IL2R α -IL2 fusion constructs was then expressed as a percentage of the calculated TRmax. Furthermore, the percentage of TRmax results was then normalized to the control antibodies (REGN1945 and REGN8512) using the following formula:

$$\text{Normalized Activity (\%)} = \frac{(\% \text{TRmax Sample} - \% \text{TRmax REGN1945})}{(\% \text{TRmax REGN8512} - \% \text{TRmax REGN1945})} \times 100$$

Results, summary, and conclusions

[0232] The anti-PD1-IL2-IL2R α fusion constructs demonstrated greatly reduced binding to hIL2R α compared to an unattenuated (IL2 only) control.

[0233] The anti-PD1-IL2R α -IL2 fusion constructs demonstrated ability to bind hIL2R β with partially reduced binding signals compared to the unattenuated (IL2 only) control. In the presence of bound hIL2R β , marginal binding of hIL2R γ was observed compared to the unattenuated IL2 control construct.

Table 12. Binding of anti-PD1 anti-PD1-IL2R α -IL2 fusion constructs to bind recombinant monomeric IL2R α protein

Ab reagent	IL2R α				
	mAb Capture (RU)	1 μ M hIL2R α .6H Bind (RU)	TRmax (RU)	% TRmax	% Normalized Activity
REGN10486	275.7	0.9	58.5	1.5	1.3
REGN10595	152.1	2.1	32.0	6.6	6.6
REGN10597	206.7	0.6	43.6	1.4	1.2
REGN8512 (Ab-linked to IL2)	585.6	144.9	151.0	96.0	100.0
Isotype control	422.3	0.3	133.3	0.2	0.0

Table 13: Binding of anti-PD1-IL2R α -IL2 fusion constructs to bind recombinant monomeric IL2R β or IL2R γ binding in the presence of pre-bound IL2R β

Ab reagent	IL2R β					IL2R γ After IL2R β			
	mAb Capture (RU)	1 μ M hIL2R β .m mH Bind (RU)	TRmax (RU)	% TRmax	% Normalized Activity	1 μ M hIL2R γ .m mH Bind (RU)	TRmax (RU)	% TRmax	% Normalized Activity
REGN10486	355.1	13.3	111.5	12.0	46.4	0.8	125.7	0.6	5.5
REGN10595	324.1	15.6	101.7	15.3	61.6	2.2	114.7	1.9	12.6
REGN10597	304.3	16.8	95.5	17.6	71.9	1.4	107.7	1.3	9.3
REGN8512 (Ab linked to IL2)	1474.0	110.3	462.6	23.8	100.0	90.0	521.7	17.3	100.0
Isotype control	611.8	3.2	192.0	1.7	0.0	-0.7	216.5	-0.3	0.0

EXAMPLE 8: Cell binding of anti-PD1-IL2R α -IL2 fusion constructs and parental mAbs characterized by a FACS binding assay

[0234] To compare hPD1 binding of ahPD1-IL2R α -IL2 proteins to their respective parental anti-hPD1 antibodies, YT/STAT5 luc/CI4 cells expressing endogenous levels of hPD1 were washed twice with FACS wash (PBS+2%FBS) and resuspended at 1×10^6 cells/ml in FACS wash. Cells (100 μ l/well) were then plated in 96 well U bottom plates. Cells were spun down (1200 RPM, 5 minutes) and resuspended in 100 μ l/well of the various antibody dilutions listed in mAb clone IDs. The antibody titrations were prepared starting at 20 μ g/ml with a 6-fold dilution using FACS wash. Cells were incubated with the antibodies for 30 minutes at 4°C. The unbound antibodies were then removed by washing the cells twice using FACS wash. The cells were then resuspended in 100 μ l/well of 1:200 dilution of APC-anti-human Fc γ secondary antibody. The cells were incubated for 30 minutes at 4°C. The unbound antibodies were then removed by washing the cells twice using FACS wash. The cells were then resuspended in 160 μ l/well of FACS wash and run to test binding using BD FACSCanto™ Flow Cytometer.

[0235] Anti-hPD1-IL2R α -IL2 proteins and their respective anti-hPD1 parental antibodies bind comparably to PD1+ cells (FIGS. 2A, 2B). Anti-hPD1-IL2R α -IL2 proteins binding shows slightly lower affinities (~2x) than the corresponding parental mAbs.

EXAMPLE 9: Fab-exchange and native mass spectrometry (MS) analysis of anti-PD1-IL2R α -IL2 fusion constructs

[0236] Fab-exchange and native mass spectrometry (MS) analysis were used to determine the conformation of the PD-1-IL2R α -IL2 fusion molecules. REGN10597, REGN8509, REGN2810, and REGN475 were each treated with peptide N-glycosidase F (PNGase F; 1 IUB milliunit per 10 μ g of protein) at 45°C for 1 hour to completely remove the glycan chains from each heavy chain constant region. The deglycosylated protein samples were then mixed according to FIG. 3A and Table 14 below, and each mixture was incubated at 37 °C in the presence of 2 mM DTT for 30 min. The treated protein mixtures were then subjected to native desalting size exclusion chromatography coupled to mass spectrometry (SEC-MS) analysis on a native LC-MS platform (refer to provisional patent #10724). Desalting SEC was performed on a BEH® SEC column (4.6 $\times$ 30 mm, 200Å, 1.7 μ m) using an isocratic flow of 150 mM ammonium acetate (pH 6.8) at a flow rate of 0.2 mL/min. Mass measurement was performed on a Thermo Q-Exactive UHMR mass spectrometer.

Table 14. Mixing of REGN10597, REGN8509, REGN2810, and REGN475 for Fab-exchange and native MS analysis

	Component		Mixture ratio (molar ratio)	Final protein concentration
Mixture 1	REGN2810	REGN475	1:1	5 mg/mL
Mixture 2	REGN8509	REGN475	1:1	5 mg/mL
Mixture 3	REGN10597	REGN475	1:1	5 mg/mL

[0237] With hinge-region disulfide bond disrupted, IgG4 molecules go through fast Fab exchange, the product of which can be monitored by native MS analysis. Control experiments using a mixture of two IgG4 antibodies (Mixture 1) or a mixture of one IgG4 antibody + anti-hCD20-IL2 (Mixture 2) displayed Fab exchange under partially reduced conditions and Fc exchange under FabRICATOR digested conditions (FIG. 3B). Fab or Fc exchange products were not observed for the mixture of PD1-IL2R α -IL2 + an IgG4 molecule (Mixture 3) under partially reduced or FabRICATOR digested conditions, respectively, suggesting the PD1-IL2R α -IL2 molecules exist primarily in the inactive form, where strong inter-chain interaction between IL2R α and IL2 prohibits Fab (or Fc) exchange (FIG. 3B). In summary, the Fab-exchange and native MS analysis indicate that the anti-PD1-IL2R α -IL2 molecule exists primarily in the trans-sequestered conformation.

EXAMPLE 10: Ability of anti-PD1-IL2R α -IL2 constructs to enhance human primary T-cell activation as evaluated by a mixed lymphocyte reaction (MLR) assay

[0238] The ability of anti-PD1-IL2R α -IL2 constructs to enhance human primary T-cell activation was evaluated using a mixed lymphocyte reaction (MLR) assay. Allogeneic donor PBMC will stimulate T-cells leading to their proliferation and release of cytokines. The addition of recombinant IL-2 can further support T-cell activation. Thus, this assay was used to evaluate how PD1 targeted IL2R α -IL2 may impact T-cell proliferation and release of IFN γ , which serve as indicators of T-cell activation.

Isolation of human primary cells

[0239] Human PBMCs were isolated from leukopaks of peripheral blood from 2 healthy donors obtained from Precision for Medicine (donor 1) or Stem Cell Technologies (donor 2) using the EasySepTM Direct Human PBMC Isolation Kit and following the manufacturer's recommended protocol. CD3⁺ T-cells from Donor 2 were isolated from PBMCs using an EasySepTM Human CD3⁺ T Cell Isolation Kit from StemCell Technologies and following the manufacturer's recommended instructions.

Assay Procedure:

[0240] Isolated, Donor 2 CD3⁺ T-cells were resuspended at a concentration of 1×10^6 cells/ml in primary culture media (X Vivo 15 media supplemented with 10% FBS, 10mM HEPES, 1mM Sodium Pyruvate, 1X non-essential amino-acids and 0.01 mM Beta-Mercaptoethanol). Donor 1 PBMCs (10×10^6 cells/ml) were treated for 1 hour at 37°C/5% CO₂ with 50ug/mL of mitomycin C diluted in primary culture media (to arrest cell growth). After washing 3 times with primary culture media, PBMC were resuspended and added to Donor 2 T-cells, such that the final ratio of T-cell to PBMC was 1:3 (1×10^6 T-cells + 3×10^6 PBMC per ml). After incubating CD3⁺ T-cell/PBMC mixtures for 6 days, T-cells were re-isolated using Miltenyi CD3⁺ Microbeads following manufacturer's instructions. Subsequently, isolated cells were rested for 24h in primary cell culture media. 100,000 T-cells were added to wells of a round bottom microtiter plate, and freshly mitomycin C treated Donor 1 PBMCs were added to wells at 300,000 cells/well. Anti-PD1-IL2R α -IL2 (REGN10597, REGN10595 or REGN10486), isotype-IL2R α -IL2 control (REGN9903), anti-PD1 (REGN2810), recombinant IL2, or matched IgG4 and IgG4s isotype controls (REGN1945 and REGN7540 respectively) were titrated 1:6 in a 9 point dilution, ranging from 1000nM to 0.595pM, with a final 10th point containing no antibody (represented by the lowest point on the curve). Each condition was performed in triplicate. After 72 hour incubation at 37°C/5% CO₂, microtiter plates were centrifuged to pellet

the cells, and 50µl of media supernatant was collected. From the collected supernatant, 5 µl was tested in a human IFN γ AlphaLISA (PerkinElmer) assay according to the manufacturer's protocol. The measurements were acquired on the multilabel plate reader Envision (PerkinElmer). The pelleted cells were resuspended in primary stimulation media containing 3H-thymidine (1.25mCi/ml), and incubated for 6 hours at 37°C/5% CO₂. Plates were processed using a Filtermate Cell Harvester (PerkinElmer) and measured using a MicroBeta2 Microplate counter (PerkinElmer). Values were recorded as counts per minute (CPM). The EC₅₀ values of the antibodies were determined from a four-parameter logistic equation over a 10-point dose-response curve using GraphPad Prism™ software.

[0241] In the presence of allogeneic PBMCs, T-cells treated with a dose titration of recombinant IL-2, anti-PD-1-IL2 α -IL2 (REGN10597, REGN10595, REGN10486) or isotype-IL2 α -IL2 control (REGN9903) lead to dose-dependent increases in IFN γ release and proliferation (FIGS. 4A, 4B and Table 15). Recombinant IL2 led to the highest maximum cytokine release and most potent proliferation, followed by the anti-PD1-IL2 α -IL2 molecules. Isotype-targeted IL2 α -IL2, in comparison to PD-1 targeted, exhibited reduced potency for cytokine release and proliferation. REGN2810 led to a minor dose-dependent increase in IFN γ only, while matched IgG4 and IgG4s isotype controls (REGN1945 and REGN7540, respectively) did not impact cytokine release or proliferation.

Table 15. Maximum and EC₅₀ values for IFN γ release and proliferation

Antibodies	IFN γ Release		Proliferation	
	Max [pg/mL]	EC ₅₀ [M]	Max [CPM]	EC ₅₀ [M]
REGN10486	1709.7	4.44E-09	22558	1.3E-10
REGN10595	1395.2	1.27E-09	25376	6.1E-11
REGN10597	1534.0	9.53E-09	26602	1.8E-10
REGN9903	1268.0	1.53E-08	21951	4.88E-10
REGN2810	158.3	NC	4671	ND
REGN1945	2.4	ND	4342	ND
rhIL-2	5552.6	NC	20711	NC
REGN7540	6.2	ND	5702	ND

Abbreviations: ND: Not Determined, because a concentration-dependent increase was not observed; NC: Not calculated because the data did not fit a 4-parameter logistic equation.

EXAMPLE 11: Comparison of anti-PD1-IL2R α -IL2 and isotype control-IL2R α -IL2 + anti-PD1 constructs in a Raji-based primary T cell stimulation assay

[0242] The ability of anti-PD1-IL2R α -IL2 constructs to enhance human primary T-cell activation was evaluated using a Raji-cell based primary T cell assay. T-cells were stimulated by coculture with Raji cells knocked out for CD80 and CD86 and over-expressing human PD-L1 (Raji/CD80 KO/CD86 KO/hPD-L1) in the presence of a CD3xCD20 bispecific antibody, leading to T cells release of cytokines. The addition of recombinant IL-2 can further support T-cell activation. Thus, this assay was used to evaluate how PD1 targeted IL2R α -IL2 may impact T-cell release of IFN γ , which serve as indicators of T-cell activation.

Isolation of human primary cells:

Human PBMCs were isolated from leukopaks of peripheral blood from a healthy donor obtained from Precision for Medicine using the EasySepTM Direct Human PBMC Isolation Kit and following the manufacturer's recommended protocol. CD3+ T-cells were isolated from PBMCs using an EasySepTM Human CD3+ T Cell Isolation Kit from StemCell Technologies and following the manufacturer's recommended instructions.

Raji-based T cell assay Procedure:

[0243] T cells were spun down, resuspended in stimulation media (X Vivo 15 media supplemented with 10% FBS, 10mM HEPES, 1mM Sodium Pyruvate, 1X non-essential amino-acids and 0.01 mM Beta-Mercaptoethanol) and 1×10^5 cells/well plated out into 96-well round-bottom plates. Raji/CD80 KO/CD86 KO/hPD-L1 cells (1×10^7 cells/ml) were treated for 1 hour at 37°C / 5% CO₂ with 20 mg/ml mitomycin C diluted in stimulation media in order to arrest cell growth. Cells were then washed 3 times with D-PBS containing 2% FBS, resuspended in stimulation media and 5×10^4 cells were added per well. Subsequently, 0.5nM of anti-CD3 x anti-CD20 bispecific antibody (REGN1979) was added, along with either 20nM constant isotype control (REGN1945) or anti-PD1 (REGN2810). A titration of either isotype control (REGN7540), isotype-IL2R α -IL2 antibody (REGN9903), anti-PD1 antibody with VRs of mAb9048 (REGN15187), anti-PD1-IL2R α -IL2 (REGN10597), or an equimolar titration of REGN9903 + REGN15187 was added to wells in a 9-point, 1:6 serial dilution ranging from 500nM to 0.3pM per molecule, with the 10th dilution point containing only 0.5nM constant of REGN1979 and 20nM REGN2810 or REGN1945. Each condition was performed in duplicate. The plates were incubated for 96 hours at 37°C / 5% CO₂. IFN γ was quantified in the cell culture supernatant using an AlphaLISA assay according to the manufacturer's protocol. The measurements were

acquired on the multilabel plate reader Envision (Perkin Elmer). The EC₅₀ values of the cytokines were determined using GraphPad Prism™ software from a four-parameter logistic equation over a 10-point dose-response curve, where the 10th dilution point contained only 0.5nM constant of REGN1979 and 20nM REGN2810 or REGN1945.

Summary of Results

[0244] In the presence of Raji/CD80 KO/CD86 KO/hPDL1, T-cells treated with a dose titration of isotype-IL2R α -IL2 (REGN9903) or anti-PD1-IL2R α -IL2 (REGN10597) in the presence or absence of REGN2810 lead to surprisingly significant dose-dependent increases in IFN γ release (Table 16 and FIGS. 5A, 5B). Anti-PD1-IL2R α -IL2 molecule led to the highest maximum cytokine release, followed by the isotype-IL2R α -IL2 molecule alone or in combination with the anti-PD1 antibody REGN15187. Matched IgG4 and IgG4s isotype controls (REGN1945 and REGN7540, respectively) did not impact IFN γ release.

Table 16. Maximum values for IFN γ Release

	Max IFN γ Release [pg/mL]	
	Constant - 20nM REGN1945	Constant - 20nM REGN2810
REGN7540	5.1	75.0
REGN9903	46.9	110.6
REGN15187	17.0	79.5
REGN9903 + REGN15187	47.6	140.0
REGN10597	133.0	258.5

EXAMPLE 12: *In vivo* anti-tumor efficacy and safety profile of anti-PD1-IL2R α -IL2 fusion constructs

[0245] In a first experiment, the *in vivo* anti-tumor efficacy of single agent REGN10597 was assessed. Human PD1 x LAG3 knock-in mice (described in Burova E. *et al.*, 2019) were inoculated s.c. with 3x10⁵ MC38 tumor cells on day 0 and were randomized on day 7 when average tumor size reached 105 mm³. Mice in each randomized group received semi-weekly intraperitoneal injections of indicated molecules at specific dose levels for four total injections. Average tumor volumes (mm³ + SD) in each treatment group were plotted (FIG. 6A). Tumor sizes were calculated as $v = a \cdot b^2 / 2$, where *a* represents the longest tumor diameter, and *b* is the perpendicular tumor diameter. Arrows in the figure indicate the days of treatment.

Individual tumor growth curves and frequency of mice that underwent complete tumor rejection in each treatment group were shown (FIG. 6B).

[0246] REGN10597 monotherapy, at either 0.5 or 1 mg/kg dose levels, demonstrated superior anti-tumor activity over the combination of 1 mg/kg REGN9904 and 10 mg/kg REGN2810, resulted in a higher frequency of complete tumor regression in treated mice. An intermediate anti-tumor efficacy was also observed with 0.2 mg/kg REGN10597 treatment (FIGS. 6A, 6B).

[0247] In a second experiment, *in vivo* activity and toxicity profiles of REGN10597 were compared to those of the comparator REGN13233, an anti-PD1-targeted IL2 mutein with abrogated binding to IL2Ra. Human PD1 x LAG3 knock-in mice (described in Burova E. *et al.*, 2019) were inoculated s.c. with 3×10^5 MC38 tumor cells on day 0 and were randomized on day 7 when the average tumor size reached 70mm³. Mice in each randomized group received semi-weekly intraperitoneal injections of indicated molecules at specific dose levels for four total injections. Average tumor volumes (mm³ + SD) (FIG. 6C), Kaplan-Meier survival curves (FIG. 6D), and percentage of body weight changes (Mean + SD) (FIG. 6E) in each treatment group were plotted. Arrows in FIG. 6A indicate the days of treatment. Frequencies of mice that underwent complete tumor rejection were indicated for select groups in (FIG. 6D). On day 13, blood from all groups was collected and analyzed by flow cytometry. Counts of total white blood cells were shown (FIG. 6F).

[0248] Both REGN10597 and REGN13233 monotherapies demonstrated superior anti-tumor efficacy over the combination of REGN2810 with their respective non-targeted controls (REGN9904 for REGN10597, REGN13234 for REGN13233), and showed complete tumor regression in the majority of the treated mice in both groups (FIGS. 6C, 6D).

[0249] However, unlike REGN10597, which caused no obvious body weight change in treated mice, the same dose of REGN13233 treatment led to significant body weight loss that was concomitant with a marked increase of circulating lymphocytes (FIG. 6E). This difference in toxicity was likely attributable to the different IL-2 moieties in REGN10597 and REGN13233, as similar body weight loss and white blood cell count increase were observed in REGN13234 treated group (FIGS. 6E, 6F). Further immunophenotyping of white blood cells by multi-parameter flow cytometry revealed that REGN10597 more selectively expanded PD-1⁺ CD4 and CD8 T cells, whereas REGN13233 not only expanded those cells but also massively enlarged CD44⁺CD62L⁺ CD8 T cell and NK cell populations.

[0250] These results indicated although REGN10597 and the comparator molecule REGN13233 both displayed robust anti-tumor efficacy, REGN10597 has a better safety profile,

likely due to its ability to more selectively expand PD1⁺ T cells *in vivo* compared to REGN13233 which expanded broader CD8⁺ T and NK cell populations.

[0251] In a third experiment, *in vivo* anti-tumor efficacy of REGN10597 was compared to that of aPD1-IL2-IL2R α , a molecule that contains all the same parts as REGN10597, but the IL2 and IL2R α moieties are fused to each other in the reversed order. Human PD1 x LAG3 knock-in mice (described in Burova E. *et al.*, 2019) were inoculated s.c. with 3x10⁵ MC38 tumor cells on day 0 and were randomized on day 9 when average tumor size reached 120mm³. Mice in each group then received weekly intraperitoneal injection of indicated molecules at specific dose levels for two total injections. (FIG. 6G). Average tumor volumes (mm³ + SD) in each treatment group were plotted. Tumor sizes were calculated as $v = a \cdot b^2/2$, where a represents the longest tumor diameter, and b is the perpendicular tumor diameter. Arrows in the figure indicate the days of treatment (FIG. 6H). Kaplan-Meier survival curves in each treatment group were also shown. Loss of survival was defined as euthanasia when tumors showed profound ulceration or when tumors reached 20mm in any dimension or 2250mm³ in total volume. Frequencies of mice that underwent complete tumor rejection were indicated for select groups.

[0252] Unlike REGN10597, which displayed robust anti-tumor activity *in vivo*, the same or a higher dose of aPD1-IL2-IL2R α showed minimal tumor growth control. This result indicated the correct order of IL2R α -IL2 fusion in REGN10597 is crucial for its potent anti-tumor activity *in vivo* (FIGS. 6G, 6H).

EXAMPLE 13: Flow binding of Ab-IL2Ra-IL2 fusion molecules to IL2 receptors

[0253] Functional IL2 receptors are formed by the differential assembly of IL2R subunits (IL2Ra = CD25, IL2Rb = CD122 and IL2Rg = CD132) leading to medium affinity IL2 receptors (IL2Rb/IL2Rg) and high affinity IL2 receptors (IL2Ra/IL2Rb/IL2Rg) expressed on immune cells (PMID: 16293754). The ability of IL2 and IL2Ra-IL2 chimeric antibodies to bind cells expressing the medium affinity IL2 receptor (IL2Rb/g, knocked out for IL2Ra) or high affinity receptor (IL2Rb/g and IL2Ra over-expressing) was assessed using flow cytometry.

[0254] While YT cells express endogenously human PD1, Over-Expressing PD-1 cells (PD1 OE) were generated. To test the binding of human IL2 and human IL2Ra-IL2 chimeric antibodies (= primary antibody) to cells, YT/STAT5-Luc/PD1 OE reporter cells expressing endogenously all three IL2 receptors subunits, were genetically modified to display either the medium affinity IL2 receptor by Knocking Out the IL2Ra subunit (CD25 KO) or the high affinity receptor by Over-Expressing IL2Ra (CD25 OE) on the cell surface. Binding of

antibodies to these cells was detected using a fluorescently-labeled secondary antibody by flow cytometry. In brief, PD1 OE YT cells that were CD25 KO or CD25 OE were resuspended in stain buffer (2% FBS in PBS), 3×10^5 cells were seeded per well into 96 well plates and incubated for 30 min on ice with 1:5 serially diluted primary antibodies [Isotype-IL2 (REGN8512) or Isotype-IL2Ra-IL2 (REGN9904)]. The final antibody concentration ranged from 768 fM to 300 nM, including a no antibody control labeled as “secondary only”. After incubation, samples were washed with ice-cold stain buffer, followed by the incubation with an AF647-labeled anti-human IgG antibody on ice for 30 minutes. Unbound secondary antibodies were removed, samples washed once with ice-cold PBS, stained with a viability dye, washed once with ice-cold stain buffer, fixed for 30 minutes at room temperature, washed in stain buffer, resuspended in stain buffer, and filtered before the analysis on the iQue Plus flow cytometer to capture the geometric Mean Fluorescent Intensity (gMFI). The following equation was used to calculate the maximal fold binding over secondary only:

$$\text{Fold induction} = \frac{\text{Highest gMFI value within tested dose range}}{\text{Mean gMFI values of no primary antibody control}}$$

Summary of Results

[0255] Table 17 summarizes max geometric MFI and fold induction values for antibody-IL2 or antibody-IL2Ra-IL2 chimeric constructs binding to CD25 knocked-out or over-expressing cells. A dose dependent binding of an isotype IL2 molecule (REGN8512) and an isotype IL2Ra-IL2 molecule (REGN9904) was observed on both engineered YT cells expressing either the intermediate affinity IL2R β / γ receptor (CD25 KO) or the high affinity IL2R α / β / γ receptor (CD25 OE) (FIGS. 7A, 7B; Table 17). As expected max fold binding was greater for both constructs on CD25 OE cells. However, binding of Ab-IL2Ra-IL2 was greatly reduced compared to Ab-IL2 on both IL2R α / β / γ - and IL2R β / γ -expressing cell lines, suggesting “masking” of IL-2 by the presence of IL2Ra in the fusion protein.

Table 17: IL2 flow binding assay - Summary of Max gMFI and Fold induction values

	Test article	Max gMFI	Fold induction*
CD25 KO /PD1 OE	Isotype-IL2 [REGN8512]	7.18E+04	31.2
	Isotype-IL2Ra-IL2 [REGN9904]	3.99E+03	1.704
CD25 OE /PD1 OE	Isotype-IL2 [REGN8512]	3.33E+06	1341
	Isotype-IL2Ra-IL2 [REGN9904]	7.76E+04	33.11

*Fold induction defined as the highest gMFI value within the tested dose-range relative to the gMFI observed in absence of antibody.

EXAMPLE 14: Bioassay to assess potency of PD1-IL2R2-IL2 molecules

[0256] Functional IL2 receptors are formed by the differential assembly of IL2R subunits (IL2Ra = CD25, IL2Rb = CD122 and IL2Rg = CD132) leading to medium affinity IL2 receptors (IL2Rβ/γ) and high affinity IL2 receptors (IL2Rα/β/γ) expressed on immune cells (PMID: 16293754).

[0257] To assess the bioactivity of IL2 or IL2Ra-IL2 or IL-2-IL2Ra chimeric antibodies, cell-based reporter assays were established leading to the activation of STAT5 driven luciferase expression upon binding of IL2 to medium affinity or high affinity IL2 receptors on engineered YT/STAT5-Luc. While YT cells express endogenously human PD1, Over-Expressing PD-1 cells (PD1 OE) were generated. YT/STAT5-Luc/PD1 OE reporter cells expressing endogenously all three IL2 receptors subunits were genetically modified to display either the medium affinity IL2 receptor by Knocking Out the IL2Ra subunit (CD25 KO) or the high affinity receptor by Over-Expressing IL2Ra (CD25 OE) on the cell surface.

[0258] RPMI1640 supplemented with 10% FBS and P/S/G was used as assay medium to prepare cell suspensions and antibody dilutions. A day prior to screening, engineered YT/STAT5-Luc reporter cells (CD25 KO/PD1 OE and CD25 OE/PD1 OE) were diluted at 3×10^5 cells/mL. On the day of the assay, cells were spun down, resuspended in assay medium, plated at 2.5×10^4 reporter cells/well in 96 well white flat bottom plates and incubated with Isotype-IL2Ra-IL2 [REGN9903 or REGN9904], Isotype-IL2-IL2Ra, Isotype-IL2(3m) [REGN13234], anti-PD1(arm 9048)-targeted-IL2Ra-IL2 [REGN10597], anti-PD1(arm 9048)-targeted-IL2-IL2Ra or anti-PD1(arm 9048)-targeted-IL2(3m) [REGN13233], serially diluted (1:5) over an 11-point titration range (200nM to 21 fM) (FIGS. 1A, 1B, 1C, 1D), or (1:4)

over an 11-point titration range (250nM to 238 fM) (FIGS. 2A, 2B), and a 12th point containing no recombinant protein. Plates were incubated for 4h 30minutes (FIGS. 1A, 1B, 1C, 1D) or 4 hours (FIGS. 2A, 2B) at 37°C / 5% CO₂ and then 100mL ONE-Glo™ (Promega) reagent was added to the wells to lyse the cells and detect luciferase activity. The emitted light was measured in RLU on a multilabel plate reader Envision (PerkinElmer). EC₅₀ values of the antibodies were determined using GraphPad Prism™ software from a four-parameter logistic equation over a 12-point dose-response curve. Fold induction was calculated using the following equation:

$$\text{Maximum Fold Induction} = \frac{\text{Highest mean RLU value within tested dose range}}{\text{Mean RLU values of no protein control}}$$

Summary of Results

[0259] Isotype IL2Ra-IL2 (REGN9903), Isotype-IL2-IL2Ra, anti-PD1 targeted-IL2Ra-IL2 (REGN10597) and anti-PD1 targeted-IL2-IL2Ra lead to dose dependent increases in STAT5-driven reporter expression both in absence (FIG. 8A) or presence (FIG. 8B) of IL2Ra expression on reporter cells (Table 18). PD-1 targeted molecules exhibited enhanced potency compared to Isotype targeted molecules. IL2-IL2Ra chimeric molecules exhibited reduced potency compared to IL2Ra-IL2 chimeric molecules, whether in presence or absence of IL2Ra expression on reporter cells.

Table 18: IL2 reporter assay comparison of Ab-IL2Ra-IL2 and Ab-IL2-IL2Ra

Summary of EC₅₀ values and Fold induction

	EC50 (M)		Max Fold induction*	
	CD25 KO / PD1 OE	CD25 OE / PD1 OE	CD25 KO / PD1 OE	CD25 OE/ PD1 OE
Isotype-IL2Ra-IL2 [REGN9903]	NC	3.313E-10	4.37	7.00
aPD1(9048)-IL2Ra-IL2 [REGN10597]	5.781E-11	3.297E-11	5.72	7.36
Isotype-IL2-IL2Ra	NC	1.408E-09	1.25	7.78
aPD1(9048)-IL2-IL2Ra	2.052E-09	1.748E-10	4.42	7.77

NC: Not calculated because the data did not fit a 4-parameter logistic equation.

*Fold induction is the highest mean RLU value within the tested dose-range relative to the mean RLU in absence of antibody.

[0260] Isotype IL2Ra-IL2 (REGN9903), Isotype-IL2(3m) (REGN13234), anti-PD1 targeted-IL2Ra-IL2 (REGN10597) and anti-PD1 targeted-IL2(3m) (REGN13233) lead to dose dependent increases in STAT5-driven reporter expression both in absence (FIG. 9A) or presence (FIG. 9B) of IL2Ra expression on reporter cells (Table 19). PD1 targeted molecules exhibited similar potency in absence of IL2Ra expression in reporter cells (REGN13233: 53pM, REGN10597: 84pM) or presence of IL2Ra on reporter cells (REGN13233: 46pM, REGN10597: 37pM). In absence of PD1 targeting, IL2Ra-IL2 chimeric molecules exhibited reduced potency compared to IL2(3m) chimeric molecules.

Table 19: IL2 reporter assay comparison of Ab-IL2Ra-IL2 and Ab-IL2(3m)
Summary of EC₅₀ values and Fold induction

	EC50 (M)		Max Fold induction*	
	CD25 KO/ PD1 OE	CD25 OE/ PD1 OE	CD25 KO/ PD1 OE	CD25 OE/ PD1 OE
Isotype-IL2(3m) [REGN13234]	1.743E-09	3.875E-10	4.25	4.49
Isotype-IL2Ra-IL2 [REGN9904]	NC	7.687E-10	3.24	4.57
aPD1(9048)-IL2(3m) [REGN13233]	5.317E-11	4.644E-11	4.24	4.62
aPD1(9048)-IL2Ra-IL2 [REGN10597]	8.383E-11	3.706E-11	4.36	5.10

NC: Not calculated because the data did not fit a 4-parameter logistic equation.

*Fold induction is the highest mean RLU value within the tested dose-range relative to the mean RLU in absence of antibody.

[0261] The present disclosure is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the disclosure in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

CLAIMS

We claim:

1. A fusion protein comprising: (i) an antigen-binding moiety that binds specifically to human programmed cell death protein 1 (PD-1) and (ii) an interleukin 2 (IL2) moiety.
2. The fusion protein of claim 1, wherein the antigen-binding moiety comprises an antibody or antigen-binding fragment thereof that binds specifically to human PD-1.
3. The fusion protein of claim 2, wherein the antibody or antigen-binding fragment thereof that binds to human PD-1 is a human monoclonal antibody.
4. The fusion protein of any one of claims 1-3, wherein the antigen-binding moiety comprises three heavy chain complementarity determining regions (HCDRs) (HCDR1, HCDR2, and HCDR3) and three light chain CDRs (LCDR1, LCDR2, and LCDR3), wherein:
 - HCDR1 comprises an amino acid sequence of SEQ ID NO: 43, 4, or 24;
 - HCDR2 comprises an amino acid sequence of SEQ ID NO: 45, 6, or 26;
 - HCDR3 comprises an amino acid sequence of SEQ ID NO: 47, 8, or 28;
 - LCDR1 comprises an amino acid sequence of SEQ ID NO: 12 or 32;
 - LCDR2 comprises an amino acid sequence of SEQ ID NO: 14; and
 - LCDR3 comprises an amino acid sequence of SEQ ID NO: 16 or 35.
5. The fusion protein of claim 4, wherein the antigen-binding moiety comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising respective amino acid sequences of (i) SEQ ID NOs: 43, 45, 47, 12, 14, and 16; (ii) SEQ ID NOs: 4, 6, 8, 12, 14, and 16; or (iii) SEQ ID NOs: 24, 26, 28, 32, 14, and 35.
6. The fusion protein of claim 4 or 5, wherein the antigen-binding moiety comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprising respective amino acid sequences of SEQ ID NOs: 43, 45, 47, 12, 14, and 16.
7. The fusion protein of any one of claims 1-6, wherein the antigen-binding moiety comprises a heavy chain variable region (HCVR) comprising an amino acid sequence of SEQ ID NO: 41,

2, or 22; and a light chain variable region (LCVR) comprising an amino acid sequence of SEQ ID NO: 10 or 30.

8. The fusion protein of claim 7, wherein the HCVR comprises the amino acid sequence of SEQ ID NO: 2 and the LCVR comprises the amino acid sequence of SEQ ID NO: 10.

9. The fusion protein of claim 7, wherein the HCVR comprises the amino acid sequence of SEQ ID NO: 22 and the LCVR comprises the amino acid sequence of SEQ ID NO: 30.

10. The fusion protein of claim 7, wherein the HCVR comprises the amino acid sequence of SEQ ID NO: 41 and the LCVR comprises the amino acid sequence of SEQ ID NO: 10.

11. The fusion protein of any one of claims 1-10, wherein the antigen-binding moiety comprises a heavy chain constant region of SEQ ID NO: 55 and a light chain constant region of SEQ ID NO: 56.

12. The fusion protein of any one of claims 1-11, wherein the antigen-binding moiety comprises a heavy chain and a light chain wherein the heavy chain comprises an amino acid sequence of SEQ ID NO: 61, 57, or 59 and the light chain comprises an amino acid sequence of SEQ ID NO: 62, 58, or 60.

13. The fusion protein of any one of claims 1-12, wherein the antigen-binding moiety comprises a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62, 57/58, or 59/60.

14. The fusion protein of any one of claims 1-13, wherein the antigen-binding moiety comprises a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62.

15. The fusion protein of any one of claims 1-14, wherein the IL2 moiety comprises (i) IL2 or a fragment thereof; and (ii) IL2 receptor alpha (IL2R α) or a fragment thereof.

16. The fusion protein of claim 15, wherein the IL2 or fragment thereof is human IL2 (hIL2) or a fragment thereof.

17. The fusion protein of claim 16, wherein the IL2R α or fragment thereof is human IL2R α (hIL2R α) or a fragment thereof.
18. The fusion protein of any one of claims 15-17, wherein the IL2 or fragment thereof comprises the amino acid sequence of SEQ ID NO: 53.
19. The fusion protein of any one of claims 15-17, wherein the IL2R α or fragment thereof comprises the amino acid sequence of SEQ ID NO: 51.
20. The fusion protein of any one of claims 15-19, wherein the IL2 or fragment thereof is connected to the C-terminal of the IL2R α or fragment thereof via a linker.
21. The fusion protein of any one of claims 1-20, wherein the IL2 moiety is connected to the C-terminal of the heavy chain constant region of the antigen-binding moiety via a linker.
22. The fusion protein of claim 20 or 21, wherein the linker comprises an amino acid sequence of one or more repeats of GGGGS (SEQ ID NO: 67).
23. The fusion protein of any one of claims 20-22, wherein the linker comprises an amino acid sequence of SEQ ID NO: 50 or 52.
24. The fusion protein of any one of claims 1-23, wherein the IL2 moiety comprises the amino acid sequence of SEQ ID NO: 54.
25. A fusion protein comprising: (i) a first polypeptide comprising a light chain variable region (LCVR) of a human antibody that binds specifically to human PD-1; and (ii) a second polypeptide that comprises (a) a heavy chain variable region (HCVR) of the antibody that binds specifically to human PD-1 and (b) an IL2 moiety.
26. The fusion protein of claim 25, wherein the HCVR comprises three heavy chain complementarity determining regions (HCDRs) (HCDR1, HCDR2, and HCDR3) and the LCVR comprises three light chain CDRs (LCDR1, LCDR2, and LCDR3), wherein:
HCDR1 comprises an amino acid sequence of SEQ ID NO: 43, 4, or 24;
HCDR2 comprises an amino acid sequence of SEQ ID NO: 45, 6, or 26;

HCDR3 comprises an amino acid sequence of SEQ ID NO: 47, 8, or 28;

LCDR1 comprises an amino acid sequence of SEQ ID NO: 12 or 32;

LCDR2 comprises an amino acid sequence of SEQ ID NO: 14; and

LCDR3 comprises an amino acid sequence of SEQ ID NO: 16 or 35.

27. The fusion protein of claim 26, wherein the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 comprising amino acid sequences of (i) SEQ ID NOs: 43, 45, 47, 12, 14, and 16; (ii) SEQ ID NOs: 4, 6, 8, 12, 14, and 16; or (iii) SEQ ID NOs: 24, 26, 28, 32, 14 and 35.

28. The fusion protein of claim 26 or 27, wherein the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 comprise the amino acid sequences of SEQ ID NOs: 43, 45, 47, 12, 14, and 16, respectively.

29. The fusion protein of any one of claims 25-28, wherein the HCVR and the LCVR comprise amino acid sequences of (i) SEQ NOs: 41 and 10; (ii) SEQ ID NOs: 2 and 10; or (iii) SEQ ID NOs: 22 and 30.

30. The fusion protein of any one of claims 25-29, wherein the first polypeptide comprises a light chain constant region linked to the LCVR.

31. The fusion protein of claim 30, wherein the light chain constant region comprises an amino acid sequence of SEQ ID NO: 56.

32. The fusion protein of any one of claims 25-31, wherein the second polypeptide comprises a heavy chain constant region linked to the HCVR and wherein the IL2 moiety is linked to the C-terminal of the heavy chain constant region.

33. The fusion protein of claim 32, wherein the heavy chain constant region comprises an amino acid sequence of SEQ ID NO: 55.

34. The fusion protein of any one of claims 25-33, wherein the first polypeptide comprises a light chain sequence of SEQ ID NO: 62, 58, or 60.

35. The fusion protein of any one of claims 25-34, wherein the second polypeptide comprises a heavy chain sequence of SEQ ID NO: 61, 57, or 59.
36. The fusion protein of any one of claims 25-35, comprising a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62, 57/58, or 59/60.
37. The fusion protein of any one of claims 25-36, comprising a heavy chain/light chain sequence pair of SEQ ID NOs: 61/62.
38. The fusion protein of any one of claims 25-37, wherein the IL2 moiety comprises (i) IL2 or a fragment thereof; and (ii) IL2R α or a fragment thereof.
39. The fusion protein of any one of claims 25-38, wherein the IL2 moiety is connected to the C-terminal of the heavy chain constant region via a linker.
40. The fusion protein of claim 38 or 39, wherein the IL2 or fragment thereof is connected to the C-terminal of IL2R α or fragment thereof via a linker.
41. The fusion protein of any one of claims 38-40, wherein the IL2 or fragment thereof is human IL2 (hIL2) or a fragment thereof.
42. The fusion protein of any one of claims 38-41, wherein the IL2R α or fragment thereof is human IL2R α (hIL2R α) or a fragment thereof.
43. The fusion protein of any one of claims 38-42, wherein the IL2 or fragment thereof comprises the amino acid sequence of SEQ ID NO: 53.
44. The fusion protein of any one of claims 38-43, wherein the IL2R α or fragment thereof comprises the amino acid sequence of SEQ ID NO: 51.
45. The fusion protein of any one of claims 40-44, wherein the linker comprises an amino acid sequence of one or more repeats of GGGGS (SEQ ID NO: 67).
46. The fusion protein of claim 45, wherein the linker comprises an amino acid sequence of SEQ ID NO: 50 or 52.

47. The fusion protein of any one of claims 25-46, wherein the IL2 moiety comprises the amino acid sequence of SEQ ID NO: 54.
48. The fusion protein of any one of claims 25-47, wherein the first polypeptide comprises the amino acid sequence of SEQ ID NO: 62, 58, or 60.
49. The fusion protein of any one of claims 25-48, wherein the second polypeptide comprises the amino acid sequence of SEQ ID NO: 63, 64, or 65.
50. The fusion protein of any one of claims 25-49, wherein the first polypeptide comprises the amino acid sequence of SEQ ID NO: 58 and the second polypeptide comprises the amino acid sequence of SEQ ID NO: 64.
51. The fusion protein of any one of claims 25-49, wherein the first polypeptide comprises the amino acid sequence of SEQ ID NO: 60 and the second polypeptide comprises the amino acid sequence of SEQ ID NO: 63.
52. The fusion protein of any one of claims 25-49, wherein the first polypeptide comprises the amino acid sequence of SEQ ID NO: 62 and the second polypeptide comprises the amino acid sequence of SEQ ID NO: 65.
53. The fusion protein of any one of claims 1-52, wherein the fusion protein forms a dimeric fusion protein.
54. The fusion protein of any one of claims 1-53, wherein the fusion protein dimerizes through their respective heavy chain constant regions.
55. The fusion protein of any one of claims 1-54, wherein the fusion protein does not cross-compete with REGN2810, pembrolizumab or nivolumab for binding to PD-1.
56. The fusion protein of any one of claims 1-55, wherein the fusion protein exhibits reduced activity in activating human IL2R α / β / γ and IL2R β / γ complexes as compared to IL2.

57. The fusion protein of any one of claims 1-56, wherein the fusion protein exhibits increased activity in activating human IL2R α as compared to a non-targeted IL2R α -IL2.
58. The fusion protein of any one of claims 1-57, wherein the fusion protein exhibits increased activity in stimulating T cells as measured by a level of IFN- γ release as compared to a wild-type human IL2.
59. The fusion protein of any one of claims 1-58, wherein the fusion protein exhibits attenuated binding to IL2R α , IL2R β and IL2R γ .
60. A nucleic acid or plurality of nucleic acids comprising a polynucleotide sequence encoding the fusion protein of any one of claims 1-59.
61. A vector comprising the nucleic acid or plurality of nucleic acids of claim 60.
62. A host cell comprising the nucleic acid or plurality of nucleic acids of claim 60 or the vector of claim 61.
63. The host cell of claim 62 expressing a first vector comprising a polynucleotide sequence that encodes the first polypeptide of the fusion protein set forth in any one of claims 25-59 and a second vector comprising a polynucleotide sequence that encodes the second polypeptide of the fusion protein set forth in any one of claims 25-59.
64. A method of producing the fusion protein of any one of claims 1-59, comprising culturing the host cell of claim 62 or 63 under conditions permitting production of the fusion protein or fragment, and recovering the fusion protein or fragment thereof so produced.
65. A pharmaceutical composition comprising the fusion protein of any one of claims 1-59.
66. The pharmaceutical composition of claim 65 further comprising an anti-PD-1 antibody or antigen-binding fragment thereof.
67. The pharmaceutical composition of claim 66, wherein the anti-PD-1 antibody or antigen-binding fragment thereof does not cross-compete with the fusion protein for binding to PD-1.

68. A method of treating cancer, comprising administering to a subject in need thereof a therapeutically effective amount of the fusion protein of any one of claims 1-59 or the pharmaceutical composition of any one of claims 65-67.

69. The method of claim 68, comprising administering to the subject the fusion protein in an amount of 0.005 mg/kg to 10 mg/kg of the subject's body weight.

70. The method of claim 68 or 69, further comprising administering a second therapeutic agent or therapy to the subject.