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(73) Patenthaver: **Alpine Immune Sciences, Inc., 188 East Blaine Street , Suite 200, Seattle, WA 98102, USA**

(72) Opfinder: **SWANSON, Ryan, 201 Elliott Avenue West, Suite 230, Seattle, Washington 98119, USA**
**KORNACKER, Michael, 201 Elliott Avenue West, Suite 230, Seattle, Washington 98119, USA**
**MAURER, Mark F., 201 Elliott Avenue West, Suite 230, Seattle, Washington 98119, USA**
**ARDOUREL, Dan, 201 Elliott Avenue West, Suite 230, Seattle, Washington 98119, USA**
**DEMONTE, Daniel William, 201 Elliott Avenue West, Suite 230, Seattle, Washington 98119, USA**
**KUIJPER, Joseph L., 201 Elliott Avenue West, Suite 230, Seattle, Washington 98119, USA**

(74) Fuldmægtig i Danmark: **RWS Group, Europa House, Chiltern Park, Chiltern Hill, Chalfont St Peter, Bucks SL9 9FG, Storbritannien**

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# DESCRIPTION

## Cross-Reference to Related Applications

**[0001]** This application claims priority from U.S. provisional application No. 62/472,554 filed March 16, 2017, entitled "PD-L1 Variant Immunomodulatory Proteins and Uses Thereof," U.S. provisional application No. 62/475,076 filed March 22, 2017, entitled "PD-L1 Ligand Variant Immunomodulatory Proteins and Uses Thereof," U.S. provisional application No. 62/537,923 filed July 27, 2017, entitled "PD-L1 Ligand Variant Immunomodulatory Proteins and Uses Thereof," and U.S. provisional application No. 62/582,249 filed November 6, 2017, entitled "PD-L1 Ligand Variant Immunomodulatory Proteins and Uses Thereof,".

## Field

**[0002]** The present disclosure relates to therapeutic compositions for modulating immune response in the treatment of cancer and immunological diseases. The present disclosure relates to particular variants of PD-L1 that exhibit improved binding, such as improved binding affinity or selectivity, for PD-1.

## Background

**[0003]** Modulation of the immune response by intervening in the processes that occur in the immunological synapse (IS) formed by and between antigen-presenting cells (APCs) or target cells and lymphocytes is of increasing medical interest. Mechanistically, cell surface proteins in the IS can involve the coordinated and often simultaneous interaction of multiple protein targets with a single protein to which they bind. IS interactions occur in close association with the junction of two cells, and a single protein in this structure can interact with both a protein on the same cell (cis) as well as a protein on the associated cell (trans), likely at the same time. Although therapeutics are known that can modulate the IS, improved therapeutics are needed. Provided are immunomodulatory proteins, including soluble proteins or transmembrane immunomodulatory proteins capable of being expressed on cells, that meet such needs.

**[0004]** WO 2017/029389 discloses several variants of murine and human PD-L1 comprising an IgV and/or and IgC domain and fragments thereof. Wang et al (J. Exp. Med. (2003) 197, p1083-1091) also discloses variants of murine PD-L1. Kerstin et al (Nature (2011) 478, p476 - 482) and Wade et al (Science (2009) 326, p865-867) disclose the sequence of rabbit and horse orthologs of PD-L1, which have substitutions N45Q and N45K respectively. Terawaki et al (Immunology (2007) 19, p881 - 890) discusses the specific and high affinity binding of PD-L1 extracellular domain to PD-1 expressing cells. Srinivasan et al (Current protein and peptide science (2005) 6, p1 - 12) discloses that the Ig domain is highly conserved in cell surface proteins due to its ability to resist proteolysis. Omid et al (Methods (2014) 65, p114 -126) describes an engineered Fc variant of an Ig eliminates all effector functions via structural perturbations. Maya et al (MABS (2013) 5, p896-903 studies the effects of altered FcyR biding on antibody pharmacokinetics in cynomolgus monkeys. Danny et al (Nature Rev. Clin. Onc. (2016) 13, p273-290) looked at the future of cancer treatment by immunomodulation, CARs and combination therapy. WO 2016/168771 describes immunomodulatory proteins, comprising at least one non-immunoglobulin affinity modified IgSF domain comprising one or more amino acid substitutions in a wild-type IgSF domain. WO 2018/022945 and WO 2018/022946 disclose variant PD-L1 polypeptides including D43G, I20L, I36T, N45D substitutions and Fc fusions.

**Summary**

**[0005]** Provided herein is a variant PD-L1 polypeptide, in accordance with claim 1.

**[0006]** The unmodified PD-L1 is a human PD-L1 or a specific binding fragment thereof. In some of any such embodiments, the unmodified PD-L1 contains (i) the sequence of amino acids set forth in SEQ ID NO:30 or 1728, (ii) a sequence of amino acids that has at least 95% sequence identity to SEQ ID NO:30 or 1728; or (iii) a portion thereof including an IgV domain or IgC domain or specific binding fragments thereof or both.

**[0007]** In some of any such embodiments, the specific binding fragment of the IgV domain or IgC domain has a length of at least 50, 60, 70, 80, 90, 100, 110 or more amino acids; or the specific binding fragment of the IgV domain contains a length that is at least 80% of the length of the IgV domain set for as amino acids 24-130 of SEQ ID NO:3 and/or the specific binding fragment of the IgC domain contains a length that is at least 80% of the length of the IgC domain set forth as amino acids 133-225 of SEQ ID NO:3. In some of any such embodiments, the variant PD-L1 contains up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid modifications, optionally amino acid substitutions, insertions and/or deletions. In some of any such embodiments, the variant PD-L1 polypeptide contains a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:30, 1728, or a specific binding fragment thereof.

**[0008]** In accordance with the invention, the variant PD-L1 polypeptide exhibits increased binding affinity to the ectodomain of PD-1 compared to the binding of the unmodified PD-L1 to the ectodomain of PD-1. In some embodiments, the variant PD-L1 polypeptide exhibits altered binding to the ectodomain of CD80 compared to the binding of the unmodified PD-L1 to the ectodomain of CD80. In some of any such embodiments, the variant PD-L1 polypeptide exhibits altered binding to the ectodomain of PD-1 compared to the unmodified PD-L1. In some embodiments, the altered binding is altered binding affinity and/or altered binding selectivity.

**[0009]** In some of any such embodiments, the one or more amino acid substitutions are selected from I20L/N45D, I36T/N45D, D43G/N45D, N45D/V58A, N45D/S75P, N45D/N78I, I20L/I36T/N45D, D43G/N45D/V58A, I20L/E27G/D43G/N45D/V58A/N78I, I20L/D43G/N45D/V58A/N78I, I20L/A33D/D43G/N45D/V58A/N78I, I20L/D43G/N45D/N78I, V11A/I20L/E27G/D43G/N45D/HS1Y/S99G, I20L/K28E/D43G/N45D/V58A/Q89R, I20L/I36T/N45D, A33D/D43G/N45D/V58A/S75P, K23R/D43G/N45D, D43G/N45D/L56Q/V58A/G101G-ins (G101GG), I20L/K23E/D43G/N45D/V58A/N78I, I20L/K23E/D43G/N45D/VSOA/N78I, I20L/K28E/D43G/N45D/V58A/Q89R/G101G-ins (G101GG), N45D, N45D/K144E, N45D/P198S, N45D/P198T, N45D/R195G, N45D/R195S, N45D/S131F, N45D/V58D, N45D/I148V/R195G, N45D/K111T/R195G, N45D/N113Y/R195S, N45D/N165Y/E170G, N45D/Q89R/I98V, N45D/S131F/P198S, N45D/S75P/P198S, N45D/V50A/R195T, E27D/N45D/T183A/I188V, K23N/N45D/S75P/N120S, N45D/I148V/R195G/N201D, N45D/K111T/T183A/I188V, N45D/Q89R/F189S/P198S, N45D/T163I/K167R/R195G, N45D/V50A/I119T/K144E, T19A/N45D/K144E/R195G, V11E/N45D/T130A/P198T, V26A/N45D/T163I/T185A, K23NIN45D/L 124S/K 167T/R195G, K23N/N45D/Q73R/T163I, K28R/N45D/K57E/I98V/R195S, K28R/N45D/V129D/T163N/R195T, M41K/D43G/N45D/R64S/R195G, M41K/D43G/N45D/R64S/S99G, N45D/R68L/F173L/D197G/P198S, N45D/V50A/I148V/R195G/N201D, M41K/D43G/K44E/N45D/R195G/N201D, N45D/VSOA/L124S/K144E/L179P/R195G.

**[0010]** In some of any such embodiments, the variant PD-L1 polypeptide contains the variant PD-L1

polypeptide comprises or consists of the PD-L1 extracellular domain (ECD); and/or the variant PD-L1 polypeptide comprises or consists of the IgV domain and the IgC domain. In some of any such embodiments, the variant PD-L1 polypeptide includes the sequence of amino acids set forth in any of SEQ ID NOS: 66, 82, 101-104, 107, 109-110, 112-113, 115-117, 131, 147, 166-169, 172, 174-175, 177-178, 180-182, 254, 270, 289-292, 295, 297-298, 300-301, 303-305, 1725-1727, 1774-1780, 1785-1793, 1795, 1798-1800, 1802, 1804-1809, 1811-1818, 1863-1869, 1874-1882, 1884, 1887-1889, 1891, 1893-1898, 1900-1907, 1920-1922, 1935-1937, 1953, 1969, 1988-1991, 1994, 1996-1997, 1999-2000, 2002-2004, 2008or a specific binding fragment thereof, or a sequence of amino acids that exhibits at least 95% sequence identity to any of SEQ ID NOS: 66, 82, 101-104, 107, 109-110, 112-113, 115-117, 131, 147, 166-169, 172, 174-175, 177-178, 180-182, 254, 270, 289-292, 295, 297-298, 300-301, 303-305, 1725-1727, 1774-1780, 1785-1793, 1795, 1798-1800, 1802, 1804-1809, 1811-1818, 1863-1869, 1874-1882, 1884, 1887-1889, 1891, 1893-1898, 1900-1907, 1920-1922, 1935-1937, 1953, 1969, 1988-1991, 1994, 1996-1997, 1999-2000, 2002-2004, 2008 or a specific binding fragment thereof and that contains the one or more of the amino acid substitutions.

**[0011]** In some of any such embodiments, the variant PD-L1 polypeptide contains the IgV domain. In some of any such embodiments, the IgV domain or specific binding fragment thereof is the only PD-L1 portion of the variant PD-L1 polypeptide. In some embodiments, the IgC domain is the only PD-L1 portion of the variant PD-L1 polypeptide.

**[0012]** In accordance with the invention, the variant PD-L1 polypeptide specifically binds to the ectodomain of PD-1 with increased affinity compared to the binding of the unmodified PD-L1 to the same ectodomain of PD-1. In some of any such embodiments, the variant PD-L1 polypeptide specifically binds to the ectodomain of PD-1 and the ectodomain of CD80 each with increased affinity compared to the binding of the unmodified PD-L1 to the same ectodomains. In some of any such embodiments, the variant PD-L1 polypeptide specifically binds to the ectodomain of PD-1 with increased affinity and specifically binds to the ectodomain of CD80 with decreased affinity compared to the binding of the unmodified PD-L1 to the same ectodomains.

**[0013]** In some of any such embodiments, the increased affinity to the ectodomain of PD-1 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold or 60-fold compared to the unmodified PD-L1. In some aspects, the increased affinity to the ectodomain of CD80 is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold or 60-fold compared to the unmodified PD-L1. In some cases, the decreased affinity to the ectodomain of CD80 is decreased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold or 60-fold compared to the unmodified PD-L1.

**[0014]** In some of any such embodiments, the variant polypeptide specifically binds to the ectodomain of PD-1 with increased selectivity compared to the unmodified PD-L1. In some instances, the increased selectivity comprises a greater ratio of binding of the variant polypeptide for PD-1 versus CD80 compared to the ratio of binding of the unmodified PD-L1 polypeptide for the same ectodomains of PD-1 versus CD80. In some examples, the ratio is greater by at least or at least about 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold, 50-fold or more.

**[0015]** In some of any such embodiments, the CD80 is a human CD80.

**[0016]** In some of any such embodiments, the binding activity is altered (increased or decreased) more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold compared to the unmodified PD-L1.

**[0017]** In some of any such embodiments, the variant PD-L1 polypeptide is a soluble protein. In some of any such embodiments, the variant PD-L1 polypeptide lacks the PD-L1 transmembrane domain and intracellular signaling domain; and/or the variant PD-L1 polypeptide is not capable of being expressed on the surface of a cell. In some of any such embodiments, the variant PD-L1 polypeptide is linked to a multimerization domain. In some of any such embodiments, the variant PD-L1 polypeptide is a multimeric polypeptide, optionally a dimeric polypeptide, comprising a first variant PD-L1 polypeptide linked to a multimerization domain and a second variant PD-L1 polypeptide linked to a multimerization domain. In some embodiments, the first variant PD-L1 polypeptide and the second variant PD-L1 polypeptide are the same or different.

**[0018]** In some of any such embodiments, the multimerization domain is an Fc domain or a variant thereof with reduced effector function. In some of any such embodiments, the variant PD-L1 polypeptide is linked to a moiety that increases biological half-life of the polypeptide. In some of any such embodiments, the variant PD-L1 polypeptide is linked to an Fc domain or a variant thereof with reduced effector function.

**[0019]** In some of any such embodiments, the Fc domain is mammalian, optionally human; or the variant Fc domain comprises one or more amino acid modifications compared to an unmodified Fc domain that is mammalian, optionally human. In some of any such embodiments, the Fc domain or variant thereof contains the sequence of amino acids set forth in SEQ ID NO: 187 or SEQ ID NO: 188 or a sequence of amino acids that exhibits at least 85% sequence identity to SEQ ID NO: 187 or SEQ ID NO: 188. In some embodiments, the Fc domain comprises one or more amino acid modifications selected from among E233P, L234A, L234V, L235A, L235E, G236del, G237A, S267K, N297G, R292C, V302C, and K447del, each by EU numbering. In some embodiments, the Fc domain comprises the amino acid modification C220S by EU numbering. In some of any such embodiments, the Fc domain comprises the sequence of amino acids set forth in any of SEQ ID NOS: 1155, 1157, 1158, 1159, 1715, 1938, 1939, and 1940 or a sequence of amino acids that exhibits at least 85% sequence identity to any of SEQ ID NOS:1155, 1157, 1158, 1159, 1715, 1938, 1939, and 1940 and exhibits reduced effector function.

**[0020]** In some of any such embodiments, the variant PD-L1 polypeptide is linked indirectly via a linker, optionally a G4S linker. In some of any such embodiments, the variant PD-L1 polypeptide is a transmembrane immunomodulatory protein further containing a transmembrane domain linked to the extracellular domain (ECD) or specific binding fragment thereof of the variant PD-L1 polypeptide.

**[0021]** In some of any such embodiments, the transmembrane domain contains the sequence of amino acids set forth as residues 239-259 of SEQ ID NO:3 or a functional variant thereof that exhibits at least 85% sequence identity to residues 239-259 of SEQ ID NO:3. In some embodiments, the variant PD-L1 polypeptide further contains a cytoplasmic signaling domain linked to the transmembrane domain. In some instances, the cytoplasmic signaling domain contains the sequence of amino acids set forth as residues 260-290 of SEQ ID NO:3 or a functional variant thereof that exhibits at least 85% sequence identity to residues 260-290 of SEQ ID NO:3.

**[0022]** In some of any of the provided embodiments, the variant PD-L1 polypeptide modulates a response of an immune cell, such as a T cell. In some embodiments, the response, e.g. T cell response, is increased or is decreased. In some of any such embodiments, the variant PD-L1 increases IFN-gamma (interferon-gamma) expression relative to the unmodified PD-L1 in an in vitro T-cell assay. In some of any such embodiments, the variant PD-L1 decreases IFN-gamma (interferon-gamma) expression relative to the unmodified PD-L1 in an *in vitro* T-cell assay.

**[0023]** In some of any such embodiments, the variant PD-L1 polypeptide is deglycosylated.

**[0024]** In some embodiments of any one of the variant PD-L1 polypeptides described herein, the variant PD-L1 polypeptide increases T cell signaling relative to the unmodified PD-L1, such as determined using a reporter assay involving a T cell (e.g. Jurkat) engineered with a reporter (e.g. luciferase) operably connected to an IL-2 promoter. In some embodiments of any one of the variant PD-L1 polypeptides described herein, the variant PD-L1 polypeptide decreases T cell signaling relative to the unmodified PD-L1, such as determined using a reporter assay involving a T cell (e.g. Jurkat) engineered with a reporter (e.g. luciferase) operably connected to an IL-2 promoter. In some of any such embodiments, the variant PD-L1 polypeptide is provided in any of a variety of formats, such as soluble or immobilized (e.g. plate-bound).

**[0025]** Also provided is an immunomodulatory polypeptide containing the variant PD-L1 according to any of the provided embodiments linked, directly or indirectly via a linker, to a second polypeptide comprising an immunoglobulin superfamily (IgSF) domain. In some cases, the IgSF domain is affinity modified and exhibits altered binding to one or more of its cognate binding partner(s) compared to the unmodified or wild-type IgSF domain to the same one or more cognate binding partner(s). In some embodiments, the affinity-modified IgSF domain contains one or more amino acid modifications compared to the unmodified or wild-type IgSF domain of the IgSF family member. In some instances, the IgSF domain exhibits increased binding to one or more of its cognate binding partner(s) compared to the unmodified or wild-type IgSF domain of the IgSF family member to the same one or more cognate binding partner(s). In some examples, the IgSF domain is affinity modified and exhibits altered binding to one or more of its cognate binding partner(s) compared to the binding of the unmodified or wild-type IgSF domain of the IgSF family member to the same one or more cognate binding partner(s). In some embodiments, the variant PD-L1 is a first PD-L1 variant and the IgSF domain of the second polypeptide is an IgSF domain from a second variant PD-L1 wherein the first and second PD-L1 variant are the same or different.

**[0026]** In some embodiments, the variant PD-L1 polypeptide is capable of specifically binding to PD-1 and the IgSF domain of the second polypeptide is capable of binding to a cognate binding partner other than one specifically bound by the PD-L1 variant polypeptide. In some embodiments, the IgSF domain is from a member of the B7 family. In some cases, the IgSF domain is a tumor-localizing moiety that binds to a ligand expressed on a tumor or to an inflammatory-localizing moiety that binds to a cell or tissue associated with an inflammatory environment. In some embodiments, the IgSF domain is a tumor-localizing moiety that binds to a ligand expressed on a tumor. In some cases, the ligand is B7H6. In some embodiments, the IgSF domain is from NKp30.

**[0027]** In some embodiments, the IgSF domain or affinity-modified IgSF domain thereof, optionally of the second or third polypeptide, is or contains an IgV domain. In some embodiments, the variant PD-L1 polypeptide is or contains an IgV domain. In some embodiments, the immunomodulatory protein comprises a multimerization domain linked to one or both of the variant PD-L1 polypeptide of the IgSF domain. In some instances, the multimerization domain is an Fc domain or a variant thereof with reduced effector function. In some embodiments, the immunomodulatory protein is dimeric. In some embodiments, the immunomodulatory protein is homodimeric. In some embodiments, the immunomodulatory protein is heterodimeric.

**[0028]** In some of any such embodiments, the IgSF domain of the second polypeptide is an IgSF domain of a ligand that binds to an inhibitory receptor, or is an affinity-modified IgSF domain thereof. In some instances, the affinity-modified IgSF domain exhibits increased binding affinity and/or binding selectivity for the inhibitory receptor compared to binding of the unmodified IgSF domain to the same inhibitory receptor. In some embodiments, the inhibitory receptor is TIGIT, PD-1 or CTLA-4; or the ligand of the inhibitory receptor is PD-L2, CD155, CD112 or CD80.

**[0029]** In some of any such embodiments, the IgSF domain of the second polypeptide is an affinity-modified IgSF domain comprising: (i) a wildtype CD155 comprising an IgSF set forth in any of SEQ ID NOS: 47, 310, or 353, or a variant CD155 polypeptide comprising an IgSF domain of any of SEQ ID NOS set forth in Table 5, optionally any of the SEQ ID NOs: 311-352, 354-665, 1505-1576, 1551-1714; (ii) a wildtype CD112 comprising an IgSF domain set forth in any of SEQ ID NOS: 48, 666, or 761, or a variant CD112 polypeptide comprising an IgSF domain of any of SEQ ID NOS set forth in Table 4, optionally any of the SEQ ID NOs: 667-760, 762-931, 1433-1504; (iii) a wildtype CD80 comprising an IgSF domain set forth in any of SEQ ID NOS: 28, 1005, or 2030, or a variant CD80 polypeptide comprising an IgSF of any of SEQ ID NOS: set forth in Table 3, optionally any of the SEQ ID NOs: 932-964, 966-1038, 1040-1078, 1080-1112, 1114-1152; (iv) a wildtype PD-L2 comprising an IgSF domain set forth in any of SEQ ID NOS: 31, 1203, or 1263, or a variant PD-L2 polypeptide comprising an IgSF domain of any of SEQ ID NOS set forth in Table 3, optionally any of the SEQ ID NOs: 1204-1254, 1256-1331, 1333-1407, 1409-1432; (v) a sequence of amino acids that exhibits at least 95% sequence identity to any of the SEQ ID NOSs in (i)-(iv) and that comprises the amino acid substitution; or (vi) a specific binding fragment of any of (i)-(v).

**[0030]** In some embodiments, the immunomodulatory protein further contains a third polypeptide comprising an IgSF domain of an IgSF family member or an affinity-modified IgSF domain thereof, said affinity-modified IgSF domain comprising one or more amino acid modifications compared to the unmodified or wild-type IgSF domain of the IgSF family member. In some cases, the third polypeptide is the same as the first and/or second polypeptide or the third polypeptide is different from the first and/or second polypeptide. In some examples, the third polypeptide is selected from (i) a wildtype CD155 comprising an IgSF set forth in any of SEQ ID NOS: 47, 310, or 353, or a variant CD155 polypeptide comprising an IgSF domain set forth in any of SEQ ID NOS:311-352, 354-665, 1505-1576, 1551-1714; (ii) a wildtype CD112 comprising an IgSF domain set forth in any of SEQ ID NOS: 48, 666, or 761, or a variant CD112 polypeptide comprising an IgSF domain set forth in any of SEQ ID NOS: 667-760, 762-931, 1433-1504; (iii) a wildtype CD80 comprising an IgSF domain set forth in any of SEQ ID NOS: 28, 1005, or 2030, or a variant CD80 polypeptide comprising an IgSF domain set forth in any of SEQ ID NOS: 932-964, 966-1038, 1040-1078, 1080-1112, 1114-1152; (iv) a wildtype PD-L2 comprising an IgSF domain set forth in any of SEQ ID NOS: 31, 1203, or 1263, or a variant PD-L2 polypeptide comprising an IgSF domain set forth in any of SEQ ID NOS: 1204-1254, 1256-1331, 1333-1407, 1409-1432; (v) a sequence of amino acids that exhibits at least 95% sequence identity to any of the SEQ ID NOSs in (i)-(iv) and that comprises the amino acid substitution; or (vi) a specific binding fragment of any of (i)-(v). In some instances, the IgSF domain or affinity-modified IgSF domain thereof, optionally of the second or third polypeptide, is or contains an IgV domain. In some cases, the IgSF domain or affinity-modified IgSF domain thereof, optionally of the second or third polypeptide, is a variant PD-L2 polypeptide that is or contains an IgV domain.

**[0031]** In some embodiments, the immunomodulatory protein further contains at least one additional polypeptide comprising an IgSF domain of an IgSF family member or an affinity-modified IgSF domain thereof, said affinity-modified IgSF domain comprising one or more amino acid modifications compared to the unmodified or wild-type IgSF domain of the IgSF family member. In some embodiments, the immunomodulatory protein further contains a multimerization domain linked to at least one of the variant PD-L1 polypeptide, or the second polypeptide. In some aspects, the immunomodulatory protein further contains a multimerization domain linked to at least one of the variant PD-L1 polypeptide, the second polypeptide and/or the third polypeptide. In some cases, the multimerization domain is an Fc domain or a variant thereof with reduced effector function. In some embodiments, the multimerization domain promotes heterodimer formation.

**[0032]** Provided is an immunomodulatory protein containing a first variant PD-L1 polypeptide in which the multimerization domain is a first multimerization domain and a second variant PD-L1 polypeptide in which

the multimerization domain is a second multimerization domain, wherein the first and second multimerization domains interact to form a multimer containing the first and second variant PD-L1 polypeptide, optionally wherein the first and second variant PD-L1 polypeptide are the same. In some cases, the multimerization domain is a first multimerization domain and interacts with a second multimerization domain to form a multimer comprising the immunomodulatory protein. In some examples, the immunomodulatory protein is a first immunomodulatory protein and a second immunomodulatory protein is linked directly or indirectly via a linker to the second multimerization domain, wherein the multimer comprises the first and second immunomodulatory protein. In some embodiments, the second immunomodulatory protein is any of the immunomodulatory proteins described and the multimerization domain is the second multimerization domain. In some cases, the multimer is dimer. In some embodiments, the second polypeptide is a variant CD155 polypeptide and the first and/or second immunomodulatory protein includes the sequence set forth in any of SEQ ID NOS: 1716-1721, or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 1716-1721; or the second polypeptide is CD112 or CD155 and the third polypeptide is the other of CD112 or CD155 and the first and/or second immunomodulatory protein comprise the sequence set forth in any of SEQ ID NOS: 1722-1724, or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to any of SEQ ID NOS: 1716-1721.

**[0033]** In some cases, the immunomodulatory protein is a homodimer. In some aspects, the immunomodulatory protein is a heterodimer, optionally wherein the first and second multimerization domain are different and/or are capable of interacting to mediate heterodimer formation.
In some embodiments, the first and/or second multimerization domain is an Fc domain or a variant thereof with reduced effector function, optionally wherein the Fc domain is of an immunoglobulin protein that is human and/or the Fc region is human, optionally wherein the Fc region is of an immunoglobulin G1 (IgG1) or an immunoglobulin G2 (IgG2), optionally set forth in SEQ ID NO: 187 or SEQ ID NO: 188, optionally wherein the Fc region exhibits one or more effector functions; or the variant Fc domain comprises one or more amino acid substitutions in a wildtype Fc region, optionally wherein the reduced effector function is reduced compared to a wildtype Fc region, optionally wherein the wildtype human Fc is of human IgG1. In some cases, the the first and second multimerization domain is the same or different.

**[0034]** In some embodiments, the variant Fc region contains the amino acid substitutions E233P, L234A, L234V, L235A, L235E, G236del, G237A, S267K, or N297G, with residue numbering according to the EU index of Kabat; or the amino acid substitutions R292C/N297G/V302C or L234A/L235E/G237A, with residue numbering according to the EU index of Kabat. In some examples, the Fc region or variant Fc region contains the amino acid substitution C220S, with residue numbering according to the EU index of Kabat. In some cases, the Fc region or variant Fc region contains K447del, with residue numbering according to the EU index of Kabat.

**[0035]** Also provided is a conjugate containing a variant PD-L1 according to any of the provided embodiments, or an immunomodulatory polypeptide according to any of the provided embodiments linked to a moiety. In some instances, the moiety is a targeting moiety that specifically binds to a molecule on the surface of a cell. In some aspects, the targeting moiety specifically binds to a molecule on the surface of an immune cell.

**[0036]** In some embodiments, the immune cell is an antigen presenting cell or a lymphocyte. In some cases, the targeting moiety is a tumor-localizing moiety that binds to a molecule on the surface of a tumor. In some examples, the moiety is a protein, a peptide, nucleic acid, small molecule or nanoparticle. In some embodiments, the moiety is an antibody or antigen-binding fragment. In some of any such embodiments, the conjugate is divalent, tetravalent, hexavalent or octavalent. In some aspects, the conjugate is a fusion

protein

**[0037]** Also provided is a nucleic acid molecule, encoding a variant PD-L1 polypeptide according to any of the provided embodiments, or an immunomodulatory polypeptide or conjugate that is a fusion protein according to any of the provided embodiments. In some embodiments, the nucleic acid molecule is synthetic nucleic acid. In some embodiments, the nucleic acid molecule is cDNA.

**[0038]** Also provided is a vector containing the nucleic acid molecule according to any of the provided embodiments. In some cases, the vector is an expression vector. In some aspects, the vector is a mammalian expression vector or a viral vector.

**[0039]** Also provided is a cell, comprising the vector according to any of the provided embodiments. In some instances, the cell is a mammalian cell. In some aspects, the cell is a human cell.

**[0040]** Also provided is a method of producing a variant PD-L1 polypeptide or an immunomodulatory protein, comprising introducing the nucleic acid molecule according to any of the provided embodiments or vector according to any of the provided embodiments into a host cell under conditions to express the protein in the cell. In some instances, the method further includes isolating or purifying the variant PD-L1 polypeptide or immunomodulatory protein from the cell. Also provided is a method of engineering a cell expressing a variant PD-L1 variant polypeptide including introducing a nucleic acid molecule encoding the variant PD-L1 polypeptide according to any of the provided embodiments into a host cell under conditions in which the polypeptide is expressed in the cell.

**[0041]** Also provided is an engineered cell, expressing the variant PD-L1 polypeptide according to any of the provided embodiments, the immunomodulatory protein according to any of the provided embodiments, the conjugate that is a fusion protein according to any of the provided embodiments, the nucleic acid molecule according to any of the provided embodiments or the vector according to any of the provided embodiments. In some cases, the variant PD-L1 polypeptide or immunomodulatory protein comprises a signal peptide. In some cases, the variant PD-L1 polypeptide or immunomodulatory protein is encoded by a nucleic acid including a sequence of nucleotides encoding comprises a signal peptide. In some embodiments, the variant PD-L1 polypeptide or immunomodulatory protein does not contain a transmembrane domain and/or is not expressed on the surface of the cell.

**[0042]** In some embodiments of the engineered cell, the variant PD-L1 polypeptide or immunomodulatory protein is secreted or capable of being secreted from the engineered cell. In some embodiments, the engineered cell contains a variant PD-L1 polypeptide that contains a transmembrane domain and/or is the transmembrane immunomodulatory protein according to any of the provided embodiments. In some embodiments, the variant PD-L1 polypeptide is expressed on the surface of the cell.

**[0043]** In some embodiments of the engineered cell, the cell is an immune cell. In some instances, the immune cell is an antigen presenting cell (APC) or a lymphocyte. In some embodiments, the cell is a primary cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the lymphocyte is a T cell. In some embodiments, the APC is an artificial APC. In some examples, the cell is a lymphocyte and the lymphocyte is a T cell. In some aspects, the cell is an APC and the APC is an artificial APC. In some of any such embodiments, the engineered cell further contains a chimeric antigen receptor (CAR) or an engineered T-cell receptor.

**[0044]** Also provided is an infectious agent, containing a nucleic acid molecule encoding a variant PD-L1 polypeptide according to any of the provided embodiments, a conjugate that is a fusion protein of any of

the provided embodiments, or an immunomodulatory polypeptide according to any of the provided embodiments. In some instances, the encoded variant PD-L1 polypeptide or immunomodulatory polypeptide does not contain a transmembrane domain and/or is not expressed on the surface of a cell in which it is expressed. In some embodiments, the encoded variant PD-L1 polypeptide or immunomodulatory polypeptide is secreted or capable of being secreted from a cell in which it is expressed. In some instances, the encoded variant PD-L1 polypeptide contains a transmembrane domain. In some embodiments, the encoded variant PD-L1 polypeptide is expressed on the surface of a cell in which it is expressed.

**[0045]** In some of any such embodiments, the infectious agent is a bacterium or a virus. In some embodiments, the virus is a lentiviral or retroviral construct or a hybrid thereof. In some aspects, the virus is an oncolytic virus. In some examples, the oncolytic virus is an adenovirus, adeno-associated virus, herpes virus, Herpes Simplex Virus, Vesticular Stomatic virus, Reovirus, Newcastle Disease virus, parvovirus, measles virus, vesticular stomatitis virus (VSV), Coxsackie virus or a Vaccinia virus. In some cases, the virus specifically targets dendritic cells (DCs) and/or is dendritic cell-tropic. In some examples, the virus is a lentiviral vector that is pseudotyped with a modified Sindbis virus envelope product. In some of any such embodiments, the infectious agent further contains a nucleic acid molecule encoding a further gene product that results in death of a target cell or that can augment or boost an immune response. In some examples, the further gene product is selected from an anticancer agent, anti-metastatic agent, an antiangiogenic agent, an immunomodulatory molecule, an immune checkpoint inhibitor, an antibody, a cytokine, a growth factor, an antigen, a cytotoxic gene product, a pro-apoptotic gene product, an anti-apoptotic gene product, a cell matrix degradative gene, genes for tissue regeneration or reprogramming human somatic cells to pluripotency.

**[0046]** Also provided is a pharmaceutical composition containing the variant PD-L1 polypeptide according to any of the provided embodiments, an immunomodulatory protein according to any of the provided embodiments, a conjugate according to any of the embodiments, an engineered cell according to any of the provided embodiments or an infectious agent according to any of the provided embodiments. In some cases, the pharmaceutical composition contains a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition is sterile.

**[0047]** Also provided is an article of manufacture containing the pharmaceutical composition according to any of the provided embodiments in a vial. In some cases, the vial is sealed.

**[0048]** Also provided is a kit containing the pharmaceutical composition according to any of the provided embodiments and instructions for use. Also provided is a kit containing the article of manufacture according to any of the provided embodiments and instructions for use.

**[0049]** Also provided is a pharmaceutical composition for use in a method of modulating an immune response, such as increasing or decreasing an immune response, in a subject, including administering the pharmaceutical composition according to any of the provided embodiments to the subject. In some embodiments, the method of modulating an immune response in a subject, includes administering the engineered cells according to any of the provided embodiments to the subject. In some cases, the engineered cells are autologous to the subject. In some instances, the engineered cells are allogenic to the subject. In some embodiments, the method that modulates the immune response treats a disease or condition in the subject. In some embodiments, the method comprises administering to the subject a soluble variant PD-L1 polypeptide according to any one of the embodiments described herein, an immunomodulatory protein according to any one of the embodiments described herein or a conjugate according to any one of the embodiments described herein. In some embodiments, the method comprises administering to the subject an infectious agent encoding a variant PD-L1 polypeptide according to any one of the embodiments described herein.

**[0050]** In some of any such embodiments, the immune response is increased. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein that is soluble is administered to the subject. In some embodiments, the variant PD-L1 polypeptide or immunomodulatory protein is an Fc fusion protein. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein that is soluble, optionally that lacks a PD-L1 transmembane and intracellular signaling domain, is administered to the subject. In some cases, the soluble immunomodulatory protein is an immunomodulatory Fc fusion protein. In some embodiments, a variant PD-L1 polypeptide according to any of the provided embodiments, the immunomodulatory protein according to any of the provided embodiments, or the conjugate according to any of the provided embodiments is administered to the subject. In some embodiments, an engineered cell containing a secretable variant PD-L1 polypeptide is administered to the subject. In some of any such embodiments, an engineered cell according to any of the provided embodiments is administered to the subject. In some aspects, an infectious agent encoding a variant PD-L1 polypeptide that is a secretable immunomodulatory protein is administered to the subject, optionally under conditions in which the infectious agent infects a tumor cell or immune cell and the secretable immunomodulatory protein is secreted from the infected cell.

**[0051]** In some embodiment, the disease or condition is a tumor or cancer. In some examples, the disease or condition is selected from melanoma, lung cancer, bladder cancer, a hematological malignancy, liver cancer, brain cancer, renal cancer, breast cancer, pancreatic cancer, colorectal cancer, spleen cancer, prostate cancer, testicular cancer, ovarian cancer, uterine cancer, gastric carcinoma, a musculoskeletal cancer, a head and neck cancer, a gastrointestinal cancer, a germ cell cancer, or an endocrine and neuroendocrine cancer.

**[0052]** In some of any such embodiments, the immune response is decreased. In some embodiments, an immunomodulatory protein or conjugate containing a variant PD-L1 polypeptide linked to an IgSF domain or a moiety that localizes to a cell or tissue of an inflammatory environment is administered to the subject. In some cases, the binding molecule comprises an antibody or an antigen-binding fragment thereof or contains a wild-type IgSF domain or variant thereof. In some embodiments, the immunomodulatory protein according to any of the provided embodiments or the conjugate according to any of the provided embodiments is administered to the subject.

**[0053]** In some embodiments, a variant PD-L1 polypeptide that is a transmembrane immunomodulatory protein is administered to the subject. In some embodiments, the engineered cell containing a variant PD-L1 polypeptide that is a transmembrane immunomodulatory protein according to any of the provided embodiments is administered to the subject. In some cases, an infectious agent encoding a variant PD-L1 polypeptide that is a transmembrane immunomodulatory protein is administered to the subject, optionally under conditions in which the infectious agent infects a tumor cell or immune cell and the transmembrane immunomodulatory protein is expressed on the surface of the infected cell.

**[0054]** In some embodiments, the disease or condition is an inflammatory or autoimmune disease or condition. In some of any such embodiments, the disease or condition is an antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, a vasculitis, an autoimmune skin disease, transplantation, a Rheumatic disease, an inflammatory gastrointestinal disease, an inflammatory eye disease, an inflammatory neurological disease, an inflammatory pulmonary disease, an inflammatory endocrine disease, or an autoimmune hematological disease. In some examples, the disease or condition is selected from inflammatory bowel disease, transplant, Crohn's disease, ulcerative colitis, multiple sclerosis, asthma, rheumatoid arthritis, or psoriasis. In some of any such embodiments, the variant PD-L 1 is administered in a format that decreases an immune response in the subject.

**Brief Description of the Drawings (embodiments which do not fall under the literal scope of the appended claims, are provided by way of background reference)**

**[0055]**

**FIG. 1A-1C** depicts various formats of the provided variant IgSF domain molecules. **FIG. 1A** depicts soluble molecules, including: (1) a variant IgSF domain (vIgD) fused to an Fc chain; (2) a stack molecule containing a first variant IgSF domain (first vIgD) and a second IgSF domain, such as a second variant IgSF domain (second vIgD); (3) a tumor targeting IgSF molecule containing a first variant IgSF domain (vIgD) and an IgSF domain that targets to a tumor antigen, such as an NKp30 IgSF domain; and (4) a variant IgSF domain (vIgD) linked to an antibody (V-mAb). **FIG. 1B** depicts a transmembrane immunomodulatory protein (TIP) containing a variant IgSF domain (vIgD) expressed on the surface of a cell. In an exemplary embodiment, the cognate binding partner of the transmembrane bound vIgD is an inhibitory receptor (e.g. PD-L1), and the TIP containing the vIgD (e.g. PD-L1 vIgD) antagonizes or blocks the negative signaling of the inhibitory receptor, thereby resulting in an activated T cell or effector T cell. In some cases, if clustering of the inhibitory receptor (PD-1) is proximal to an activating receptor (e.g. CD28) then agonizing activity by the TIP may be realized. **FIG. 1C** depicts a secreted immunomodulatory protein (SIP) in which a variant IgSF domain (vIgD) is secreted from a cell, such as a first T cell (e.g. CAR T cell). In an exemplary embodiment, the cognate binding partner of the secreted vIgD is an inhibitory receptor (e.g., PD-1), which can be expressed by the first cell (e.g., T cell, such as a CAR T cell) and/or on a second cell (e.g. T cell; either endogenous or engineered, such as a CAR T cell). Upon binding of the SIP with its cognate binding partner, the SIP antagonizes or blocks the negative signaling via the inhibitory receptor, thereby resulting in an activated T cell or effector T cell. In all cases, the vIgD can be a V-domain (IgV) only, the combination of the V-domain (IgV) and C-domain (IgC), including the entire extracellular domain (ECD), or any combination of Ig domains of the IgSF superfamily member.

**FIG. 2** depicts an exemplary schematic of the activity of a variant IgSF domain (vIgD) fused to an Fc (vIgD-Fc) in which the vIgD is a variant of an IgSF domain of PD-L1. As shown, a soluble vIgD of PD-L1 interacts with its cognate binding partners to block interactions of PD-L1 or PD-L2 with PD-1, thereby blocking the PD-1 inhibitory receptor, and, in some cases, allowing the T cell to differentiate into an effector phenotype.

**FIG. 3** depicts an exemplary schematic of a stack molecule that is a multi-target checkpoint antagonist containing a first variant IgSF domain (first vIgD) that is a PD-L1 or PD-L2 vIgD and a second IgSF domain (e.g. a second vIgD) that binds to a second inhibitory receptor. In the exemplary schematic, the second IgSF domain (e.g. second vIgD) is a CD112 or CD155 vIgD. As shown, the first vIgD and second vIgD interact with their cognate binding partners to block interactions of PD-L1 or PD-L2 with PD-1 and block interactions of CD155 or CD112 with TIGIT and/or CD112R, respectively, thereby blocking multiple inhibitory receptors.

**FIG. 4** depicts an exemplary schematic of a stack molecule for localizing the variant IgSF (vIgD) to a tumor cell. In this format, the stack molecule contains a first variant IgSF domain (first vIgD) and a second IgSF domain (e.g. a second vIgD) in which the second IgSF domain (e.g a second vIgD) is a tumor-targeted IgSF domain that binds to a tumor antigen. An exemplary tumor-targeted IgSF domain is an IgSF domain of NKp30, which binds to the tumor antigen B7-H6. In this depiction, the variant IgSF domain (vIgD) is a variant of an IgSF domain of PD-L1. As shown, binding of tumor-targeted IgSF domain to the surface of the tumor cell localizes the first variant IgSF domain on the tumor cell surface where it can interact with one or more of its cognate binding partners expressed on the surface of an adjacent immune cell (e.g. T cell) to antagonize PD-1 inhibitory activity and facilitate T cell activation.

**FIG. 5A** depicts various exemplary configurations of a stack molecule containing a first variant IgSF domain

(first vIgD) and a second IgSF domain, such as a second variant IgSF domain (second vIgD). As shown, the first vIgD and second IgSF domain are independently linked, directly or indirectly, to the N- or C-terminus of an Fc region. For generating a homodimeric Fc molecule, the Fc region is one that is capable of forming a homodimer with a matched Fc region by co-expression of the individual Fc region in a cell. For generating a heterodimeric Fc molecule, the individual Fc region contain mutations (e.g. "knob-into-hole" mutations in the CH3 domain), such that formation of the heterodimer is favored compared to homodimers when the individual Fc region are co-expressed in a cell.

**FIG. 5B** depicts various exemplary configurations of a stack molecule containing a first variant IgSF domain (first vIgD), a second IgSF domain, such as a second variant IgSF domain (second vIgD), and a third IgSF domain, such as a third variant IgSF domain (third vIgD). As shown, the first vIgD, second IgSF, and third IgSF domains are independently linked, directly or indirectly, to the N- or C-terminus of an Fc region. For generating a homodimeric Fc molecule, the Fc region is one that is capable of forming a homodimer with a matched Fc region by co-expression of the individual Fc region in a cell.

**FIG. 6** depicts an exemplary schematic of the activity of a variant IgSF domain (vIgD) -conjugated to an antibody (V-Mab) in which the antibody (e.g. anti-HER2 antibody) binds to an antigen on the surface of the tumor cell to localize the vIgD to the cell. As shown, binding of the antibody to the surface of the tumor cell localizes the vIgD on the tumor cell surface where it can interact with one or more of its cognate binding partners expressed on the surface of an adjacent immune cell (e.g. T cell) to agonize or antagonize receptor signaling. In an exemplary embodiment as shown, the variant IgSF domain (vIgD) is a variant of an IgSF domain of PD-L1 that binds, such as has increased affinity for, the inhibitory receptor PD-1. Binding of the PD-L1 vIgD to the PD-1 inhibitory receptor antagonizes or blocks the negative signaling of the inhibitory receptor, thereby resulting in an activated T cell or effector T cell. In some cases, if clustering of the inhibitory receptor (PD-1) is proximal to an activating receptor (e.g. CD28) then agonizing of the inhibitory receptor activity by the TIP may be realized.

**FIG. 7A-7C** depicts various exemplary configurations of a variant IgSF-antibody conjugate (V-Mab). **FIG. 7A** shows various configurations in which a variant IgSF domain is linked, directly or indirectly, to the N- and/or C-terminus of the light chain of an antibody. **FIG. 7B** shows various configurations in which a variant IgSF domain is linked, directly or indirectly, to the N- and/or C-terminus of the heavy chain of an antibody. **FIG. 7C** depicts the results V-Mab configurations when a light chain of **FIG. 7A** and a heavy chain of **FIG. 7B** are co-expressed in a cell.

**FIG. 8** and **FIG. 9** depict the results for soluble variant PD-L1 IgV-Fc bioactivity tested in a human Mixed Lymphocyte Reaction (MLR). Approximately, 10,000 matured DC and 100,000 purified allogeneic CD3+ T cells were co-cultured with various increasing concentrations of variant PD-L1 IgV-Fc fusion proteins. Irrelevant human IgG or media only (designated "No Add") were used as negative controls. Control proteins, PDL1-Fc (full wild-type PD-L1 extracellular domain), wildtype PD-L1 IgV-Fc and positive control anti-PD-1 monoclonal antibody (nivolumab) were assessed. **FIG. 8** and **FIG. 9** sets forth the calculated levels of IFN-gamma in culture supernatants (pg/mL) at the indicated concentration of variant IgV-Fc fusion molecule.

**FIG. 10** depicts proliferation studies for T cells transduced with exemplary tested variant PD-L1 SIP.

**FIG. 11** depicts dose response for binding of the indicated variant IgV-Fc fusion molecules, PD-L1/CD155 stack Fc fusion molecule, or PD-L1/CD155/CD112 stack Fc fusion molecule to exhausted T cells.

**FIG. 12** sets forth the calculated levels of IFN-gamma in culture supernatants (pg/mL) of exhausted T cells at the indicated concentration of variant IgV-Fc fusion molecule, PD-L1/CD155 stack Fc fusion molecule, or antibody controls.

## Detailed Description

**[0056]** Provided herein are immunomodulatory proteins that are or comprise variants or mutants of Programmed cell death 1 ligand 1 or PD-L1 (also known as cluster of differentiation 274, CD274. B7 homolog 1 or B7-H1) or specific binding fragments thereof that exhibit activity to bind to at least one target ligand cognate binding partner (also called counter-structure protein). The variant PD-L1 polypeptides contain at least the amino acid substitution N45D, with reference to the numbering of SEQ ID NO:30,compared to an unmodified human PD-L1 polypeptide. -The variant PD-L1 polypeptide and immunomodulatory proteins exhibits increased binding affinity for PD-1. In some embodiments, the immunomodulatory proteins are soluble. In some embodiments, the immunomodulatory proteins are transmembrane immunomodulatory proteins capable of being expressed on the surface of cells. In some embodiments, also provided herein are one or more other immunomodulatory proteins that are conjugates or fusions containing a variant PD-L1 polypeptide provided herein and one or more other moiety or polypeptide.

**[0057]** In some embodiments, the variant PD-L1 polypeptides and immunomodulatory proteins modulate an immunological immune response, such an increase or decrease an immune response. In some embodiments, the variant PD-L1 polypeptides and immunomodulatory proteins provided herein can be used for the treatment of diseases or conditions that are associated with a dysregulated immune response.

**[0058]** In some embodiments, the provided variant PD-L1 polypeptides modulate T cell activation via interactions with costimulatory and/or coinhibitory signaling molecules. In general, antigen specific T-cell activation generally requires two distinct signals. The first signal is provided by the interaction of the T-cell receptor (TCR) with major histocompatibility complex (MHC) associated antigens present on antigen presenting cells (APCs). The second signal is costimulatory to TCR engagement and is necessary for T cell proliferation, differentiation and/or survival, including, in some cases, to avoid T-cell apoptosis or anergy.

**[0059]** In some embodiments, under normal physiological conditions, the T cell-mediated immune response is initiated by antigen recognition by the T cell receptor (TCR) and is regulated by a balance of co-stimulatory and inhibitory signals (e.g., immune checkpoint proteins). The immune system relies on immune checkpoints to prevent autoimmunity (i.e., self- tolerance) and to protect tissues from excessive damage during an immune response, for example during an attack against a pathogenic infection. In some cases, however, these immunomodulatory proteins can be dysregulated in diseases and conditions, including tumors, as a mechanism for evading the immune system.

**[0060]** In some embodiments, among known T-cell costimulatory receptors is Programmed cell death protein 1 or PD-1, which is the T-cell costimulatory receptor for the ligands PD-L1 (also known as cluster of differentiation 274, CD274. B7 homolog 1 or B7-H1) and Programmed cell death 1 ligand 2 or PD-L2 (also known as PDCD1L2, PDCD1LG2, cluster of differentiation 273, CD273. or B7-DC). PD-L1 and PD-L2 are normally expressed on the surface of T cells, B cells, and myeloid cells. PD-L1 and PD-L2 are negative regulators of immune activation and are capable of down-modulating the immune response via interactions with programmed death 1 (PD-1) receptor. In some aspects, PD-1 is expressed on NK cells and T cells, including CD4+ and CD8+ T cells, whereby engagement of PD-1 can inhibit activation cell activation, proliferation, and/or expansion.

**[0061]** However, PD-L1 ligands can also bind to Cluster of differentiation 80 (also known as CD80 or B7-1). The binding of PD-L1 to CD80 can block the interaction between PD-L1 and PD-1, and thereby potentiate

or enhance the immune response. Thus, in some cases, interaction of PD-L1 with CD80 and PD-L1 with PD-1 yields opposing effects in modulating immune responses. Thus, PD-1 and CD80 may play opposing roles in immune responses to modulate pro-inflammatory or anti-inflammatory response, which, in some cases, are associated with a number of diseases and conditions.

**[0062]** In some embodiments, PD-1 and CD80 may play complementary roles in modeling an immune response. In some embodiments, enhancement or suppression of the activity of PD-1 receptor has clinical significance for treatment of inflammatory and autoimmune disorders, cancer, and viral infections. In some cases, however, therapies to intervene and alter the immunomodulatory effects of such receptors are constrained by the spatial orientation requirements as well as size limitations imposed by the confines of the immunological synapse. In some aspects, existing therapeutic drugs, including antibody drugs, may not be able to interact simultaneously with the multiple target proteins involved in modulating these interactions. In addition, in some cases, existing therapeutic drugs may only have the ability to antagonize but not agonize an immune response. Additionally, pharmacokinetic differences between drugs that independently target one of these receptors can create difficulties in properly maintaining a desired blood concentration of such drug combinations throughout the course of treatment.

**[0063]** In some embodiments, the provided variant PD-L1 polypeptides or immunomodulatory proteins modulate (e.g. increase or decrease) immunological activity associated PD-1. Thus, the provided polypeptides overcome these constraints by providing variant PD-L1 with increased binding affinities to PD-1, thereby agonizing or antagonizing the effects of the receptor. In some embodiments, the provided polypeptides overcome these constraints by providing variant PD-L1 with altered (e.g. increased or decreased) binding affinities to CD80, thereby modulating the effects of the interaction between PD-1 and PD-L1. Methods of making and using these variant PD-L1 are also provided.

**[0064]** If a definition set forth herein is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein referencedthe definition set forth herein prevails over the definition that is referenced.

**[0065]** The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

**I. DEFINITIONS**

**[0066]** Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as is commonly understood by one of ordinary skill in the art to which the claimed subject matter pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

**[0067]** The terms used throughout this specification are defined as follows unless otherwise limited in specific instances. As used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms, acronyms, and abbreviations used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Unless indicated otherwise, abbreviations and symbols for chemical and biochemical names is per IUPAC-IUB nomenclature. Unless indicated otherwise, all numerical ranges are inclusive of the values defining the

range as well as all integer values inbetween.

**[0068]** The term "affinity modified" as used in the context of an immunoglobulin superfamily domain, means a mammalian immunoglobulin superfamily (IgSF) domain having an altered amino acid sequence (relative to the corresponding wild-type parental or unmodified IgSF domain) such that it has an increased or decreased binding affinity or avidity to at least one of its cognate binding partners (alternatively "counter-structures") compared to the parental wild-type or unmodified (i.e., non-affinity modified) IgSF control domain. Included in this context is an affinity modified PD-L1 IgSF domain. In some embodiments, the affinity-modified IgSF domain can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acid differences, such as amino acid substitutions, in a wildtype or unmodified IgSF domain. An increase or decrease in binding affinity or avidity can be determined using well known binding assays such as flow cytometry. Larsen et al., American Journal of Transplantation, Vol 5: 443-453 (2005). See also, Linsley et al., Immunity, Vol 1(9): 793-801 (1994). An increase in a protein's binding affinity or avidity to its cognate binding partner(s) is to a value at least 10% greater than that of the wild-type IgSF domain control and in some embodiments, at least 20%, 30%, 40%, 50%, 100%, 200%, 300%, 500%, 1000%, 5000%, or 10000% greater than that of the wild-type IgSF domain control value. A decrease in a protein's binding affinity or avidity to at least one of its cognate binding partner is to a value no greater than 90% of the control but no less than 10% of the wild-type IgSF domain control value, and in some embodiments no greater than 80%, 70% 60%, 50%, 40%, 30%, or 20% but no less than 10% of the wild-type IgSF domain control value. An affinity-modified protein is altered in primary amino acid sequence by substitution, addition, or deletion of amino acid residues. The term "affinity modified IgSF domain" is not to be construed as imposing any condition for any particular starting composition or method by which the affinity-modified IgSF domain was created. Thus, the affinity modified IgSF domains of the present invention are not limited to wild type IgSF domains that are then transformed to an affinity modified IgSF domain by any particular process of affinity modification. An affinity modified IgSF domain polypeptide can, for example, be generated starting from wild type mammalian IgSF domain sequence information, then modeled in silico for binding to its cognate binding partner, and finally recombinantly or chemically synthesized to yield the affinity modified IgSF domain composition of matter. In but one alternative example, an affinity modified IgSF domain can be created by site-directed mutagenesis of a wild-type IgSF domain. Thus, affinity modified IgSF domain denotes a product and not necessarily a product produced by any given process. A variety of techniques including recombinant methods, chemical synthesis, or combinations thereof, may be employed.

**[0069]** The term "allogeneic" as used herein means a cell or tissue that is removed from one organism and then infused or adoptively transferred into a genetically dissimilar organism of the same species. In some embodiments of the invention, the species is murine or human.

**[0070]** The term "autologous" as used herein means a cell or tissue that is removed from the same organism to which it is later infused or adoptively transferred. An autologous cell or tissue can be altered by, for example, recombinant DNA methodologies, such that it is no longer genetically identical to the native cell or native tissue which is removed from the organism. For example, a native autologous T-cell can be genetically engineered by recombinant DNA techniques to become an autologous engineered cell expressing a transmembrane immunomodulatory protein and/or chimeric antigen receptor (CAR), which in some cases involves engineering a T-cell or TIL (tumor infiltrating lymphocyte). The engineered cells are then infused into a patient from which the native T-cell was isolated. In some embodiments, the organism is human or murine.

**[0071]** The terms "binding affinity," and "binding avidity" as used herein means the specific binding affinity and specific binding avidity, respectively, of a protein for its counter-structure under specific binding conditions. In biochemical kinetics avidity refers to the accumulated strength of multiple affinities of

individual non-covalent binding interactions, such as between PD-L1 and its counter-structures PD-1 and/or CD80. As such, avidity is distinct from affinity, which describes the strength of a single interaction. An increase or attenuation in binding affinity of a variant PD-L1 containing an affinity modified PD-L1 IgSF domain to its counter-structure is determined relative to the binding affinity of the unmodified PD-L1, such as an unmodified PD-L1 containing the native or wild-type IgSF domain, such as IgV domain. Methods for determining binding affinity or avidity are known in art. See, for example, Larsen et al., American Journal of Transplantation, Vol 5: 443-453 (2005). In some embodiments, a variant PD-L1 of the invention (i.e. a PD-L1 protein containing an affinity modified IgSF domain) specifically binds to PD-1 and/or CD80 measured by flow cytometry with a binding affinity that yields a Mean Fluorescence Intensity (MFI) value at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater than a wild-type PD-L1 control in a binding assay.

**[0072]** The term "biological half-life" refers to the amount of time it takes for a substance, such as an immunomodulatory polypeptide comprising a variant PD-L1 of the present invention, to lose half of its pharmacologic or physiologic activity or concentration. Biological half-life can be affected by elimination, excretion, degradation (e.g., enzymatic) of the substance, or absorption and concentration in certain organs or tissues of the body. In some embodiments, biological half-life can be assessed by determining the time it takes for the blood plasma concentration of the substance to reach half its steady state level ("plasma half-life"). Conjugates that can be used to derivatize and increase the biological half-life of polypeptides of the invention are known in the art and include, but are not limited to, polyethylene glycol (PEG), hydroxyethyl starch (HES), XTEN (extended recombinant peptides; see, WO2013130683), human serum albumin (HSA), bovine serum albumin (BSA), lipids (acylation), and poly-Pro-Ala-Ser (PAS), polyglutamic acid (glutamylation).

**[0073]** The term "chimeric antigen receptor" or "CAR" as used herein refers to an artificial (i.e., man-made) transmembrane protein expressed on a mammalian cell comprising at least an ectodomain, a transmembrane, and an endodomain. Optionally, the CAR protein includes a "spacer" which covalently links the ectodomain to the transmembrane domain. A spacer is often a polypeptide linking the ectodomain to the transmembrane domain via peptide bonds. The CAR is typically expressed on a mammalian lymphocyte. In some embodiments, the CAR is expressed on a mammalian cell such as a T-cell or a tumor infiltrating lymphocyte (TIL). A CAR expressed on a T-cell is referred to herein as a "CAR T-cell" or "CAR-T." In some embodiments the CAR-T is a T helper cell, a cytotoxic T-cell, a natural killer T-cell, a memory T-cell, a regulatory T-cell, or a gamma delta T-cell. When used clinically in, e.g. adoptive cell transfer, a CAR-T with antigen binding specificity to the patient's tumor is typically engineered to express on a T-cell obtained from the patient. The engineered T-cell expressing the CAR is then infused back into the patient. The CAR-T is thus often an autologous CAR-T although allogeneic CAR-T are included within the scope of the invention. The ectodomain of a CAR comprises an antigen binding region, such as an antibody or antigen binding fragment thereof (e.g. scFv), that specifically binds under physiological conditions with a target antigen, such as a tumor specific antigen. Upon specific binding a biochemical chain of events (i.e., signal transduction) results in modulation of the immunological activity of the CAR-T. Thus, for example, upon specific binding by the antigen binding region of the CAR-T to its target antigen can lead to changes in the immunological activity of the T-cell activity as reflected by changes in cytotoxicity, proliferation or cytokine production. Signal transduction upon CAR-T activation is achieved in some embodiments by the CD3-zeta chain ("CD3-z") which is involved in signal transduction in native mammalian T-cells. CAR-Ts can further comprise multiple signaling domains such as CD28, 41BB or OX40, to further modulate immunomodulatory response of the T-cell. CD3-z comprises a conserved motif known as an immunoreceptor tyrosine-based activation motif (ITAM) which is involved in T-cell receptor signal transduction.

**[0074]** The term "collectively" or "collective" when used in reference to cytokine production induced by the

presence of two or more variant PD-L1 of the invention in an in vitro assay, means the overall cytokine expression level irrespective of the cytokine production induced by individual variant PD-L1. In some embodiments, the cytokine being assayed is IFN-gamma, such as in an in vitro primary T-cell assay.

**[0075]** The term "cognate binding partner" (used interchangeably with "counter-structure") in reference to a polypeptide, such as in reference to an IgSF domain of a variant PD-L1, refers to at least one molecule (typically a native mammalian protein) to which the referenced polypeptide specifically binds under specific binding conditions. In some aspects, a variant PD-L1 containing an affinity modified IgSF domain specifically binds to the counter-structure of the corresponding native or wildtype PD-L1 but with increased or attenuated affinity. A species of ligand recognized and specifically binding to its cognate receptor under specific binding conditions is an example of a counter-structure or cognate binding partner of that receptor. A "cognate cell surface binding partner" is a cognate binding partner expressed on a mammalian cell surface. A "cell surface molecular species" is a cognate binding partner of ligands of the immunological synapse (IS), expressed on and by cells, such as mammalian cells, forming the immunological synapse.

**[0076]** As used herein, "conjugate," "conjugation" or grammatical variations thereof refers the joining or linking together of two or more compounds resulting in the formation of another compound, by any joining or linking methods known in the art. It can also refer to a compound which is generated by the joining or linking together two or more compounds. For example, a variant PD-L1 polypeptide linked directly or indirectly to one or more chemical moieties or polypeptide is an exemplary conjugate. Such conjugates include fusion proteins, those produced by chemical conjugates and those produced by any other methods.

**[0077]** The term "competitive binding" as used herein means that a protein is capable of specifically binding to at least two cognate binding partners but that specific binding of one cognate binding partner inhibits, such as prevents or precludes, simultaneous binding of the second cognate binding partner. Thus, in some cases, it is not possible for a protein to bind the two cognate binding partners at the same time. Generally, competitive binders contain the same or overlapping binding site for specific binding but this is not a requirement. In some embodiments, competitive binding causes a measurable inhibition (partial or complete) of specific binding of a protein to one of its cognate binding partner due to specific binding of a second cognate binding partner. A variety of methods are known to quantify competitive binding such as ELISA (enzyme linked immunosorbent assay) assays.

**[0078]** The term "conservative amino acid substitution" as used herein means an amino acid substitution 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). Examples of groups of amino acids that have side chains with similar chemical properties include 1) aliphatic side chains: glycine, alanine, valine, leucine, and isoleucine; 2) aliphatic-hydroxyl side chains: serine and threonine; 3) amide-containing side chains: asparagine and glutamine; 4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; 5) basic side chains: lysine, arginine, and histidine; 6) acidic side chains: aspartic acid and glutamic acid; and 7) sulfurcontaining side chains: cysteine and methionine. Conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamateaspartate, and asparagine-glutamine.

**[0079]** The term, "corresponding to" with reference to positions of a protein, such as recitation that nucleotides or amino acid positions "correspond to" nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence based on structural sequence alignment or using a standard alignment algorithm, such as the GAP algorithm. For example, corresponding residues can be determined by alignment of a reference sequence with the sequence of wild-type PD-L1 set forth in SEQ ID NO:30 or 1728 (ECD domain) or set forth in SEQ ID NO: 55 or 309 (IgV domain) by structural alignment methods as

described herein. By aligning the sequences, one skilled in the art can identify corresponding residues, for example, using conserved and identical amino acid residues as guides.

**[0080]** The terms "decrease" or "attenuate" "or suppress" as used herein means to decrease by a statistically significant amount. A decrease can be at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%.

**[0081]** The terms "derivatives" or "derivatized" refer to modification of a protein by covalently linking it, directly or indirectly, to a composition so as to alter such characteristics as biological half-life, bioavailability, immunogenicity, solubility, toxicity, potency, or efficacy while retaining or enhancing its therapeutic benefit. Derivatives of immunomodulatory polypeptides of the invention are within the scope of the invention and can be made by, for example, glycosylation, pegylation, lipidation, or Fc-fusion.

**[0082]** As used herein, "domain" (typically a sequence of three or more, generally 5 or 7 or more amino acids, such as 10 to 200 amino acid residues) refers to a portion of a molecule, such as a protein or encoding nucleic acid,that is structurally and/or functionally distinct from other portions of the molecule and is identifiable. For example, domains include those portions of a polypeptide chain that can form an independently folded structure within a protein made up of one or more structural motifs and/or that is recognized by virtue of a functional activity, such as binding activity. A protein can have one, or more than one, distinct domains. For example, a domain can be identified, defined or distinguished by homology of the primary sequence or structure to related family members, such as homology to motifs. In another example, a domain can be distinguished by its function, such as an ability to interact with a biomolecule, such as a cognate binding partner. A domain independently can exhibit a biological function or activity such that the domain independently or fused to another molecule can perform an activity, such as, for example binding. A domain can be a linear sequence of amino acids or a non-linear sequence of amino acids. Many polypeptides contain a plurality of domains. Such domains are known, and can be identified by those of skill in the art. For exemplification herein, definitions are provided, but it is understood that it is well within the skill in the art to recognize particular domains by name. If needed appropriate software can be employed to identify domains.

**[0083]** The term "ectodomain" as used herein refers to the region of a membrane protein, such as a transmembrane protein, that lies outside the vesicular membrane. Ectodomains often comprise binding domains that specifically bind to ligands or cell surface receptors, such as via a binding domain that specifically binds to the ligand or cell surface receptor. The ectodomain of a cellular transmembrane protein is alternately referred to as an extracellular domain.

**[0084]** The terms "effective amount" or "therapeutically effective amount" refer to a quantity and/or concentration of a therapeutic composition of the invention, including a protein composition or cell composition, that when administered ex vivo (by contact with a cell from a patient) or in vivo (by administration into a patient) either alone (i.e., as a monotherapy) or in combination with additional therapeutic agents, yields a statistically significant decrease in disease progression as, for example, by ameliorating or eliminating symptoms and/or the cause of the disease. An effective amount may be an amount that relieves, lessens, or alleviates at least one symptom or biological response or effect associated with a disease or disorder, prevents progression of the disease or disorder, or improves physical functioning of the patient. In the case of cell therapy, the effective amount is an effective dose or number of cells administered to a patient by adoptive cell therapy. In some embodiments the patient is a mammal such as a nonhuman primate or human patient.

**[0085]** The term "endodomain" as used herein refers to the region found in some membrane proteins, such as transmembrane proteins,that extends into the interior space defined by the cell surface

membrane. In mammalian cells, the endodomain is the cytoplasmic region of the membrane protein. In cells, the endodomain interacts with intracellular constituents and can be play a role in signal transduction and thus, in some cases, can be an intracellular signaling domain. The endodomain of a cellular transmembrane protein is alternately referred to as a cytoplasmic domain, which, in some cases, can be a cytoplasmic signaling domain.

**[0086]** The terms "enhanced" or "increased" as used herein in the context of increasing immunological activity of a mammalian lymphocyte means to increase one or more activities the lymphocyte. An increased activity can be one or more of increase cell survival, cell proliferation, cytokine production, or T-cell cytotoxicity, such as by a statistically significant amount. In some embodiments, reference to increased immunological activity means to increase interferon gamma (IFN-gamma) production, such as by a statistically significant amount. In some embodiments, the immunological activity can be assessed in a mixed lymphocyte reaction (MLR) assay. Methods of conducting MLR assays are known in the art. Wang et al., Cancer Immunol Res. 2014 Sep: 2(9):846-56. Other methods of assessing activities of lymphocytes are known in the art, including any assay as described herein. In some embodiments an enhancement can be an increase of at least 10%, 20%, 30%, 40%, 50%, 75%, 100%, 200%, 300%, 400%, or 500% greater than a non-zero control value.

**[0087]** The term "engineered cell" as used herein refers to a mammalian cell that has been genetically modified by human intervention such as by recombinant DNA methods or viral transduction. In some embodiments, the cell is an immune cell, such as a lymphocyte (e.g. T cell, B cell, NK cell) or an antigen presenting cell (e.g. dendritic cell). The cell can be a primary cell from a patient or can be a cell line. In some embodiments, an engineered cell of the invention comprises a variant PD-L1 of the invention engineered to modulate immunological activity of a T-cell expressing PD-1 and optionally CD80 to which the variant PD-L1 specifically binds. In some embodiments, the variant PD-L1 is a transmembrane immunomodulatory protein (hereinafter referred to as "TIP") containing the extracellular domain or a portion thereof containing the IgV or ECD domain linked to a transmembrane domain (e.g. a PD-L1 transmembrane domain) and, optionally, an intracellular signaling domain. In some cases, the TIP is formatted as a chimeric receptor containing a heterologous cytoplasmic signaling domain or endodomain. In some embodiments, an engineered cell is capable of expressing and secreting an immunomodulatory protein as described herein. Among provided engineered cells also are cells further containing an engineered T-cell receptor (TCR) or chimeric antigen receptor (CAR).

**[0088]** The term "engineered T-cell" as used herein refers to a T-cell such as a T helper cell, cytotoxic T-cell (alternatively, cytotoxic T lymphocyte or CTL), natural killer T-cell, regulatory T-cell, memory T-cell, or gamma delta T-cell, that has been genetically modified by human intervention such as by recombinant DNA methods or viral transduction methods. An engineered T-cell comprises a variant PD-L1 transmembrane immunomodulatory protein (TIP) or secreted immunomodulatory protein (SIP) of the present invention that is expressed on the T-cell and is engineered to modulate immunological activity of the engineered T-cell itself, or a mammalian cell to which the variant PD-L1 expressed on the T-cell specifically binds.

**[0089]** The term "engineered T-cell receptor" or "engineered TCR" refers to a T-cell receptor (TCR) engineered to specifically bind with a desired affinity to a major histocompatibility complex (MHC)/peptide target antigen that is selected, cloned, and/or subsequently introduced into a population of T-cells, often used for adoptive immunotherapy. In contrast to engineered TCRs, CARs are engineered to bind target antigens in a MHC independent manner.

**[0090]** The term "expressed on" as used herein is used in reference to a protein expressed on the surface of a cell, such as a mammalian cell. Thus, the protein is expressed as a membrane protein. In some

embodiments, the expressed protein is a transmembrane protein. In some embodiments, the protein is conjugated to a small molecule moiety such as a drug or detectable label. Proteins expressed on the surface of a cell can include cell-surface proteins such as cell surface receptors that are expressed on mammalian cells.

**[0091]** The term "half-life extending moiety" refers to a moiety of a polypeptide fusion or chemical conjugate that extends the half-life of a protein circulating in mammalian blood serum compared to the half-life of the protein that is not so conjugated to the moiety. In some embodiments, half-life is extended by greater than or greater than about 1.2-fold, 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5.0-fold, or 6.0-fold. In some embodiments, half-life is extended by more than 6 hours, more than 12 hours, more than 24 hours, more than 48 hours, more than 72 hours, more than 96 hours or more than 1 week after *in vivo* administration compared to the protein without the half-life extending moiety. The half-life refers to the amount of time it takes for the protein to lose half of its concentration, amount, or activity. Half-life can be determined for example, by using an ELISA assay or an activity assay. Exemplary half-life extending moieties include an Fc domain, a multimerization domain, polyethylene glycol (PEG), hydroxyethyl starch (HES), XTEN (extended recombinant peptides; see, WO2013130683), human serum albumin (HSA), bovine serum albumin (BSA), lipids (acylation), and poly-Pro-Ala-Ser (PAS), and polyglutamic acid (glutamylation).

**[0092]** The term "immunological synapse" or "immune synapse" as used herein means the interface between a mammalian cell that expresses MHC I (major histocompatibility complex) or MHC II, such as an antigen-presenting cell or tumor cell, and a mammalian lymphocyte such as an effector T cell or Natural Killer (NK) cell.

**[0093]** An Fc (fragment crystallizable) region or domain of an immunoglobulin molecule (also termed an Fc polypeptide) corresponds largely to the constant region of the immunoglobulin heavy chain, and is responsible for various functions, including the antibody's effector function(s). The Fc domain contains part or all of a hinge domain of an immunoglobulin molecule plus a CH2 and a CH3 domain. The Fc domain can form a dimer of two polypeptide chains joined by one or more disulfide bonds. In some embodiments, the Fc is a variant Fc that exhibits reduced (e.g. reduced greater than 30%, 40%, 50%, 60%, 70%, 80%, 90% or more) activity to facilitate an effector function. In some embodiments, reference to amino acid substitutions in an Fc region is by EU numbering system unless described with reference to a specific SEQ ID NO. EU numbering is known and is according to the most recently updated IMGT Scientific Chart (IMGT®, the international ImMunoGeneTics information system®, http://www.imgt.org/IMGTScientificChart/Numbering/Hu_IGHGnber.html (created: 17 May 2001 , last updated: 10 Jan 2013) and the EU index as reported in Kabat, E.A. et al. Sequences of Proteins of Immunological interest. 5th ed. US Department of Health and Human Services, NIH publication No. 91-3242 (1991).

**[0094]** An immunoglobulin Fc fusion ("Fc-fusion"), such as an immunomodulatory Fc fusion protein, is a molecule comprising one or more polypeptides (or one or more small molecules) operably linked to an Fc region of an immunoglobulin. An Fc-fusion may comprise, for example, the Fc region of an antibody (which facilitates effector functions and pharmacokinetics) and a variant PD-L1. An immunoglobulin Fc region may be linked indirectly or directly to one or more variant PD-L1 or small molecules (fusion partners). Various linkers are known in the art and can optionally be used to link an Fc to a fusion partner to generate an Fc-fusion. Fc-fusions of identical species can be dimerized to form Fc-fusion homodimers, or using non-identical species to form Fc-fusion heterodimers. In some embodiments, the Fc is a mammalian Fc such as a murine or human Fc.

**[0095]** The term "host cell" refers to a cell that can be used to express a protein encoded by a recombinant expression vector. A host cell can be a prokaryote, for example, E. coli, or it can be a eukaryote, for example, a single-celled eukaryote (e.g., a yeast or other fungus), a plant cell (e.g., a tobacco or tomato plant cell), an animal cell (e.g., a human cell, a monkey cell, a hamster cell, a rat cell, a mouse cell, or an insect cell) or a hybridoma. Examples of host cells include Chinese hamster ovary (CHO) cells or their derivatives such as Veggie CHO and related cell lines which grow in serum-free media or CHO strain DX-B11, which is deficient in DHFR. Another example is Human Endothelial Kidney 293 cells or their derivatives. In some embodiments, a host cell is a mammalian cell (e.g., a human cell, a monkey cell, a hamster cell, a rat cell, a mouse cell, or an insect cell).

**[0096]** The term "immunoglobulin" (abbreviated "Ig") as used herein refers to a mammalian immunoglobulin protein including any of the five human classes of antibody: IgA (which includes subclasses IgA1 and IgA2), IgD, IgE, IgG (which includes subclasses IgG1, IgG2, IgG3, and IgG4), and IgM. The term is also inclusive of immunoglobulins that are less than full-length, whether wholly or partially synthetic (e.g., recombinant or chemical synthesis) or naturally produced, such as antigen binding fragment (Fab), variable fragment (Fv) containing $V_H$ and $V_L$, the single chain variable fragment (scFv) containing $V_H$ and $V_L$ linked together in one chain, as well as other antibody V region fragments, such as Fab', $F(ab)_2$, $F(ab')_2$, dsFv diabody, Fc, and Fd polypeptide fragments. Bispecific antibodies, homobispecific and heterobispecific, are included within the meaning of the term.

**[0097]** The term "immunoglobulin superfamily" or "IgSF" as used herein means the group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. Molecules are categorized as members of this superfamily based on shared structural features with immunoglobulins (i.e., antibodies); they all possess a domain known as an immunoglobulin domain or fold. Members of the IgSF include cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules, certain cytokine receptors and intracellular muscle proteins. They are commonly associated with roles in the immune system. Proteins in the immunological synapse are often members of the IgSF. IgSF can also be classified into "subfamilies" based on shared properties such as function. Such subfamilies typically consist of from 4 to 30 IgSF members.

**[0098]** The terms "IgSF domain" or "immunoglobulin domain" or "Ig domain" as used herein refers to a structural domain of IgSF proteins. Ig domains are named after the immunoglobulin molecules. They contain about 70-110 amino acids and are categorized according to their size and function. Ig-domains possess a characteristic Ig-fold, which has a sandwich-like structure formed by two sheets of antiparallel beta strands. Interactions between hydrophobic amino acids on the inner side of the sandwich and highly conserved disulfide bonds formed between cysteine residues in the B and F strands, stabilize the Ig-fold. One end of the Ig domain has a section called the complementarity determining region that is important for the specificity of antibodies for their ligands. The Ig like domains can be classified (into classes) as: IgV, IgC (which either can be an IgC1 or IgC2), or IgI. Most Ig domains are either variable (IgV) or constant (IgC). IgV domains with 9 beta strands are generally longer than IgC domains with 7 beta strands. Ig domains of some members of the IgSF resemble IgV domains in the amino acid sequence, yet are similar in size to IgC domains. These are called IgC2 domains, while standard IgC domains are called IgC1 domains. T-cell receptor (TCR) chains contain two Ig domains in the extracellular portion; one IgV domain at the N-terminus and one IgC1 domain adjacent to the cell membrane. PD-L1 contains two Ig domains: one IgV and one IgC domain.

**[0099]** The term "IgSF species" as used herein means an ensemble of IgSF member proteins with identical or substantially identical primary amino acid sequence. Each mammalian immunoglobulin superfamily

(IgSF) member defines a unique identity of all IgSF species that belong to that IgSF member. Thus, each IgSF family member is unique from other IgSF family members and, accordingly, each species of a particular IgSF family member is unique from the species of another IgSF family member. Nevertheless, variation between molecules that are of the same IgSF species may occur owing to differences in post-translational modification such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. Additionally, minor sequence differences within a single IgSF species owing to gene polymorphisms constitute another form of variation within a single IgSF species as do wild type truncated forms of IgSF species owing to, for example, proteolytic cleavage. A "cell surface IgSF species" is an IgSF species expressed on the surface of a cell, generally a mammalian cell.

**[0100]** The term "immunological activity" as used herein in the context of mammalian lymphocytes such as T-cells refers to one or more cell survival, cell proliferation, cytokine production (e.g. interferon-gamma), or T-cell cytotoxicity activities. In some cases, an immunological activity can mean the cell expression of cytokines, such as chemokines or interleukins. Assays for determining enhancement or suppression of immunological activity include the MLR (mixed lymphocyte reaction) assays measuring interferon-gamma cytokine levels in culture supernatants (Wang et al., Cancer Immunol Res. 2014 Sep: 2(9):846-56), SEB (staphylococcal enterotoxin B) T cell stimulation assay (Wang et al., Cancer Immunol Res. 2014 Sep: 2(9):846-56), and anti-CD3 T cell stimulation assays (Li and Kurlander, J Transl Med. 2010: 8: 104). Since T cell activation is associated with secretion of IFN-gamma cytokine, detecting IFN-gamma levels in culture supernatants from these in vitro human T cell assays can be assayed using commercial ELISA kits (Wu et al, Immunol Lett 2008 Apr 15; 117(1): 57-62). Induction of an immune response results in an increase in immunological activity relative to quiescent lymphocytes. An immunomodulatory protein, such as a variant PD-L1 polypeptide containing an affinity modified IgSF domain, as provided herein can in some embodiments increase or, in alternative embodiments, decrease IFN-gamma (interferon-gamma) expression in a primary T-cell assay relative to a wild-type IgSF member or IgSF domain control. Those of skill will recognize that the format of the primary T-cell assay used to determine an increase in IFN-gamma expression can differ from that employed to assay for a decrease in IFN-gamma expression. In assaying for the ability of an immunomodulatory protein or affinity modified IgSF domain of the invention to alter IFN-gamma expression in a primary T-cell assay, a Mixed Lymphocyte Reaction (MLR) assay can be used. Conveniently, in some cases, a soluble form of an affinity modified IgSF domain of the invention can be employed to determine its ability to increase or decrease the IFN-gamma expression in a MI,R. Alternatively, a co-immobilization assay can be used. In a co-immobilization assay, a T-cell receptor signal, provided in some embodiments by anti-CD3 antibody, is used in conjunction with a co-immobilized affinity modified IgSF domain, such as variant PD-L1, to determine the ability to increase or decrease IFN-gamma expression relative to a wild-type IgSF domain control. Methods to assay the immunological activity of engineered cells, including to evaluate the activity of a variant PD-L1 transmembrane immunomodulatory protein, are known in the art and include, but are not limited to, the ability to expand T cells following antigen stimulation, sustain T cell expansion in the absence of re- stimulation, and anti-cancer activities in appropriate animal models. Assays also include assays to assess cytotoxicity, including a standard $^{51}$Cr-release assay (see e.g. Milone et al., (2009) Molecular Therapy 17: 1453-1464) or flow based cytotoxicity assays, or an impedance based cytotoxicity assay (Peper et al. (2014) Journal of Immunological Methods, 405:192-198).

**[0101]** An "immunomodulatory polypeptide" or "immunomodulatory protein" is a polypeptide or protein molecule that modulates immunological activity. By "modulation" or "modulating" an immune response is meant that immunological activity is either increased or decreased. An immunomodulatory protein can be a single polypeptide chain or a multimer (dimers or higher order multimers) of at least two polypeptide chains covalently bonded to each other by, for example, interchain disulfide bonds. Thus, monomeric, dimeric, and higher order multimeric polypeptides are within the scope of the defined term. Multimeric polypeptides can

be homomultimeric (of identical polypeptide chains) or heteromultimeric (of non-identical polypeptide chains). An immunomodulatory protein of the invention comprises a variant PD-L1.

**[0102]** The term "increase" as used herein means to increase by a statistically significant amount. An increase can be at least 5%, 10%, 20%, 30%, 40%, 50%, 75%, 100%, or greater than a non-zero control value.

**[0103]** An "isoform" of PD-L1 is one of a plurality naturally occurring PD-L1 polypeptides that differ in amino acid sequence. Isoforms can be the product of splice variants of an RNA transcript expressed by a single gene, or the expression product of highly similar but different genes yielding a functionally similar protein such as may occur from gene duplication. As used herein, the term "isoform" of PD-L1 also refers to the product of different alleles of a PD-L1 gene.

**[0104]** The term "lymphocyte" as used herein means any of three subtypes of white blood cell in a mammalian immune system. They include natural killer cells (NK cells) (which function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity). T cells include: T helper cells, cytotoxic T-cells, natural killer T-cells, memory T-cells, regulatory T-cells, or gamma delta T-cells. Innate lymphoid cells (ILC) are also included within the definition of lymphocyte.

**[0105]** The terms "mammal," or "patient" specifically includes reference to at least one of a: human, chimpanzee, rhesus monkey, cynomolgus monkey, dog, cat, mouse, or rat.

**[0106]** The term "membrane protein" as used herein means a protein that, under physiological conditions, is attached directly or indirectly to a lipid bilayer. A lipid bilayer that forms a membrane can be a biological membrane such as a eukaryotic (e.g., mammalian) cell membrane or an artificial (i.e., man-made) membrane such as that found on a liposome. Attachment of a membrane protein to the lipid bilayer can be by way of covalent attachment, or by way of non-covalent interactions such as hydrophobic or electrostatic interactions. A membrane protein can be an integral membrane protein or a peripheral membrane protein. Membrane proteins that are peripheral membrane proteins are non-covalently attached to the lipid bilayer or non-covalently attached to an integral membrane protein. A peripheral membrane protein forms a temporary attachment to the lipid bilayer such that under the range of conditions that are physiological in a mammal, peripheral membrane protein can associate and/or disassociate from the lipid bilayer. In contrast to peripheral membrane proteins, integral membrane proteins form a substantially permanent attachment to the membrane's lipid bilayer such that under the range of conditions that are physiological in a mammal, integral membrane proteins do not disassociate from their attachment to the lipid bilayer. A membrane protein can form an attachment to the membrane by way of one layer of the lipid bilayer (monotopic), or attached by way of both layers of the membrane (polytopic). An integral membrane protein that interacts with only one lipid bilayer is an "integral monotopic protein". An integral membrane protein that interacts with both lipid bilayers is an "integral polytopic protein" alternatively referred to herein as a "transmembrane protein".

**[0107]** The terms "modulating" or "modulate" as used herein in the context of an immune response, such as a mammalian immune response, refer to any alteration, such as an increase or a decrease, of existing or potential immune responses that occurs as a result of administration of an immunomodulatory polypeptide comprising a variant PD-L1 of the present invention or as a result of administration of engineered cells expresses an immunomodulatory protein, such as a variant PD-L1 transmembrane immunomodulatory protein of the present invention. Thus, it refers to an alteration, such as an increase or decrease, of an immune response as compared to the immune response that occurs or is present in the absence of the administration of the immunomodulatory protein comprising the variant PD-L1 or cells

expressing such an immunomodulatory polypeptide. Such modulation includes any induction, activation, suppression or alteration in degree or extent of immunological activity of an immune cell. Immune cells include B cells, T cells, NK (natural killer) cells, NK T cells, professional antigen-presenting cells (APCs), and non-professional antigen-presenting cells, and inflammatory cells (neutrophils, macrophages, monocytes, eosinophils, and basophils). Modulation includes any change imparted on an existing immune response, a developing immune response, a potential immune response, or the capacity to induce, regulate, influence, or respond to an immune response. Modulation includes any alteration in the expression and/or function of genes, proteins and/or other molecules in immune cells as part of an immune response. Modulation of an immune response or modulation of immunological activity includes, for example, the following: elimination, deletion, or sequestration of immune cells; induction or generation of immune cells that can modulate the functional capacity of other cells such as autoreactive lymphocytes, antigen presenting cells, or inflammatory cells; induction of an unresponsive state in immune cells (i.e., anergy); enhancing or suppressing the activity or function of immune cells, including but not limited to altering the pattern of proteins expressed by these cells. Examples include altered production and/or secretion of certain classes of molecules such as cytokines, chemokines, growth factors, transcription factors, kinases, costimulatory molecules, or other cell surface receptors or any combination of these modulatory events. Modulation can be assessed, for example, by an alteration in IFN-gamma (interferon gamma) expression relative to the wild-type PD-L1 control in a primary T cell assay (see, Zhao and Ji, Exp Cell Res. 2016 Jan1; 340(1): 132-138). Modulation can be assessed, for example, by an alteration of an immunological activity of engineered cells, such as an alteration in in cytotoxic activity of engineered cells or an alteration in cytokine secretion of engineered cells relative to cells engineered with a wild-type PD-L1 transmembrane protein

**[0108]** The term "molecular species" as used herein means an ensemble of proteins with identical or substantially identical primary amino acid sequence. Each mammalian immunoglobulin superfamily (IgSF) member defines a collection of identical or substantially identical molecular species. Thus, for example, human PD-L1 is an IgSF member and each human PD-L1 molecule is a molecular species of PD-L1. Variation between molecules that are of the same molecular species may occur owing to differences in post-translational modification such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. Additionally, minor sequence differences within a single molecular species owing to gene polymorphisms constitute another form of variation within a single molecular species as do wild type truncated forms of a single molecular species owing to, for example, proteolytic cleavage. A "cell surface molecular species" is a molecular species expressed on the surface of a mammalian cell. Two or more different species of protein, each of which is present exclusively on one or exclusively the other (but not both) of the two mammalian cells forming the IS, are said to be in "cis" or "cis configuration" with each other. Two different species of protein, the first of which is exclusively present on one of the two mammalian cells forming the IS and the second of which is present exclusively on the second of the two mammalian cells forming the IS, are said to be in "trans" or "trans configuration." Two different species of protein each of which is present on both of the two mammalian cells forming the IS are in both cis and trans configurations on these cells.

**[0109]** The term, a "multimerization domain" refers to a sequence of amino acids that promotes stable interaction of a polypeptide molecule with one or more additional polypeptide molecules, each containing a complementary multimerization domain (e.g. a first multimerization domain and a second multimerization domain), which can be the same or a different multimerization domain. The interactions between complementary multimerization domains, e.g. interaction between a first multimerization domain and a second multimerization domain, form a stable protein-protein interaction to produce a multimer of the polypeptide molecule with the additional polypeptide molecule. In some cases, the multimerization domain is the same and interacts with itself to form a stable protein-protein interaction between two polypeptide chains.. Generally, a polypeptide is joined directly or indirectly to the multimerization domain. Exemplary

multimerization domains include the immunoglobulin sequences or portions thereof, leucine zippers, hydrophobic regions, hydrophilic regions, and compatible protein-protein interaction domains. The multimerization domain, for example, can be an immunoglobulin constant region or domain, such as, for example, the Fc domain or portions thereof from IgG, including IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD and IgM and modified forms thereof.

**[0110]** The terms "nucleic acid" and "polynucleotide" are used interchangeably to refer to a polymer of nucleic acid residues (e.g., deoxyribonucleotides or ribonucleotides) in either singleor double-stranded form. Unless specifically limited, the terms encompass nucleic acids containing known analogues of natural nucleotides and that have similar binding properties to it and are metabolized in a manner similar to naturally-occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary nucleotide sequences as well as the sequence explicitly indicated (a "reference sequence"). Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues. The term nucleic acid or polynucleotide encompasses cDNA or mRNA encoded by a gene.

**[0111]** The term "non-competitive binding" as used herein means the ability of a protein to specifically bind simultaneously to at least two cognate binding partners. Thus, the protein is able to bind to at least two different cognate binding partners at the same time, although the binding interaction need not be for the same duration such that, in some cases, the protein is specifically bound to only one of the cognate binding partners. In some embodiments, the binding occurs under specific binding conditions. In some embodiments, the simultaneous binding is such that binding of one cognate binding partner does not substantially inhibit simultaneous binding to a second cognate binding partner. In some embodiments, non-competitive binding means that binding a second cognate binding partner to its binding site on the protein does not displace the binding of a first cognate binding partner to its binding site on the protein. Methods of assessing non-competitive binding are well known in the art such as the method described in Perez de La Lastra et al., Immunology, 1999 Apr: 96(4): 663-670. In some cases, in non-competitive interactions, the first cognate binding partner specifically binds at an interaction site that does not overlap with the interaction site of the second cognate binding partner such that binding of the second cognate binding partner does not directly interfere with the binding of the first cognate binding partner. Thus, any effect on binding of the cognate binding partner by the binding of the second cognate binding partner is through a mechanism other than direct interference with the binding of the first cognate binding partner. For example, in the context of enzyme-substrate interactions, a non-competitive inhibitor binds to a site other than the active site of the enzyme. Non-competitive binding encompasses uncompetitive binding interactions in which a second cognate binding partner specifically binds at an interaction site that does not overlap with the binding of the first cognate binding partner but binds to the second interaction site only when the first interaction site is occupied by the first cognate binding partner.

**[0112]** The term "pharmaceutical composition" refers to a composition suitable for pharmaceutical use in a mammalian subject, often a human. A pharmaceutical composition typically comprises an effective amount of an active agent (e.g., an immunomodulatory polypeptide comprising a variant PD-L1 or engineered cells expressing a variant PD-L1 transmembrane immunomodulatory protein) and a carrier, excipient, or diluent. The carrier, excipient, or diluent is typically a pharmaceutically acceptable carrier, excipient or diluent, respectively.

**[0113]** The terms "polypeptide" and "protein" are used interchangeably herein and refer to a molecular chain of two or more amino acids linked through peptide bonds. The terms do not refer to a specific length of the product. Thus, "peptides," and "oligopeptides," are included within the definition of polypeptide. The

terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. The terms also include molecules in which one or more amino acid analogs or non-canonical or unnatural amino acids are included as can be synthesized, or expressed recombinantly using known protein engineering techniques. In addition, proteins can be derivatized.

**[0114]** The term "primary T-cell assay" as used herein refers to an in vitro assay to measure interferon-gamma ("IFN-gamma") expression. A variety of such primary T-cell assays are known in the art. In a preferred embodiment, the assay used is an anti-CD3 coimmobilizaton assay. In this assay, primary T cells are stimulated by anti-CD3 immobilized with or without additional recombinant proteins. Culture supernatants are harvested at timepoints, usually 24-72 hours. In another embodiment, the assay used is the MLR. In this assay, primary T cells are stimulated with allogeneic APC. Culture supernatants are harvested at timepoints, usually 24-72 hours. Human IFN-gamma levels are measured in culture supernatants by standard ELISA techniques. Commercial kits are available from vendors and the assay is performed according to manufacturer's recommendation.

**[0115]** The term "purified" as applied to nucleic acids, such as encoding immunomodulatory proteins of the invention, generally denotes a nucleic acid or polypeptide that is substantially free from other components as determined by analytical techniques well known in the art (e.g., a purified polypeptide or polynucleotide forms a discrete band in an electrophoretic gel, chromatographic eluate, and/or a media subjected to density gradient centrifugation). For example, a nucleic acid or polypeptide that gives rise to essentially one band in an electrophoretic gel is "purified." A purified nucleic acid or protein of the invention is at least about 50% pure, usually at least about 75%, 80%, 85%, 90%, 95%, 96%, 99% or more pure (e.g., percent by weight or on a molar basis).

**[0116]** The term "recombinant" indicates that the material (e.g., a nucleic acid or a polypeptide) has been artificially (i.e., non-naturally) altered by human intervention. The alteration can be performed on the material within,or removed from, its natural environment or state. For example, a "recombinant nucleic acid" is one that is made by recombining nucleic acids, e.g., during cloning, affinity modification, DNA shuffling or other well-known molecular biological procedures. A "recombinant DNA molecule," is comprised of segments of DNA joined together by means of such molecular biological techniques. The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule. A "recombinant host cell" is a cell that contains and/or expresses a recombinant nucleic acid or that is otherwise altered by genetic engineering, such as by introducing into the cell a nucleic acid molecule encoding a recombinant protein, such as a transmembrane immunomodulatory protein provided herein. Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, i.e., promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. The terms "in operable combination," "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner or orientation that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced.

**[0117]** The term "recombinant expression vector" as used herein refers to a DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host cell. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and

polyadenylation signals. A secretory signal peptide sequence can also, optionally, be encoded by the recombinant expression vector, operably linked to the coding sequence for the recombinant protein, such as a recombinant fusion protein, so that the expressed fusion protein can be secreted by the recombinant host cell, for easier isolation of the fusion protein from the cell, if desired. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Among the vectors are viral vectors, such as lentiviral vectors.

**[0118]** The term "selectivity" refers to the preference of a subject protein, or polypeptide, for specific binding of one substrate, such as one cognate binding partner, compared to specific binding for another substrate, such as a different cognate binding partner of the subject protein. Selectivity can be reflected as a ratio of the binding activity (e.g. binding affinity) of a subject protein and a first substrate, such as a first cognate binding partner, (e.g., $K_{d1}$) and the binding activity (e.g. binding affinity) of the same subject protein with a second cognate binding partner (e.g., $K_{d2}$).

**[0119]** The term "sequence identity" as used herein refers to the sequence identity between genes or proteins at the nucleotide or amino acid level, respectively. "Sequence identity" is a measure of identity between proteins at the amino acid level and a measure of identity between nucleic acids at nucleotide level. The protein sequence identity may be determined by comparing the amino acid sequence in a given position in each sequence when the sequences are aligned. Similarly, the nucleic acid sequence identity may be determined by comparing the nucleotide sequence in a given position in each sequence when the sequences are aligned. Methods for the alignment of sequences for comparison are well known in the art, such methods include GAP, BESTFIT, BLAST, FASTA and TFASTA. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Center for Biotechnology Information (NCBI) website.

**[0120]** The term "soluble" as used herein in reference to proteins, means that the protein is not a membrane protein. In general, a soluble protein contains only the extracellular domain of an IgSF family member receptor, or a portion thereof containing an IgSF domain or domains or specific-binding fragments thereof, but does not contain the transmembrane domain. In some cases, solubility of a protein can be improved by linkage or attachment, directly or indirectly via a linker, to an Fc domain, which, in some cases, also can improve the stability and/or half-life of the protein. In some aspects, a soluble protein is an Fc fusion protein.

**[0121]** The term "species" as used herein with respect to polypeptides or nucleic acids means an ensemble of molecules with identical or substantially identical sequences. Variation between polypeptides that are of the same species may occur owing to differences in post-translational modification such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. Slightly truncated sequences of polypeptides that differ (or encode a difference) from the full length species at the amino-terminus or carboxy-terminus by no more than 1, 2, or 3 amino acid residues are considered to be of a single species. Such microheterogeneities are a common feature of manufactured proteins.

**[0122]** The term "specifically binds" as used herein means the ability of a protein, under specific binding conditions, to bind to a target protein such that its affinity or avidity is at least 5 times as great, but optionally at least 10, 20, 30, 40, 50, 100, 250 or 500 times as great, or even at least 1000 times as great as the average affinity or avidity of the same protein to a collection of random peptides or polypeptides of sufficient statistical size. A specifically binding protein need not bind exclusively to a single target molecule but may specifically bind to a non-target molecule due to similarity in structural conformation between the target and non-target (e.g., paralogs or orthologs). Those of skill will recognize that specific binding to a

molecule having the same function in a different species of animal (i.e., ortholog) or to a non-target molecule having a substantially similar epitope as the target molecule (e.g., paralog) is possible and does not detract from the specificity of binding which is determined relative to a statistically valid collection of unique non-targets (e.g., random polypeptides). Thus, a polypeptide of the invention may specifically bind to more than one distinct species of target molecule due to cross-reactivity. Solid-phase ELISA immunoassays, ForteBio Octet, or Biacore measurements can be used to determine specific binding between two proteins. Generally, interactions between two binding proteins have dissociation constants ($K_d$) less than $1\times10^{-5}$ M, and often as low as $1 \times 10^{-12}$ M. In certain embodiments of the present disclosure, interactions between two binding proteins have dissociation constants of less than or less than about $1\times10^{-6}$ M, $1\times10^{-7}$ M, $1\times10^{-8}$ M, $1\times10^{-9}$ M, $1\times10^{-10}$ M or $1\times10^{-11}$ M or less.

**[0123]** The terms "surface expresses" or "surface expression" in reference to a mammalian cell expressing a polypeptide means that the polypeptide is expressed as a membrane protein. In some embodiments, the membrane protein is a transmembrane protein.

**[0124]** As used herein, "synthetic," with reference to, for example, a synthetic nucleic acid molecule or a synthetic gene or a synthetic peptide refers to a nucleic acid molecule or polypeptide molecule that is produced by recombinant methods and/or by chemical synthesis methods.

**[0125]** The term "targeting moiety" as used herein refers to a composition that is covalently or non-covalently attached to, or physically encapsulates, a polypeptide comprising a variant PD-L1 of the present invention. The targeting moiety has specific binding affinity for a desired counter-structure such as a cell surface receptor (e.g., PD-1), or a tumor antigen such as tumor specific antigen (TSA) or a tumor associated antigen (TAA) such as B7-H6. Typically, the desired counter-structure is localized on a specific tissue or cell-type. Targeting moieties include: antibodies, antigen binding fragment (Fab), variable fragment (Fv) containing $V_H$ and $V_L$, the single chain variable fragment (scFv) containing $V_H$ and $V_L$ linked together in one chain, as well as other antibody V region fragments, such as Fab', $F(ab)_2$, $F(ab')_2$, dsFv diabody, nanobodies, soluble receptors, receptor ligands, affinity matured receptors or ligands, as well as small molecule (<500 dalton) compositions (e.g., specific binding receptor compositions). Targeting moieties can also be attached covalently or non-covalently to the lipid membrane of liposomes that encapsulate a polypeptide of the present invention.

**[0126]** The term "transmembrane protein" as used herein means a membrane protein that substantially or completely spans a lipid bilayer such as those lipid bilayers found in a biological membrane such as a mammalian cell, or in an artificial construct such as a liposome. The transmembrane protein comprises a transmembrane domain ("transmembrane domain") by which it is integrated into the lipid bilayer and by which the integration is thermodynamically stable under physiological conditions. Transmembrane domains are generally predictable from their amino acid sequence via any number of commercially available bioinformatics software applications on the basis of their elevated hydrophobicity relative to regions of the protein that interact with aqueous environments (e.g., cytosol, extracellular fluid). A transmembrane domain is often a hydrophobic alpha helix that spans the membrane. A transmembrane protein can pass through the both layers of the lipid bilayer once or multiple times. A transmembrane protein includes the provided transmembrane immunomodulatory proteins described herein. In addition to the transmembrane domain, a transmembrane immunomodulatory protein of the invention further comprises an ectodomain and, in some embodiments, an endodomain.

**[0127]** The terms "treating," "treatment," or "therapy" of a disease or disorder as used herein mean slowing, stopping or reversing the disease or disorders progression, as evidenced by decreasing, cessation or elimination of either clinical or diagnostic symptoms, by administration of a therapeutic composition (e.g.

containing an immunomodulatory protein or engineered cells) of the invention either alone or in combination with another compound as described herein. "Treating," "treatment," or "therapy" also means a decrease in the severity of symptoms in an acute or chronic disease or disorder or a decrease in the relapse rate as for example in the case of a relapsing or remitting autoimmune disease course or a decrease in inflammation in the case of an inflammatory aspect of an autoimmune disease. As used herein in the context of cancer, the terms "treatment" or, "inhibit," "inhibiting" or "inhibition" of cancer refers to at least one of: a statistically significant decrease in the rate of tumor growth, a cessation of tumor growth, or a reduction in the size, mass, metabolic activity, or volume of the tumor, as measured by standard criteria such as, but not limited to, the Response Evaluation Criteria for Solid Tumors (RECIST), or a statistically significant increase in progression free survival (PFS) or overall survival (OS). "Preventing," "prophylaxis," or "prevention" of a disease or disorder as used in the context of this invention refers to the administration of an immunomodulatory polypeptide or engineered cells of the invention, either alone or in combination with another compound, to prevent the occurrence or onset of a disease or disorder or some or all of the symptoms of a disease or disorder or to lessen the likelihood of the onset of a disease or disorder.

**[0128]** The term "tumor specific antigen" or "TSA" as used herein refers to a counter-structure that is present primarily on tumor cells of a mammalian subject but generally not found on normal cells of the mammalian subject. A tumor specific antigen need not be exclusive to tumor cells but the percentage of cells of a particular mammal that have the tumor specific antigen is sufficiently high or the levels of the tumor specific antigen on the surface of the tumor are sufficiently high such that it can be targeted by anti-tumor therapeutics, such as immunomodulatory polypeptides of the invention, and provide prevention or treatment of the mammal from the effects of the tumor. In some embodiments, in a random statistical sample of cells from a mammal with a tumor, at least 50% of the cells displaying a TSA are cancerous. In other embodiments, at least 60%, 70%, 80%, 85%, 90%, 95%, or 99% of the cells displaying a TSA are cancerous.

**[0129]** The term "variant" (also "modified" or mutant") as used in reference to a variant PD-L1 means a PD-L1, such as a mammalian (e.g., human or murine) PD-L1 created by human intervention. The variant PD-L1 is a polypeptide comprising an amino acid substitution N45D, with reference to the numbering of SEQ ID NO:30, relative to an unmodified human PD-L1. The variant PD-L1 is a polypeptide which differs from an unmodified PD-L1 isoform sequence by one or more amino acid substitutions, deletions, additions, or combinations thereof. For purposes herein, the variant PD-L1 contains at least one affinity modified domain, whereby one or more of the amino acid differences occurs in an IgSF domain (e.g. IgV domain or ECD). A variant PD-L1 can contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acid differences, such as amino acid substitutions. A variant PD-L1 polypeptide generally exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a corresponding wildtype or unmodified PD-L1, such as to the sequence of SEQ ID NO:3, a mature sequence thereof (lacking the signal sequence) or a portion thereof containing the extracellular domain or an IgSF domain thereof. In some embodiments, a variant PD-L1 polypeptide exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a corresponding wild-type or unmodified PD-L1 comprising the sequence set forth in SEQ ID NO:30 or 1728 or SEQ ID NO: 55 or 309. Non-naturally occurring amino acids as well as naturally occurring amino acids are included within the scope of permissible substitutions or additions. A variant PD-L1 is not limited to any particular method of making and includes, for example, de novo chemical synthesis, de novo recombinant DNA techniques, or combinations thereof. A variant PD-L1 of the invention specifically binds to at least PD-1 of a mammalian species. In some embodiments, the altered amino acid sequence results in an altered (i.e., increased or decreased) binding affinity or avidity to PD-1 and/or CD80 compared to the wildtype or unmodified PD-L1 protein. An increase or decrease in binding affinity or avidity can be determined using well known binding assays such as flow cytometry. Larsen et al., American Journal of Transplantation, Vol 5: 443-453 (2005). See also, Linsley et al.,

Immunity, Vol 1(9): 793-801 (1994). An increase in variant PD-L1 binding affinity or avidity to PD-1 and/or CD80 is to a value at least 5% greater than that of the wild-type or unmodified PD-L1 and in some embodiments, at least 10%, 15%, 20%, 30%, 40%, 50%, 100% greater than that of the wild-type or unmodified PD-L1 control value. A decrease in PD-L1 binding affinity or avidity to PD-1 and/or CD80 is to a value no greater than 95% of the wild-type or unmodified control values, and in some embodiments no greater than 80%, 70% 60%, 50%, 40%, 30%, 20%, 10%, 5%, or no detectable binding affinity or avidity of the wild-type or unmodified control values. A variant PD-L1 is altered in primary amino acid sequence by substitution, addition, or deletion of amino acid residues. The term "variant" in the context of variant PD-L1 is not to be construed as imposing any condition for any particular starting composition or method by which the variant PD-L1 is created. A variant PD-L1 can, for example, be generated starting from wild type mammalian PD-L1 sequence information, then modeled in silico for binding to PD-1 and/or CD80, and finally recombinantly or chemically synthesized to yield a variant PD-L1 of the present invention. In but one alternative example, a variant PD-L1 can be created by site-directed mutagenesis of a wild-type PD-L1. Thus, variant PD-L1 denotes a composition and not necessarily a product produced by any given process. A variety of techniques including recombinant methods, chemical synthesis, or combinations thereof, may be employed.

**[0130]** The term "wild-type" or "natural" or "native" as used herein is used in connection with biological materials such as nucleic acid molecules, proteins (e.g., PD-L1), IgSF members, host cells, and the like, refers to those which are found in nature and not modified by human intervention.

**II. VARIANT PD-L1 POLYPEPTIDES**

**[0131]** In addition to binding PD-1, provided herein are variant PD-L1 polypeptides that exhibitincreased binding affinity for one or more further PD-L1 cognate binding partners. In some embodiments, the PD-L1 cognate binding partner is CD80. The variant PD-L1 polypeptide contains one or more amino acids modifications, such as one or more substitutions (alternatively, "mutations" or "replacements"), deletions or addition, in an immunoglobulin superfamily (IgSF) domain (IgD) relative to a wild-type or unmodified PD-L1 polypeptide or a portion of a wildtype or unmodified PD-L1 containing the IgD or a specific binding fragment thereof. Thus, a provided variant PD-L1 polypeptide is or comprises a variant IgD (hereinafter called "vIgD") in which the one or more amino acid modifications (e.g. substitutions) is in an IgD.

**[0132]** In some embodiments, the IgD comprises an IgV domain or both an IgV domain and an IgC (e.g. IgC2) domain. In some embodiments, the IgD can be an IgV only, the combination of the IgV and IgC, including the entire extracellular domain (ECD), or any combination of Ig domains of PD-L1. Table 2 provides exemplary residues that correspond to IgV or IgC regions of PD-L1. In some embodiments, the variant PD-L1 polypeptide contains an IgV domain or both an IgV domain and an IgC domain in which at least one amino acid modifications (e.g. substitutions) is in the IgV domain. In some embodiments, by virtue of the altered binding activity or affinity, the altered IgV domain or IgC (e.g. IgC2) domain is an affinity-modified IgSF domain.

**[0133]** In some embodiments, the variant is modified in one more IgSF domains relative to the sequence of an unmodified PD-L1 sequence. In some embodiments, the unmodified PD-L1 sequence is a wild-type PD-L1. In some embodiments, the unmodified or wild-type PD-L1 has the sequence of a native PD-L1 or an ortholog thereof. In some embodiments, the unmodified PD-L1 is or comprises the extracellular domain (ECD) of PD-L1 or a portion thereof containing one or more IgSF domain (see Table 2). In some embodiments, the extracellular domain of an unmodified or wild-type PD-L1 polypeptide comprises an IgV domain and an IgC (e.g. IgC2) domain or domains. However, the variant PD-L1 polypeptide need not

comprise both the IgV domain and the IgC (e.g. IgC2) domain or domains. In some embodiments, the variant PD-L1 polypeptide comprises or consists essentially of the IgV domain. In some embodiments, the variant PD-L1 polypeptide comprises one or both of the IgC (e.g. IgC2) domain In some embodiments, the variant PD-L1 polypeptide comprises the IgV domain, and the first and second IgC (e.g. IgC2) domains. In some embodiments, the variant PD-L1 is soluble and lacks a transmembrane domain. In some embodiments, the variant PD-L1 further comprises a transmembrane domain and, in some cases, also a cytoplasmic domain.

**[0134]** In some embodiments, the unmodified PD-L1 sequence has (i) the sequence of amino acids set forth in SEQ ID NO:3 or a mature form thereof lacking the signal sequence, (ii) a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:3 or the mature form thereof, or (iii) is a portion of (i) or (ii) containing an IgV domain and optionally IgC (e.g. IgC2) domain.

**[0135]** In some embodiments, the wild-type or unmodified PD-L1 sequence is or comprises an extracellular domain of the PD-L1 or a portion thereof. In some embodiments, the unmodified or wild-type PD-L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 30 or 1728, or an ortholog thereof. In some cases, the unmodified or wild-type PD-L1 polypeptide can comprise (i) the sequence of amino acids set forth in SEQ ID NO: 30 or 1728, (ii) a sequence of amino acids that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 30 or 1728, or (iii) is a specific binding fragment of the sequence of (i) or (ii) comprising an IgV domain or an IgC (e.g. IgC2) domain.

**[0136]** In some embodiments, the wild-type or unmodified PD-L1 polypeptide comprises an IgV domain or an IgC (e.g. IgC2) domain or domains, or a specific binding fragment thereof. In some embodiments, the IgV domain of the wild-type or unmodified PD-L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 55 or 309(corresponding to amino acid residues24-130 of SEQ ID NO: 3), or an ortholog thereof. For example, the IgV domain of the unmodified or wild-type PD-L1 polypeptide can contain (i) the sequence of amino acids set forth in SEQ ID NO: 55 or 309, (ii) a sequence of amino acids that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 55 or 309, or (iii) a specific binding fragment of the sequence of (i) or (ii). In some embodiments, the wild-type or unmodified IgV domain is capable of binding one or more PD-L1 cognate binding proteins, such as one or more of PD-1 or CD80.

**[0137]** In some embodiments, a first IgC2 domain of the wild-type or unmodified PD-L1 polypeptide comprises the amino acid sequence set forth as residues 133-225 of SEQ ID NO: 3, or an ortholog thereof. For example, an IgC2 domain of the unmodified or wild-type PD-L1 polypeptide can contain (i) the sequence of amino acids set forth as residues 133-225 of SEQ ID NO: 3, (ii) a sequence of amino acids that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to residues 133-225 of SEQ ID NO: 3, or (iii) a specific binding fragment of (i) or (ii). In some embodiments, the wild-type or unmodified IgC domain is capable of binding one or more PD-L1 cognate binding proteins in addition to PD-1.

**[0138]** In some embodiments, the wild-type or unmodified PD-L1 polypeptide contains a specific binding fragment of PD-L1, such as a specific binding fragment ofthe IgC (e.g. IgC2) domain. In some embodiments the specific binding fragment can bind PD-1 and/or CD80. The specific binding fragment can have an amino acid length of at least 50 amino acids, such as at least 60, 70, 80, 90, 100, or 110 amino acids. In some embodiments, a specific binding fragment of an IgC (e.g. IgC2) domain comprises an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the length of the IgC domain set forth as amino acids 133-225 of SEQ ID NO: 3.

**[0139]** In some embodiments, the variant PD-L1 polypeptide comprises the ECD domain or a portion thereof comprising one or more affinity modified IgSF domains. In some embodiments, the variant PD-L1 polypeptides can comprise an IgV domain and optionally an IgC (e.g. IgC2) domain or domains. In some embodiments, the variant PD-L1 polypeptides can comprise an IgV domain and an IgC (e.g. IgC2) domain or domains. The variant PD-L1 polypeptide comprises a full-length IgV domain. In some embodiments, the variant PD-L1 polypeptide comprises a full-length IgC (e.g. IgC2) domain or domains. In some embodiments, the variant PD-L1 polypeptide comprises a specific binding fragment of the IgC (e.g. IgC2) domain or domains. In some embodiments, the variant PD-L1 polypeptide comprises a full-length IgV domain and a full-length IgC (e.g. IgC2) domain or domains. In some embodiments, the variant PD-L1 polypeptide comprises a full-length IgV domain and a specific binding fragment of an IgC (e.g. IgC2) domain or domains.

**[0140]** In any of such embodiments, one or more amino acid modifications (e.g. substitutions) of the variant PD-L1 polypeptides can be located in any one or more of the PD-L1 polypeptide IgSF domains. For example, in some embodiments, one or more amino acid modifications (e.g. substitutions) are located in the extracellular domain of the variant PD-L1 polypeptide. In some embodiments, one or more amino acid modifications (e.g. substitutions) are located in the IgV domain. In some embodiments, one or more amino acid modifications (e.g. substitutions) are located in an IgC (e.g. IgC2) domain or specific binding fragment of an IgC (e.g. IgC2) domain.

**[0141]** Generally, each of the various attributes of polypeptides are separately disclosed below (e.g., soluble and membrane bound polypeptides, affinity of PD-L1 for PD-1 and CD80, number of variations per polypeptide chain, number of linked polypeptide chains, the number and nature of amino acid alterations per variant PD-L1, etc.). However, as will be clear to the skilled artisan, any particular polypeptide can comprise a combination of these independent attributes. It is understood that reference to amino acids, including to a specific sequence set forth as a SEQ ID NO used to describe domain organization of an IgSF domain are for illustrative purposes and are not meant to limit the scope of the embodiments provided. It is understood that polypeptides and the description of domains thereof are theoretically derived based on homology analysis and alignments with similar molecules. Thus, the exact locus can vary, and is not necessarily the same for each protein. Hence, the specific IgSF domain, such as specific IgV domain or IgC domain, can be several amino acids (such as one, two, three or four) longer or shorter.

**[0142]** Further, various embodiments of the invention as discussed below are frequently provided within the meaning of a defined term as disclosed above. Thus, the headings, the order of presentation of the various aspects and embodiments, and the separate disclosure of each independent attribute is not meant to be a limitation to the scope of the present disclosure.

**A. Exemplary Modifications**

**[0143]** Provided herein are variant PD-L1 polypeptides containing at least one affinity-modified IgV domain relative to an IgV domain contained in an unmodified PD-L1 polypeptide such that the variant PD-L1 polypeptide exhibits increased binding affinity for PD-1 compared tounmodified human PD-L1 polypeptide. In some embodiments, a variant PD-L1 polypeptide has a binding affinity for PD-1 or PD-1 and CD80 that differs from that of anunmodified PD-L1 polypeptide control sequence as determined by, for example, solid-phase ELISA immunoassays, flow cytometry, ForteBio Octet or Biacore assays. In some embodiments, the variant PD-L1 polypeptide has an increased binding affinity for PD-1 and CD80. In some embodiments, the variant PD-L1 polypeptide has a decreased binding affinity for CD80, relative to an unmodified PD-L1

polypeptide.

**[0144]** Binding affinities for each of the cognate binding partners are independent; that is, in some embodiments, a variant PD-L1 polypeptide has an increased binding affinity for both of PD-1 and CD80, or a decreased binding affinity for CD80, relative to anunmodified PD-L1 polypeptide.

**[0145]** In some embodiments, the variant PD-L1 polypeptide has an increased binding affinity for CD80, relative to anunmodified PD-L1 polypeptide. In some embodiments, the variant PD-L1 polypeptide has a decreased binding affinity for CD80, relative to anunmodified PD-L1polypeptide.

**[0146]** In some embodiments, the variant PD-L1 polypeptide has an increased binding affinity for PD-1 and CD80, relative to an unmodified PD-L1 polypeptide. In some embodiments, the variant PD-L1 polypeptide has an increased binding affinity for PD-1 and a decreased binding affinity for CD80, relative to an unmodified PD-L1 polypeptide.

**[0147]** In some embodiments, a variant PD-L1 polypeptide with increased or greater binding affinity to PD-1 and optionally CD80 will have an increase in binding affinity relative to the unmodified PD-L1 polypeptide control of at least about 5%, such as at least about 10%, 15%, 20%, 25%, 35%, or 50% for the PD-1 and/or CD80. In some embodiments, the increase in binding affinity relative to the wild-type or unmodified PD-L1 polypeptide is more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold. In such examples, the unmodified PD-L1 polypeptide has the same sequence as the variant PD-L1 polypeptide except that it does not contain the one or more amino acid modifications (e.g. substitutions).

**[0148]** In some embodiments, a variant PD-L1 polypeptide with reduced or decreased binding affinity to CD80 will have decrease in binding affinity relative to the unmodified PD-L1 polypeptide control of at least 5%, such as at least about 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more for the CD80. In some embodiments, the decrease in binding affinity relative to theunmodified PD-L1 polypeptide is more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold. In such examples, the unmodified PD-L1 polypeptide has the same sequence as the variant PD-L1 polypeptide except that it does not contain the one or more amino acid modifications (e.g. substitutions).

**[0149]** In some embodiments, the equilibrium dissociation constant ($K_d$) of any of the foregoing embodiments to PD-1 and/or CD80 can be less than $1\times10^{-5}$ M, $1\times10^{-6}$ M, $1\times10^{-7}$ M, $1\times10^{-8}$ M, $1\times10^{-9}$ M, $1\times10^{-10}$ M or $1\times10^{-11}$M, or $1\times10^{-12}$ M or less.

**[0150]** The unmodified PD-L1 sequence does not necessarily have to be used as a starting composition to generate variant PD-L1 polypeptides described herein. Therefore, use of the term "modification", such as "substitution", does not imply that the present embodiments are limited to a particular method of making variant PD-L1 polypeptides. Variant PD-L1 polypeptides can be made, for example, by *de novo* peptide synthesis and thus does not necessarily require a modification, such as a "substitution", in the sense of altering a codon to encode for the modification, e.g. substitution. This principle also extends to the terms "addition" and "deletion" of an amino acid residue, which likewise do not imply a particular method of making. The means by which the variant PD-L1 polypeptides are designed or created is not limited to any particular method. In some embodiments, however, a wild-type or unmodified PD-L1 encoding nucleic acid is mutagenized from wild-type or unmodified PD-L1 genetic material and screened for desired specific binding affinity and/or induction of IFN-gamma expression or other functional activity. In some embodiments, a variant PD-L1 polypeptide is synthesized *de novo* utilizing protein or nucleic acid

sequences available at any number of publicly available databases and then subsequently screened. The National Center for Biotechnology Information provides such information and its website is publicly accessible via the internet as is the UniProtKB database as discussed previously.

**[0151]** Unless stated otherwise, as indicated throughout the present disclosure, the amino acid modification(s) are designated by amino acid position number corresponding to the numbering of positions of the unmodified ECD sequence set forth in SEQ ID NO:30 or 1728 or also, where applicable, the unmodified IgV sequence set forth in SEQ ID NO: 309 (containing residues 1-114, respectively, of SEQ ID NO:30) as follows:

FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEED
LKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGADYKRIT
VKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTTTT
NSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNER
(SEQ ID NO:30)

FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEED
LKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGADYKRIT
VKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTTTT
NSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNERT
(SEQ ID NO:1728)

FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFV
HGEEDLKVQHSSYRQRARLL KDQLSLGNAALQITDVKLQDAGVYRCMISY
GGADYKRITVKVNA (SEQ ID NO:309)

**[0152]** Modifications provided herein can be in an unmodified PD-L1 polypeptide set forth in SEQ ID NO:30, 309 or 1728. In some cases, modifications also can be in an unmodified IgV set forth in SEQ ID NO:55. In accordance with the invention, the unmodified PD-L1 polypeptide has 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO: 30, 55, 309 or 1728.

PKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEEDLKVQHSS
YRQRARLL KDQLSLGNAALQITDVKLQDAGVYRCMISY GGADYKRITVKV (SEQ ID
NO:55)

**[0153]** It is within the level of a skilled artisan to identify the corresponding position of a modification, e.g. amino acid substitution, in a PD-L1 polypeptide, including portion thereof containing an IgSF domain (e.g. ECD or IgV) thereof, such as by alignment of a reference sequence with SEQ ID NO:30, or SEQ ID NO:309. In the listing of modifications throughout this disclosure, the amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution listed after the number. If the modification is a deletion of the position a "del" is indicated and if the modification is an insertion at the position an "ins" is indicated. In some cases, an insertion is listed with the amino acid position indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before and after the number and the identified variant amino acid insertion listed after the unmodified (e.g. wild-type) amino acid.

**[0154]** In some embodiments, the variant PD-L1 polypeptide has one or more amino acid modifications, e.g. substitutions, in anunmodified PD-L1 sequence. The one or more amino acid modifications, e.g.

substitutions, can be in the ectodomain (extracellular domain) of theunmodified PD-L1 sequence. In some embodiments, the one or more amino acid modifications, e.g. substitutions, are in the IgV domain or specific binding fragment thereof. In some embodiments, the one or more amino acid modifications, e.g. substitutions, are in the ECD domain or specific binding fragment thereof. In some embodiments, the one or more amino acid modifications, e.g. substitutions, are in an IgC (e.g. IgC2) domain or specific binding fragment thereof. In some embodiments of the variant PD-L1 polypeptide, some of the one or more amino acid modifications, e.g. substitutions, are in the IgV domain, and some of the one or more amino acid modifications, e.g. substitutions, are in an IgC domain or domains (e.g. IgC2) or a specific binding fragment thereof.

**[0155]** In some embodiments, the variant PD-L1 polypeptide has up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid modifications, e.g. substitutions. The modifications (e.g. substitutions) can be in the IgV domain and optionally the IgC (e.g. IgC2) domain or domains. In some embodiments, the variant PD-L1 polypeptide has up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid modifications, e.g. substitutions, in the IgV domain. In some embodiments, the variant PD-L1 polypeptide has up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid modifications, e.g. substitutions, in the IgC (e.g. IgC2) domain or domains or specific binding fragment thereof. In some embodiments, the variant PD-L1 polypeptide has at least about 85%, 86%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with theunmodified PD-L1 polypeptide or specific binding fragment thereof, such as with the amino acid sequence of SEQ ID NO: 30, 1728, 55 or 309.

**[0156]** In some embodiments, as well as the N45D mutation, the variant PD-L1 polypeptide has one or more amino acid modifications, e.g. substitutions, in an unmodified PD-L1 or specific binding fragment thereof corresponding to position(s) 6, 10, 11, 14, 15, 16, 17, 18, 19, 20, 22, 23, 26, 27, 28, 33, 35, 36, 40, 41, 43, 44, 45, 46, 47, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 60, 64, 65, 68, 71, 72, 73, 74, 75, 78, 79, 83, 89, 90, 93, 97, 98, 99, 101, 102, 103, 104, 106, 110, 111, 112, 113, 117, 119, 120, 121, 124, 129, 130, 131, 134, 137, 138, 144, 148, 149, 150, 155, 158, 160, 163, 165, 167, 170, 171, 173, 175, 176, 177, 179, 180, 183, 185, 188, 189, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 206, 207, 213, or 221, with reference to positions set forth in SEQ ID NO: 30 or 1728. In some embodiments, such variant PD-L1 polypeptides exhibit increased binding affinity to PD-1 and optionally CD80 compared to the unmodified PD-L1 polypeptide. For example, in some embodiments, the variant PD-L1 polypeptide exhibits increased binding affinity to PD-1 and CD80 compared to an unmodified PD-L1 polypeptide.

**[0157]** In some embodiments, the amino acid modifications, e.g. amino acid substitutions, include I20L/N45D, I36T/N45D, D43G/N45D, N45D/V58A, N45D/S75P, N45D/N78I, I20L/I36T/N45D, D43G/N45D/V58A, I20L/E27G/D43G/N45D/V58A/N78I, I20L/D43G/N45D/V58A/N78I, I20L/A33D/D43G/N45D/V58A/N78I, I20L/D43G/N45D/N78I, V11A/I20L/E27G/D43G/N45D/H51Y/S99G I20L/K28E/D43G/N45D/V58A/Q89R, I20L/I36T/N45D, A33D/D43G/N45D/V58A/S75P, K23R/D43G/N45D, D43G/N45D/L56Q/V58A/G101G-ins (G101GG), I20L/K23E/D43G/N45D/V58A/N78I, I20L/K23E/D43G/N45D/V50A/N78L I20L/K28E/D43G/N45D/V58A/Q89R/G101G-ins (G101GG), N45D, N45D/K144E, N45D/P198S, N45D/P198T, N45D/R195G, N45D/R195S, N45D/S131F, N45D/V58D, N45D/I148V/R195G, N45D/K111T/R195G, N45D/N113Y/R195S, N45D/N165Y/E170G, N45D/Q89R/I98V, N45D/S131F/P198S, N45D/S75P/P198S, N45D/V50A/R195T, E27D/N45D/T183A/I188V, K23N/N45D/S75P/N120S, N45D/I148V/R195G/N201D, N45D/K111T/T183A/I188V, N45D/Q89R/F189S/P198S, N45D/T163I/K167R/R195G, N45D/V50A/I119T/K144E, T19A/N45D/K144E/R195G, V11E/N45D/T130A/P198T, V26A/N45D/T163I/T185A, K23N/N45D/L124S/K167T/R195G, K23N/N45D/Q73R/T163I, K28R/N45D/K57E/I98V/R195S, K28R/N45D/V129D/T163N/R195T, M41K/D43G/N45D/R64S/R195G, M41K/D43G/N45D/R64S/S99G, N45D/R68L/F 1 73L/D 1 97G/P 1985, N45D/V50A/I148V/R195G/N201D,

M41K/D43G/K44E/N45D/R195G/N201D, N45D/V50A/L124S/K144E/L179P/R195G.

**[0158]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 20, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution I20L. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 27, 33, 36, 43, 50, 58, 75, 78, 99, 195 or 198. In some embodiments, the one or more amino acid modification is one or more amino acid substitutions E27G, A33D, I36T, D43G, V50A, V58A, S75P, N78I, S99G, R195G, P198S or P198T. In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications I20L/N45D.

**[0159]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 27, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution E27G. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 33, 36, 43, 50, 58, 75, 78, 99, 195 or 198.

**[0160]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 33, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution A33D. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 36, 43, 50, 58, 75, 78, 99, 195 or 198.

**[0161]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 36, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution I36T. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 43, 50, 58, 75, 78, 99, 195 or 198. In some embodiments, the one or more amino acid modification is one or more amino acid substitutions I20L, E27G, A33D, D43G, N45D, N45T, V50A, V58A, S75P, N78I, M97L, S99G, R195G, P198S or P198T, or a conservative amino acid substitution thereof. In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications I36T/N45D.

**[0162]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 43, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution D43G. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 50, 58, 75, 78, 99, 195 or 198. In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications D43G/N45D. In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications D43G/N45D/V58A.

**[0163]** In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications I20L/N45D, I36T/N45D, D43G/N45D, N45D/V58A, N45D/S75P, N45D/R195G, or N45D/P198S.

**[0164]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 50, with

reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution V50A. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 58, 75, 78, 99, 195 or 198.

**[0165]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 58, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution V58A. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 50, 75, 78, 99, 195 or 198.In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications N45D/V58D.

**[0166]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 75, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution S75P. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 50, 58, 78, 99, 195 or 198.

**[0167]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 78, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution N78I. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 50, 58, 75, 99, 195 or 198. In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications N45D/N78I.

**[0168]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 99, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution S99G. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 50, 58, 75, 78, 195 or 198.

**[0169]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 195, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution R195G. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 50, 58, 75, 78, 99 or 198. In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications N45D/R195G,.

**[0170]** In some embodiments, the variant PD-L1 polypeptide comprises amino acid modifications in an unmodified PD-L1 or specific binding fragment thereof at a position corresponding to position 198, with reference to numbering of positions set forth in SEQ ID NO:30. In some embodiments, the amino acid modification is the amino acid substitution P198S or P198T. In some embodiments, the variant PD-L1 polypeptide further contains one or more amino acid modifications, e.g. amino acid substitutions, at one or more positions 20, 27, 33, 36, 43, 50, 58, 75, 78, 99 or 195.In some embodiments, the variant PD-L1 polypeptide comprises the amino acid modifications N45D/P198S, or N45D/P198T..

**[0171]** In some embodiments, the variant PD-L1 polypeptide comprises any of the substitutions (mutations) listed in Table 1, which comprise N45D. Table 1 also provides exemplary sequences by reference to SEQ ID NO for the extracellular domain (ECD) or IgV domain of wild-type PD-L1 or exemplary variant PD-L1 polypeptides. As indicated, the exact locus or residues corresponding to a given domain can vary, such as depending on the methods used to identify or classify the domain. Also, in some cases, adjacent N- and/or C-terminal amino acids of a given domain (e.g. ECD or IgV) also can be included in a sequence of a variant IgSF polypeptide, such as to ensure proper folding of the domain when expressed. Thus, it is understood that the exemplification of the SEQ ID NOSs in Table 1 is not to be construed as limiting. For example, the particular domain, such as the ECD or IgV domain, of a variant PD-L1 polypeptide can be several amino acids longer or shorter, such as 1-10, e.g. 1, 2, 3, 4, 5, 6 or 7 amino acids longer or shorter, than the sequence of amino acids set forth in the respective SEQ ID NO.

**TABLE 1: Exemplary variant PD-L1 polypeptides**

| Mutation(s) | ECD SEQ ID NO | IgV SEQ ID NO |
|---|---|---|
| Wild-type | 30, 1728 | 55, 309 |
| K28N/M41V/N45T/H51N/K57E | 56, 1943 | 121, 244 |
| I20L/I36T/N45D/I47T | 57, 1944 | 122, 245 |
| I20L/M41K/K44E | 58, 1945 | 123, 246 |
| P6S/N45T/N78I/I83T | 59, 1946 | 124, 247 |
| N78I | 60, 1947 | 125, 248 |
| M41K/N78I | 61, 1948 | 126, 249 |
| N45T/N78I | 62, 1949 | 127, 250 |
| I20L/N45T | 63, 1950 | 128, 251 |
| N45T | 64, 1951 | 129, 252 |
| M41K | 65, 1952 | 130, 253 |
| I20L/I36T/N45D | 66, 1953 | 131, 254 |
| N17D/N45T/V50A/D72G | 67, 1954 | 132, 255 |
| I20L/F49S | 68, 1955 | 133, 256 |
| N45T/V50A | 69, 1956 | 134, 257 |
| I20L/N45T/N78I | 70, 1957 | 135, 258 |
| I20L/N45T/V50A | 71, 1958 | 136, 259 |
| M41V/N45T | 72, 1959 | 137, 260 |
| M41K/N45T | 73, 1960 | 138, 261 |
| A33D/S75P/D85E | 74, 1961 | 139, 262 |
| M18I/M41K/D43G/H51R/N78I | 75, 1962 | 140, 263 |
| V11E/I20L/I36T/N45D/H60R/S75P | 76, 1963 | 141, 264 |
| A33D/V50A | 77, 1964 | 142, 265 |
| S16G/A33D/K71E/S75P | 78, 1965 | 143, 266 |
| E27G/N45T/M97I | 79, 1966 | 144, 267 |
| E27G/N45T/K57R | 80, 1967 | 145, 268 |
| A33D/E53V | 81, 1968 | 146, 269 |
| D43G/N45D/V58A | 82, 1969 | 147, 270 |

| TABLE 1: Exemplary variant PD-L1 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| E40G/D43V/N45T/V50A | 83, 1970 | 148, 271 |
| Y14S/K28E/N45T | 84, 1971 | 149, 272 |
| A33D/N78S | 85, 1972 | 150, 272 |
| A33D/N78I | 86, 1973 | 151, 274 |
| A33D/N45T | 87, 1974 | 152, 275 |
| A33D/N45T/N78I | 88, 1975 | 153, 276 |
| E27G/N45T/V50A | 89, 1976 | 154, 277 |
| N45T/V50A/N78S | 90, 1977 | 155, 278 |
| I20L/N45T/V110M | 91, 1978 | 156, 279 |
| I20L/I36T/N45T/V50A | 92, 1979 | 157, 280 |
| N45T/L74P/S75P | 93, 1980 | 158, 281 |
| N45T/S75P | 94, 1981 | 159, 282 |
| S75P/K106R | 95, 1982 | 160, 283 |
| S75P | 96, 1983 | 161, 284 |
| A33D/S75P | 97, 1984 | 162, 285 |
| A33D/S75P/D104G | 98, 1985 | 163, 286 |
| A33D/S75P | 99, 1986 | 164, 287 |
| I20L/E27G/N45T/V50A | 100, 1987 | 165, 288 |
| I20L/E27G/D43G/N45D/V58A/N78I | 101, 1988 | 166, 289 |
| I20L/D43G/N45D/V58A/N78I | 102, 1989 | 167, 290 |
| I20L/A33D/D43G/N45D/V58A/N78I | 103, 1990 | 168, 291 |
| I20L/D43G/N45D/N78I | 104, 1991 | 169, 292 |
| E27G/N45T/V50A/N78I | 105, 1992 | 170, 293 |
| N45T/V50A/N78I | 106, 1993 | 171,294 |
| V11A/I20L/E27G/D43G/N45D/H51Y/S99G | 107, 1994 | 172, 295 |
| I20L/E27G/D43G/N45TN 50A | 108, 1995 | 173, 296 |
| I20L/K28E/D43G/N45D/V58A/Q89R | 109, 1996 | 174, 297 |
| I20L/I36T/N45D | 110, 1997 | 175, 298 |
| I20L/K28E/D43G/N 45D/E5 3GN 5 8A/N78I | 111, 1998 | 176, 299 |
| A33D/D43G/N45D/V58A/S75P | 112, 1999 | 177, 300 |
| K23R/D43G/N45D | 113, 2000 | 178, 301 |
| I20L/D43G/N45D/V58A/N78I/D90G/G101D | 114,2001 | 179, 302 |
| D43G/N45D/L56Q/V58A/ G101G-ins (G101GG) | 115, 2002 | 180, 303 |
| I20L/K23E/D43G/N45D/V58A/N78I | 116, 2003 | 181, 304 |
| I20L/K23E/D43G/N45DN 50A/N78I | 117, 2004 | 182, 305 |
| T19I/E27G/N45I/V50A/N78I/M97K | 118, 2005 | 183, 306 |
| I20L/M41K/D43G/N45D | 119, 2006 | 184, 307 |
| K23R/N45T/N78I | 120,2007 | 185, 308 |

| TABLE 1: Exemplary variant PD-L1 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| I20L/K28E/D43G/N45D/V58A/Q89R/G101G-ins (G101GG) | 1725, 2008 | 1726, 1727 |
| K57R/S99G | 1729, 1819 | 1908, 1923 |
| K57R/S99G/F189L | 1730, 1820 | |
| M18V/M97L/F193S/R195G/E200K/H202Q | 1731, 1821 | |
| I36S/M41K/M97L/K144Q/R195G/E200K/H202Q/L206F | 1732, 1822 | |
| C22R/Q65L/L124S/K144Q/R195G/E200N/H202Q/T221L | 1733 | |
| M18V/I98L/L124S/P198T/L206F | 1734, 1823 | |
| S99G/N117S/I148V/K171R/R180S | 1735, 1824 | |
| I36T/M97L/A103V/Q155H | 1736, 1825 | |
| K28I/S99G | 1737, 1826 | 1909, 1924 |
| R195S | 1738, 1827 | |
| A79T/S99G/T185A/R195G/E200K/H202Q/L206F | 1739, 1828 | |
| K57R/S99G/L124S/K144Q | 1740, 1829 | |
| K57R/S99G/R195G | 1741, 1830 | |
| D55V/M97L/S99G | 1742, 1831 | 1910, 1925 |
| E27G/I36T/D55N/M97L/K111E | 1743, 1832 | 1911, 1926 |
| E54G/M97L/S99G | 1744, 1833 | 1912, 1927 |
| G15A/I36T/M97L/K111E/H202Q | 1745, 1834 | |
| G15A/I36T/V129D | 1746, 1835 | |
| G15A/I36T/V129D/R195G | 1747, 1836 | |
| G15A/V129D | 1748, 1837 | |
| I36S/M97L | 1749, 1838 | 1913, 1928 |
| I36T/D55N/M97L/K111E/A204T | 1750, 1839 | |
| I36T/DSSN/M97L/K111E/V 129A/F173L | 1751, 1840 | |
| I36T/DSSS/M97L/K111E/I148V/R180S | 1752, 1841 | |
| I36T/G52R/M97L/V112A/K144E/V175A/P198T | 1753, 1842 | |
| I36T/I46V/D55G/M97L/K106E/K144E/T185A/R195G | 1754, 1843 | |
| I36T/I83T/M97L/K144E/P198T | 1755, 1844 | |
| I36T/M97L/K111E | 1756, 1845 | 1914, 1929 |
| I36T/M97L/K144E/P198T | 1757, 1846 | |
| I36T/M97L/Q155H/F193S/N201Y | 1758, 1847 | |
| I36T/M97L/V129D | 1759, 1848 | |
| L35P/I36S/M97L/K111E | 1760, 1849 | 1915, 1930 |
| M18I/I36T/E53G/M97L/K144E/E199G/V207A | 1761, 1850 | |
| M18T/I36T/DSSN/M97L/K111E | 1762, 1851 | 1916, 1931 |
| M18V/M97L/T176N/R195G | 1763, 1852 | |
| M97L/S99G | 1764, 1853 | 1917, 1932 |

| TABLE 1: Exemplary variant PD-L1 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| N17D/M97L/S99G | 1765, 1854 | 1918, 1933 |
| S99G/T185A/R195G/P198T | 1766, 1855 | |
| V129D/H202Q | 1767, 1856 | |
| V129D/P198T | 1768, 1857 | |
| V129D/T150A | 1769, 1858 | |
| V93E/V129D | 1770, 1859 | |
| Y10F/M18V/S99G/Q138R/T203A | 1771, 1860 | |
| N45D | 1772, 1861 | 1919, 1934 |
| K160M/R195G | 1773, 1862 | |
| N45D/K144E | 1774, 1863 | |
| N45D/P198S | 1775, 1864 | |
| N45D/P198T | 1776, 1865 | |
| N45D/R195G | 1777, 1866 | |
| N45D/R195S | 1778, 1867 | |
| N45D/S131F | 1779, 1868 | |
| N45D/V58D | 1780, 1869 | 1920, 1935 |
| V129D/R195S | 1781, 1870 | |
| I98T/F173Y/L196S | 1782, 1871 | |
| N45D/E134G/L213P | 1783, 1872 | |
| N45D/F173I/S177C | 1784, 1873 | |
| N45D/I148V/R195G | 1785, 1874 | |
| N45D/K111T/R195G | 1786, 1875 | |
| N45D/N113Y/R195S | 1787, 1876 | |
| N45D/N165Y/E170G | 1788, 1877 | |
| N45D/Q89R/I98V | 1789, 1878 | 1921, 1936 |
| N45D/S131F/P198S | 1790, 1879 | |
| N45D/S75P/P198S | 1791, 1880 | |
| N45D/V50A/R195T | 1792, 1881 | |
| E27D/N45D/T183A/I188V | 1793, 1882 | |
| F173Y/T183I/L196S/T203A | 1794, 1883 | |
| K23N/N45D/S75P/N120S | 1795, 1884 | |
| N45D/G102D/R194W/R195G | 1796, 1885 | |
| N45D/G52V/Q121L/P198S | 1797, 1886 | |
| N45D/I148V/R195G/N201D | 1798, 1887 | |
| N45D/K111T/T183A/I188V | 1799, 1888 | |
| N45D/Q89R/F189S/P198S | 1800, 1889 | |
| N45D/S99G/C137R/V207A | 1801, 1890 | |
| N45D/T163I/K167R/R195G | 1802, 1891 | |

| TABLE 1: Exemplary variant PD-L1 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| N45D/T183A/T192S/R194G | 1803, 1892 | |
| N45D/V50A/I119T/K144E | 1804, 1893 | |
| T19A/N45D/K144E/R195G | 1805, 1894 | |
| V11E/N45D/T130A/P198T | 1806, 1895 | |
| V26A/N45D/T163I/T185A | 1807, 1896 | |
| K23N/N45D/L124S/K167T/R195G | 1808, 1897 | |
| K23N/N45D/Q73R/T163I | 1809, 1898 | |
| K28E/N45D/W149R/S158G/P198T | 1810, 1899 | |
| K28R/N45D/K57E/I98V/R195S | 1811, 1900 | |
| K28R/N45D/V129D/T163N/R195T | 1812, 1901 | |
| M41K/D43G/N45D/R64S/R195G | 1813, 1902 | |
| M41K/D43G/N45D/R64S/S99G | 1814, 1903 | 1922, 1937 |
| N45D/R68L/F173L/D197G/P198S | 1815, 1904 | |
| N45D/V50A/I148V/R195G/N201D | 1816, 1905 | |
| M41K/D43G/K44E/N45D/R195G/N201D | 1817, 1906 | |
| N45D/V50A/L124S/K144E/L179P/R195G | 1818, 1907 | |

**[0172]** In some embodiments, the variant PD-L1 polypeptide exhibits increased affinity for the ectodomain of PD-1 compared to the wild-type or unmodified PD-L1 polypeptide, comprising the sequence set forth in SEQ ID NO: 30, 1728, 55 or 309. In some embodiments, the PD-L1 polypeptide exhibits increased affinity for the ectodomain of CD80 compared to the wild-type or unmodified PD-L1, such as comprising the sequence set forth in SEQ ID NO: 30, 1728, 55 or 309. In some embodiments, the PD-L1 polypeptide exhibits increased affinity for the ectodomain of PD-1 and the ectodomain of CD80 compared to the wild-type or unmodified PD-L1, comprising the sequence set forth in SEQ ID NO: 30, 1728, 55 or 309.

**[0173]** In some embodiments, the variant PD-L1 polypeptide exhibits increased affinity for the ectodomain of PD-1, and decreased affinity for the ectodomain of CD80, compared to unmodified PD-L1 polypeptide, comprising the sequence set forth in SEQ ID NO: 30, 1728, 55 or 309.

**[0174]** In some embodiments, a variant PD-L1 polypeptide exhibits increased selectivity for PD-1 versus CD80 compared to the ratio of binding of the unmodified PD-L1 polypeptide (e.g. set forth in SEQ ID NO: 30, 55 or 309) for binding PD-1 versus CD80, such as indicated by a ratio of PD-1binding to CD80 binding (PD-ECD80 binding ratio) that is greater than 1. In some embodiments, the variant PD-L1 polypeptide exhibits a ratio of binding PD-1 versus CD80 that is greater than or greater than about or 1.1, 1.2, 1.3, 1.4, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, or more.

### III. FORMAT OF VARIANT POLYPEPTIDES

**[0175]** The immunomodulatory polypeptide comprising a variant PD-L1 provided herein in which is contained a vIgD can be formatted in a variety of ways, including as a soluble protein, membrane bound

protein or secreted protein. In some embodiments, the particular format can be chosen for the desired therapeutic application. In some cases, an immunomodulatory polypeptide comprising a variant PD-L1 polypeptide is provided in a format to antagonize or block activity of its cognate binding partner, e.g. PD-1. In some embodiments, antagonism of PD-1 may be useful to promote immunity in oncology. In some cases, an immunomodulatory polypeptide comprising a variant PD-L1 polypeptide is provided in a format to agonize or stimulate activity of its cognate binding partner, e.g. PD-1. In some embodiments, agonism of PD-1 may be useful for treating inflammation or autoimmunity. A skilled artisan can readily determine the activity of a particular format, such as for antagonizing or agonizing one or more specific cognate binding partner. Exemplary methods for assessing such activities are provided herein, including in the examples.

**[0176]** In some aspects, provided are immunomodulatory proteins comprising a vIgD of PD-L1 in which such proteins are soluble, e.g. fused to an Fc chain. In some aspects, one or more additional IgSF domain, such as one or more additional vIgD, may be linked to a vIgD of PD-L1 as provided herein (hereinafter called a "stack" or "stacked" immunomodulatory protein). In some embodiments, the modular format of the provided immunomodulatory proteins provides flexibility for engineering or generating immunomodulatory proteins for modulating activity of multiple counterstrucutres (multiple cognate binding partners). In some embodiments, such "stack" molecules can be provided in a soluble format or, in some cases, may be provided as membrane bound or secreted proteins. In some embodiments, a variant PD-L1 immunomodulatory protein is provided as a conjugate in which is contained a vIgD of PD-L1 linked, directly or indirectly, to a targeting agent or moiety, e.g. to an antibody or other binding molecules that specifically binds to a ligand, e.g. an antigen, for example, for targeting or localizing the vIgD to a specific environment or cell, such as when administered to a subject. In some embodiments, the targeting agent, e.g. antibody or other binding molecule, binds to a tumor antigen, thereby localizing the variant PD-L1 containing the vIgD to the tumor microenvironment, for example, to modulate activity of tumor infiltrating lymphocytes (TILs) specific to the tumor microenvironment. In some embodiments, the targeting agent, e.g. antibody or other binding molecule, binds to an antigen expressed on antigen presenting cells or normal tissue in an inflammatory environment, thereby localizing the variant PD-L1 containing the vIgD to areas of unwanted autoimmune inflammation, for example, to modulate activity of T cells targeting self antigen.

**[0177]** In some embodiments, provided immunomodulatory proteins are expressed in cells and provided as part of an engineered cellular therapy (ECT). In some embodiments, the variant PD-L1 polypeptide is expressed in a cell, such as an immune cell (e.g. T cell or antigen presenting cell), in membrane-bound form, thereby providing a transmembrane immunomodulatory protein (hereinafter also called a "TIP"). In some embodiments, depending on the cognate binding partner recognized by the TIP, engineered cells expressing a TIP can agonize a cognate binding partner by providing a costimulatory signal, either positive to negative, to other engineered cells and/or to endogenous T cells. In some aspects, the variant PD-L1 polypeptide is expressed in a cell, such as an immune cell (e.g. T cell or antigen presenting cell), in secretable form to thereby produce a secreted or soluble form of the variant PD-L1 polypeptide (hereinafter also called a "SIP"), such as when the cells are administered to a subject. In some aspects, a SIP can antagonize a cognate binding partner in the environment (e.g. tumor microenvironment) in which it is secreted. In some embodiments, a variant PD-L1 polypeptide is expressed in an infectious agent (e.g. viral or bacterial agent) which, upon administration to a subject, is able to infect a cell in vivo, such as an immune cell (e.g. T cell or antigen presenting cell), for delivery or expression of the variant polypeptide as a TIP or a SIP in the cell.

**[0178]** In some embodiments, a soluble immunomodulatory polypeptide, such as a variant PD-L1 containing a vIgD, can be encapsulated within a liposome which itself can be conjugated to any one of or any combination of the provided conjugates (e.g., a targeting moiety). In some embodiments, the soluble or membrane bound immunomodulatory polypeptides of the invention are deglycosylated. In more specific embodiments, the variant PD-L1 sequence is deglycosylated. In even more specific embodiments, the IgV

and/or IgC (e.g. IgC2) domain or domains of the variant PD-L1 is deglycosylated.

**[0179]** Non-limiting examples of provided formats are described in FIGS. 1A-1C and further described below.

**A. Soluble Protein**

**[0180]** In some embodiments, the immunomodulatory protein containing a variant PD-L1 polypeptide is a soluble protein. Those of skill will appreciate that cell surface proteins typically have an intracellular, transmembrane, and extracellular domain (ECD) and that a soluble form of such proteins can be made using the extracellular domain or an immunologically active subsequence thereof. Thus, in some embodiments, the immunomodulatory protein containing a variant PD-L1 polypeptide lacks a transmembrane domain or a portion of the transmembrane domain. In some embodiments, the immunomodulatory protein containing a variant PD-L1 lacks the intracellular (cytoplasmic) domain or a portion of the intracellular domain. In some embodiments, the immunomodulatory protein containing the variant PD-L1 polypeptide only contains the vIgD portion containing the ECD domain or a portion thereof containing an IgV domain and optionallyr IgC (e.g. IgC2) domain or domainscontaining the amino acid modification(s).

**[0181]** In some embodiments, the immunomodulatory protein is or contains a variant PD-L1 polypeptide that is in monomer form and/or that exhibits monovalent binding to its binding partner. In some aspects, a variant PD-L1 polypeptide as described, such as a variant PD-L1 that is soluble and/or that lacks a transmembrane domain and intracellular signaling domain, is linked, directly or indirectly, to a further moiety. In some embodiments, the further moiety is a protein, peptide, small molecule or nucleic acid. In some embodiments, the monovalent immunomodulatory protein is a fusion protein. In some embodiments, the moiety is a half-life extending molecule. Exemplary of such half-life extending molecules include, but are not limited to, albumin, an albumin-binding polypeptide, Pro/Ala/Ser (PAS), a C-terminal peptide (CTP) of the beta subunit of human chorionic gonadotropin, polyethylene glycol (PEG), long unstructured hydrophilic sequences of amino acids (XTEN), hydroxyethyl starch (HES), an albumin-binding small molecule, or a combination thereof.

**[0182]** In some embodiments, the immunomodulatory polypeptide comprising a variant PD-L1 can be linked to a moiety that includes conformationally disordered polypeptide sequences composed of the amino acids Pro, Ala, and Ser (See e.g., WO2008/155134, SEQ ID NO: 2025). In some cases, the amino acid repeat is at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more amino acid residues, wherein each repeat comprises (an) Ala, Ser, and Pro residue(s). Thus, provided herein is an immunomodulatory protein is a PASylated protein wherein the variant PD-L1 polypeptide is linked, directly or indirectly via a linker, to Pro/Ala/Ser (PAS). In some embodiments, one or more additional linker structures may be used.

**[0183]** In some embodiments, the moiety facilitates detection or purification of the variant PD-L1 polypeptide. In some cases, the immunomodulatory polypeptide comprises a tag or fusion domain, e.g. affinity or purification tag, linked, directly or indirectly, to the N- and/or c-terminus of the PD-L1 polypeptide. Various suitable polypeptide tags and/or fusion domains are known, and include but are not limited to, a poly-histidine (His) tag, a FLAG-tag (SEQ ID NO: 2010), a Myc-tag, and fluorescent protein-tags (e.g., EGFP, set forth in SEQ ID NOs: 2027-2029). In some cases, the immunomodulatory polypeptide comprising a variant PD-L1 comprises at least six histidine residues (set forth in SEQ ID NO: 2011). In some cases, the immunomodulatory polypeptide comprising a variant PD-L1 further comprises various

combinations of moieties. For example, the immunomodulatory polypeptide comprising a variant PD-L1 further comprises one or more polyhistidine-tag and FLAG tag.

**[0184]** In some embodiments, the PD-L1 polypeptide is linked to a modified immunoglobulin heavy chain constant region (Fc) that remains in monovalent form such as set forth in SEQ ID NO: 1187.

**[0185]** In some embodiments, the immunomodulatory protein contains a variant PD-L1 polypeptide that is linked, directly or indirectly via a linker to a multimerization domain. In some aspects, the multimerization domain inreases half-life of the molecule. Interaction of two or more variant PD-L1 polypeptides can be facilitated by their linkage, either directly or indirectly, to any moiety or other polypeptide that are themselves able to interact to form a stable structure. For example, separate encoded variant PD-L1 polypeptide chains can be joined by multimerization, whereby multimerization of the polypeptides is mediated by a multimerization domain. Typically, the multimerization domain provides for the formation of a stable protein-protein interaction between a first variant PD-L1 polypeptide and a second variant PD-L1 polypeptide.

**[0186]** Homo- or heteromultimeric polypeptides can be generated from co-expression of separate variant PD-L1 polypeptides. The first and second variant PD-L1 polypeptides can be the same or different. In particular embodiments, the first and second variant PD-L1 polypeptide are the same in a homodimer, and each are linked to a multimerization domain that is the same. In other embodiments, heterodimers can be formed by linking first and second variant PD-L1 polypeptides that are different. In such embodiments, in some aspects the first and second variant PD-L1 polypeptide are linked to different multimerization domains capable of promoting heterodimer formation.

**[0187]** In some embodiments, a multimerization domain includes any capable of forming a stable protein-protein interaction. The multimerization domains can interact via an immunoglobulin sequence (e.g. Fc domain; see e.g., International Patent Pub. Nos. WO 93/10151 and WO 2005/063816 US; U.S. Pub. No. 2006/0024298; U.S. Pat. No. 5,457,035); leucine zipper (e.g. from nuclear transforming proteins fos and jun or the proto-oncogene c-myc or from General Control of Nitrogen (GCN4)) (ee e.g., Busch and Sassone-Corsi (1990) Trends Genetics, 6:36-40; Gentz et al., (1989) Science, 243:1695-1699); a hydrophobic region; a hydrophilic region; or a free thiol which forms an intermolecular disulfide bond between the chimeric molecules of a homo- or heteromultimer. In addition, a multimerization domain can include an amino acid sequence comprising a protuberance complementary to an amino acid sequence comprising a hole, such as is described, for example, in U.S. Pat. No. 5,731,168; International Patent Pub. Nos. WO 98/50431 and WO 2005/063816; Ridgway et al. (1996) Protein Engineering, 9:617-621. Such a multimerization region can be engineered such that steric interactions not only promote stable interaction, but further promote the formation of heterodimers over homodimers from a mixture of chimeric monomers. Generally, protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g., tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are optionally created on the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). Exemplary multimerization domains are described below.

**[0188]** The variant PD-L1 polypeptide can be joined anywhere, but typically via its N- or C-terminus, to the N- or C-terminus of a multimerization domain to form a chimeric polypeptide. The linkage can be direct or indirect via a linker. The chimeric polypeptide can be a fusion protein or can be formed by chemical linkage, such as through covalent or non-covalent interactions. For example, when preparing a chimeric polypeptide containing a multimerization domain, nucleic acid encoding all or part of a variant PD-L1 polypeptide can be operably linked to nucleic acid encoding the multimerization domain sequence, directly or indirectly or optionally via a linker domain. In some cases, the construct encodes a chimeric protein

where the C-terminus of the variant PD-L1 polypeptide is joined to the N-terminus of the multimerization domain. In some instances, a construct can encode a chimeric protein where the N-terminus of the variant PD-L1 polypeptide is joined to the C-terminus of the multimerization domain.

**[0189]** A polypeptide multimer contains multiple, such as two, chimeric proteins created by linking, directly or indirectly, two of the same or different variant PD-L1 polypeptides directly or indirectly to a multimerization domain. In some examples, where the multimerization domain is a polypeptide, a gene fusion encoding the variant PD-L1 polypeptide and multimerization domain is inserted into an appropriate expression vector. The resulting chimeric or fusion protein can be expressed in host cells transformed with the recombinant expression vector, and allowed to assemble into multimers, where the multimerization domains interact to form multivalent polypeptides. Chemical linkage of multimerization domains to variant PD-L1 polypeptides can be effected using heterobifunctional linkers.

**[0190]** The resulting chimeric polypeptides, such as fusion proteins, and multimers formed therefrom, can be purified by any suitable method such as, for example, by affinity chromatography over Protein A or Protein G columns. Where two nucleic acid molecules encoding different polypeptides are transformed into cells, formation of homo- and heterodimers will occur. Conditions for expression can be adjusted so that heterodimer formation is favored over homodimer formation.

**[0191]** In some embodiments, the multimerization domain is an Fc domain or portions thereof from an immunoglobulin. In some embodiments, the immunomodulatory protein comprises a variant PD-L1 polypeptide attached to an immunoglobulin Fc (yielding an "immunomodulatory Fc fusion," such as a "PD-L1-Fc variant fusion," also termed a PD-L1 vIgD-Fc fusion). In some embodiments, the attachment of the variant PD-L1 polypeptide is at the N-terminus of the Fc. In some embodiments, the attachment of the variant PD-L1 polypeptide is at the C-terminus of the Fc. In some embodiments, two or more PD-L1 variant polypeptides (the same or different) are independently attached at the N-terminus and at the C-terminus.

**[0192]** In some embodiments, the Fc is murine or human Fc. In some embodiments, the Fc is a mammalian or human IgGl, IgG2, IgG3, or IgG4 Fc regions. In some embodiments, the Fc is derived from IgG1, such as human IgG1. In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 187 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 187.

**[0193]** In some embodiments, the Fc region contains one more modifications to alter (e.g. reduce) one or more of its normal functions. In general, the Fc region is responsible for effector functions, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC), in addition to the antigen-binding capacity, which is the main function of immunoglobulins. In some cases, effector functions of an Fc region can include programmed cell death and cellular phagocytosis. Additionally, the FcRn sequence present in the Fc region plays the role of regulating the IgG level in serum by increasing the in vivo half-life by conjugation to an in vivo FcRn receptor. In some embodiments, such functions can be reduced or altered in an Fc for use with the provided Fc fusion proteins.

**[0194]** In some embodiments, one or more amino acid modifications may be introduced into the Fc region of a PD-L1-Fc variant fusion provided herein, thereby generating an Fc region variant. In some embodiments, the Fc region variant has decreased effector function. There are many examples of changes or mutations to Fc sequences that can alter effector function. For example, WO 00/42072, WO2006019447, WO2012125850, WO2015/107026, US2016/0017041 and Shields et al. J Biol. Chem. 9(2): 6591-6604 (2001) describe exemplary Fc variants with improved or diminished binding to FcRs.

**[0195]** In some embodiments, the provided variant PD-L1-Fc fusions comprise an Fc region that exhibits

reduced effector functions, which makes it a desirable candidate for applications in which the half-life of the PD-L1-Fc variant fusion *in vivo* is important yet certain effector functions (such as CDC and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the PD-L1-Fc variant fusion lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 2 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9:457-492 (1991). Non-limiting examples *of in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Pat. No. 5,500,362 (see, *e.g.* Hellstrom, I. et al. Proc. Nat'l Acad. Sci. USA 83:7059-7063 (1986)) and Hellstrom, I et al., Proc. Nat'l Acad. Sci. USA 82:1499-1502 (1985); U.S. Pat. No. 5,821,337 (see Bruggemann, M. et al., J. Exp. Med. 166:1351-1361 (1987)). Alternatively, non-radioactive assay methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, Calif.; and CytoTox 96™ non-radioactive cytotoxicity assay (Promega, Madison, Wis.). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo, e.g.,* in an animal model such as that disclosed in Clynes et al. Proc. Nat'l Acad. Sci. USA 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the PD-L1-Fc variant fusion is unable to bind C1q and hence lacks CDC activity. See, *e.g.,* C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996); Cragg, M. S. et al., Blood 101:1045-1052 (2003); and Cragg, M. S. and M. J. Glennie, Blood 103:2738-2743 (2004)). FcRn binding and *in vivo* clearance/half-life determinations can also be performed using methods known in the art (see, *e.g.,* Petkova, S. B. et al., Int'l. Immunol. 18(12):1759-1769 (2006)).

**[0196]** PD-L1-Fc variant fusions with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 by EU numbering (U.S. Pat. No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327 by EU numbering, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Pat. No. 7,332,581).

**[0197]** In some embodiments, the Fc region of PD-L1-Fc variant fusions has an Fc region in which any one or more of amino acids at positions 234, 235, 236, 237, 238, 239, 270, 297, 298, 325, and 329 (indicated by EU numbering) are substituted with different amino acids compared to the native Fc region. Such alterations of Fc region are not limited to the above-described alterations, and include, for example, alterations such as deglycosylated chains (N297A and N297Q), IgG1-N297G, IgG1-L234A/L235A, IgG1-L234A/L235E/G237A, IgG1-A325A/A330S/P331S, IgG1-C226S/C229S, IgG1-C226S/C229S/E233P/L234V/L235A, IgG1-E233P/L234V/L235A/G236del/ S267K, IgG1-L234F/L235E/P331S, IgG1-S267E/L328F, IgG2-V234A/G237A, IgG2-H268Q/V309L/A330S/A331S, IgG4-L235A/G237A/E318A, and IgG4-L236E described in Current Opinion in Biotechnology (2009) 20 (6), 685-691; alterations such as G236R/L328R, L235G/G236R, N325A/L328R, and N325LL328R described in WO 2008/092117; amino acid insertions at positions 233, 234, 235, and 237 (indicated by EU numbering); and alterations at the sites described in WO 2000/042072.

**[0198]** Certain Fc variants with improved or diminished binding to FcRs are described. (See, *e.g.,* U.S. Pat. No. 6,737,056; WO 2004/056312, WO2006019447 and Shields et al., J. Biol. Chem. 9(2): 6591-6604 (2001).)

**[0199]** In some embodiments, there is provided a PD-L1-Fc variant fusion comprising a variant Fc region

comprising one or more amino acid substitutions which increase half-life and/or improve binding to the neonatal Fc receptor (FcRn). Antibodies with increased half-lives and improved binding to FcRn are described in US2005/0014934A1 (Hinton et al.) or WO2015107026. Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434 by EU numbering, e.g., substitution of Fc region residue 434 (U.S. Pat. No. 7,371,826).

**[0200]** In some embodiments, the Fc region of a PD-L1-Fc variant fusion comprises one or more amino acid substitution E356D and M358L by EU numbering. In some embodiments, the Fc region of a PD-L1-Fc variant fusion comprises one or more amino acid substitutions C220S, C226S and/or C229S by EU numbering. In some embodiments, the Fc region of a PD-L1 variant fusion comprises one or more amino acid substitutions R292C and V302C. *See* also Duncan & Winter, Nature 322:738-40 (1988); U.S. Pat. No. 5,648,260; U.S. Pat. No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

**[0201]** In some embodiments, alterations are made in the Fc region that result in diminished C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in U.S. Pat. No. 6,194,551, WO 99/51642, and Idusogie et al., J. Immunol. 164: 4178-4184 (2000).

**[0202]** In some embodiments, there is provided a PD-L1-Fc variant fusion comprising a variant Fc region comprising one or more amino acid modifications, wherein the variant Fc region is derived from IgG1, such as human IgG1. In some embodiments, the variant Fc region is derived from the amino acid sequence set forth in SEQ ID NO: 187. In some embodiments, the Fc contains at least one amino acid substitution that is N82G by numbering of SEQ ID NO: 187 (corresponding to N297G by EU numbering). In some embodiments, the Fc further contains at least one amino acid substitution that is R77C or V87C by numbering of SEQ ID NO: 187 (corresponding to R292C or V302C by EU numbering). In some embodiments, the variant Fc region further comprises a C5S amino acid modification by numbering of SEQ ID NO: 187 (corresponding to C220S by EU numbering). For example, in some embodiments, the variant Fc region comprises the following amino acid modifications: V297G and one or more of the following amino acid modifications C220S, R292C or V302C by EU numbering (corresponding to N82G and one or more of the following amino acid modifications C5S, R77C or V87C with reference to SEQ ID NO:187), e.g., the Fc region comprises the sequence set forth in SEQ ID NO:1157. In some embodiments, the variant Fc region comprises one or more of the amino acid modifications C220S, L234A, L235E or G237A, e.g. the Fc region comprises the sequence set forth in SEQ ID NO:1158. In some embodiments, the variant Fc region comprises one or more of the amino acid modifications C220S, L235P, L234V, L235A, G236del or S267K, e.g. the Fc region comprises the sequence set forth in SEQ ID NO:1159. In some embodiments, the variant Fc comprises one or more of the amino acid modifications C220S, L234A, L235E, G237A, E356D or M358L, e.g. the Fc region comprises the sequence set forth in SEQ ID NO:1155.

**[0203]** In some embodiments, the Fc region lacks the C-terminal lysine corresponding to position 232 of the wild-type or unmodified Fc set forth in SEQ ID NO: 187 (corresponding to K447del by EU numbering). In some aspects, such an Fc region can additionally include one or more additional modifications, e.g. amino acid substitutions, such as any as described. Exemplary of such an Fc region is set forth in SEQ ID NO: 1938, 1939, 1940, or 1715.

**[0204]** In some embodiments, there is provided a PD-L1-Fc variant fusion comprising a variant Fc region in which the variant Fc comprises the sequence of amino acids set forth in any of SEQ ID NOS:1155, 1157, 1158, 1159, 1715, 1938, 1939, or 1940 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 1155, 1157, 1158, 1159, 1715, 1938, 1939, or 1940.

**[0205]** In some embodiments, the Fc is derived from IgG2, such as human IgG2. In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 188 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 188.

**[0206]** In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 1200 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 1200. In some embodiments, the IgG4 Fc is a stabilized Fc in which the CH3 domain of human IgG4 is substituted with the CH3 domain of human IgG1 and which exhibits inhibited aggregate formation, an antibody in which the CH3 and CH2 domains of human IgG4 are substituted with the CH3 and CH2 domains of human IgG1, respectively, or an antibody in which arginine at position 409 indicated in the EU index proposed by Kabat et al. of human IgG4 is substituted with lysine and which exhibits inhibited aggregate formation (see e.g. U.S. Patent No. 8,911,726. In some embodiments, the Fc is an IgG4 containing the S228P mutation, which has been shown to prevent recombination between a therapeutic antibody and an endogenous IgG4 by Fab-arm exchange (see e.g. Labrijin et al. (2009) Nat. Biotechnol., 27(8): 767-71.) In some embodiments, the Fc comprises the amino acid sequence set forth in SEQ ID NO: 1201 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 1201.

**[0207]** In some embodiments, the variant PD-L1 polypeptide is directly linked to the Fc sequence. In some embodiments, the variant PD-L1 polypeptide is indirectly linked to the Fc sequence, such as via a linker. In some embodiments, one or more "peptide linkers" link the variant PD-L1 polypeptide and the Fc domain. In some embodiments, a peptide linker can be a single amino acid residue or greater in length. In some embodiments, the peptide linker has at least one amino acid residue but is no more than 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues in length. In some embodiments, the linker is three alanines (AAA). In some embodiments, the linker is a flexible linker. In some embodiments, the linker is (in one-letter amino acid code): GGGGS ("4GS" or "$G_4S$"; SEQ ID NO: 1942) or multimers of the 4GS linker, such as repeats of 2, 3, 4, or 5 4GS linkers, such as set forth in SEQ ID NO: 240 (2xGGGGS) or SEQ ID NO:239 (3xGGGGS). In some embodiments, the linker (in one-letter amino acid code) is GSGGGGS (SEQ ID NO:1941). In some embodiments, the linker also can include a series of alanine residues alone or in addition to another peptide linker (such as a 4GS linker or multimer thereof). In some embodiments, the number of alanine residues in each series is: 2, 3, 4, 5, or 6 alanines. In some embodiments, the linker is a rigid linker. For example, the linker is an α-helical linker. In some embodiments, the linker is (in one-letter amino acid code): EAAAK or multimers of the EAAAK linker, such as repeats of 2, 3, 4, or 5 EAAAK linkers, such as set forth in SEQ ID NO: 2022 (1xEAAAK), SEQ ID NO: 2023 (3xEAAAK) or SEQ ID NO: 2024 (5xEAAAK). In some embodiments, the linker can further include amino acids introduced by cloning and/or from a restriction site, for example the linker can include the amino acids GS (in one-letter amino acid code) as introduced by use of the restriction site BAMHI. For example, in some embodiments, the linker (in one-letter amino acid code) is GSGGGGS (SEQ ID NO:1941), $GS(G_4S)_3$ (SEQ ID NO: 2031), or $GS(G_4S)_5$ (SEQ ID NO: 2032). In some examples, the linker is a 2xGGGGS followed by three alanines (GGGGSGGGGSAAA; SEQ ID NO: 241). In some cases, the immunomodulatory polypeptide comprising a variant PD-L1 comprises various combinations of peptide linkers.

**[0208]** In some embodiments, the variant PD-L1-Fc fusion protein is a dimer formed by two variant PD-L1 Fc polypeptides linked to an Fc domain. In some embodiments, the dimer is a homodimer in which the two variant PD-L1 Fc polypeptides are the same. In some embodiments, the dimer is a heterodimer in which

the two variant PD-L1 Fc polypeptides are different.

**[0209]** Also provided are nucleic acid molecules encoding the variant PD-L1-Fc fusion protein. In some embodiments, for production of an Fc fusion protein, a nucleic acid molecule encoding a variant PD-L1-Fc fusion protein is inserted into an appropriate expression vector. The resulting variant PD-L1-Fc fusion protein can be expressed in host cells transformed with the expression where assembly between Fc domains occurs by interchain disulfide bonds formed between the Fc moieties to yield dimeric, such as divalent, variant PD-L1-Fc fusion proteins.

**[0210]** The resulting Fc fusion proteins can be easily purified by affinity chromatography over Protein A or Protein G columns. For the generation of heterodimers, additional steps for purification can be necessary. For example, where two nucleic acids encoding different variant PD-L1 polypeptides are transformed into cells, the formation of heterodimers must be biochemically achieved since variant PD-L1 molecules carrying the Fc-domain will be expressed as disulfide-linked homodimers as well. Thus, homodimers can be reduced under conditions that favor the disruption of interchain disulfides, but do no effect intra-chain disulfides. In some cases, different variant-PD-L1 Fc monomers are mixed in equimolar amounts and oxidized to form a mixture of homo- and heterodimers. The components of this mixture are separated by chromatographic techniques. Alternatively, the formation of this type of heterodimer can be biased by genetically engineering and expressing Fc fusion molecules that contain a variant PD-L1 polypeptide using knob-into-hole methods described below.

**B. Stack Molecules with Additional IgSF Domains**

**[0211]** In some embodiments, the immunomodulatory proteins can contain any of the variant PD-L1 polypeptides provided herein linked, directly or indirectly, to one or more other immunoglobulin superfamily (IgSF) domain ("stacked" immunomodulatory protein construct and also called a "Type II" immunomodulatory protein). In some aspects, this can create unique multi-domain immunomodulatory proteins that bind two or more, such as three or more, cognate binding partners, thereby providing a multi-targeting modulation of the immune synapse.

**[0212]** In some embodiments, an immunomodulatory protein comprises a combination (a "non-wild-type combination") and/or arrangement (a "non-wild type arrangement" or "non-wild-type permutation") of a variant PD-L1 domain with one or more other affinity modified and/or non-affinity modified IgSF domain sequences of another IgSF family member (e.g. a mammalian IgSF family member) that are not found in wild-type IgSF family members. In some embodiments, the immunomodulatory protein contains 2, 3, 4, 5 or 6 immunoglobulin superfamily (IgSF) domains, where at least one of the IgSF domain is a variant PD-L1 IgSF domain (vIgD of PD-L1) according to the provided description.

**[0213]** In some embodiments, the sequences of the additional IgSF domains can be a modified IgSF domain that contains one or more amino acid modifications, e.g. substitutions, compared to a wildtype or unmodified IgSF domain. In some embodiments, the IgSF domain can be non-affinity modified (e.g., wild-type) or have been affinity modified. In some embodiments, the unmodified or wild-type IgSF domain can be from mouse, rat, cynomolgus monkey, or human origin, or combinations thereof. In some embodiments, the additional IgSF domains can be an IgSF domain of an IgSF family member set forth in Table 2. In some embodiments, the additional IgSF domain can be an affinity-modified IgSF domain containing one or more amino acid modifications, e.g. substitutions, compared to an IgSF domain contained in an IgSF family member set forth in Table 2.

**[0214]** In some embodiments, the additional IgSF domain is an affinity or non-affinity modified IgSF domain contained in an IgSF family member of a family selected from Signal-Regulatory Protein (SIRP) Family, Triggering Receptor Expressed On Myeloid Cells Like (TREML) Family, Carcinoembryonic Antigen-related Cell Adhesion Molecule (CEACAM) Family, Sialic Acid Binding Ig-Like Lectin (SIGLEC) Family, Butyrophilin Family, B7 family, CD28 family, V-set and Immunoglobulin Domain Containing (VSIG) family, V-set transmembrane Domain (VSTM) family, Major Histocompatibility Complex (MHC) family, Signaling lymphocytic activation molecule (SLAM) family, Leukocyte immunoglobulin-like receptor (LIR), Nectin (Nec) family, Nectin-like (NECL) family, Poliovirus receptor related (PVR) family, Natural cytotoxicity triggering receptor (NCR) family, T cell immunoglobulin and mucin (TIM) family or Killer-cell immunoglobulin-like receptors (KIR) family. In some embodiments, the additional IgSF domains are independently derived from an IgSF protein selected from the group consisting of CD80(B7-1), CD86(B7-2), CD274 (PD-L1, B7-H1), PDCD1LG2(PD-L2, CD273), ICOSLG(B7RP1, CD275, ICOSL, B7-H2), CD276(B7-H3), VTCN1(B7-H4), CD28, CTLA4, PDCD1(PD-1), ICOS, BTLA(CD272), CD4, CD8A(CD8-alpha), CD8B(CD8-beta), LAG3, HAVCR2(TIM-3), CEACAM1, TIGIT, PVR(CD155), PVRL2(CD112), CD226, CD2, CD160, CD200, CD200R1(CD200R), NCR3 (NKp30), and VSIG8.

**[0215]** The first column of Table 2 provides the name and, optionally, the name of some possible synonyms for that particular IgSF member. The second column provides the protein identifier of the UniProtKB database, a publicly available database accessible via the internet at uniprot.org or, in some cases, the GenBank Number. The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data. The UniProt databases include the UniProt Knowledgebase (UniProtKB). UniProt is a collaboration between the European Bioinformatics Institute (EMBL-EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR) and supported mainly by a grant from the U.S. National Institutes of Health (NIH). GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (Nucleic Acids Research, 2013 Jan;41(D1):D36-42). The third column provides the region where the indicated IgSF domain is located. The region is specified as a range where the domain is inclusive of the residues defining the range. Column 3 also indicates the IgSF domain class for the specified IgSF region. Column 4 provides the region where the indicated additional domains are located (signal peptide, S; extracellular domain, E; transmembrane domain, T; cytoplasmic domain, C). It is understood that description of domains can vary depending on the methods used to identify or classify the domain, and may be identified differently from different sources. The description of residues corresponding to a domain in Table 2 is for exemplification only and can be several amino acids (such as one, two, three or four) longer or shorter. Column 5 indicates for some of the listed IgSF members, some of its cognate cell surface binding partners.

**TABLE 2. IgSF members according to the present disclosure.**

| IgSF Member (Synonyms) | NCBI Protein Accession Number/ UniProtKB Protein Identifier | IgSF Region & Domain Class | Other Domains | Cognate Cell Surface Binding Partners | IgSF Member Amino Acid Sequence (SEQ ID NO) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Precursor (mature residues) | Mature | ECD |
| CD80 (B7-1) | NP_005182.1 P33681 | 35-135, 35-138,37-138, or 35-141 IgV, 145-230 or 154-232 IgC | S: 1-34, E: 35-242, T: 243-263, C: 264-288 | CD28, CTLA4, PD-L1 | SEQ ID NO: 1 (35-288) | SEQ ID NO: 189 | SEQ ID NO: 28 |
| CD86 (B7- | P42081.2 | 33-131 IgV, | S: 1-23, | CD28, CTLA4 | SEQ ID | SEQ | SEQ |

TABLE 2. IgSF members according to the present disclosure.

| IgSF Member (Synonyms) | NCBI Protein Accession Number/ UniProtKB Protein Identifier | IgSF Region & Domain Class | Other Domains | Cognate Cell Surface Binding Partners | IgSF Member Amino Acid Sequence (SEQ ID NO) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Precursor (mature residues) | Mature | ECD |
| 2) | | 150-225 IgC2 | E: 24-247, T: 248-268, C: 269-329 | | NO: 2 (24-329) | ID NO: 190 | ID NO: 29 |
| CD274 (PD-L1, B7-H1) | Q9NZQ7.1 NP_054862.1 | 24-130 or 19-127 IgV, 133-225 IgC2 | S: 1-18, E: 19-238, T: 239-259, C: 260-290 | PD-1, B7-1 | SEQ ID NO: 3 (19-290) | SEQ ID NO: 191 | SEQ ID NO: 30 |
| **IgSF Member (Synonyms)** | **NCBI Protein Accession Number/ UniProtKB Protein Identifier** | **IgSF Region & Domain Class** | **Other Domains** | **Cognate Cell Surface Binding Partners** | **IgSF Member Amino Acid Sequence (SEQ ID NO)** | | |
| | | | | | **Precursor (mature residues)** | **Mature** | **ECD** |
| PDCD1LG2 (PD-L2, CD273) | Q9BQ51.2 | 21-118 IgV, 122-203 IgC2 | S: 1-19, E: 20-220, T: 221-241, C: 242-273 | PD-1, RGMb | SEQ ID NO: 4 (20-273) | SEQ ID NO: 192 | SEQ ID NO: 31 |
| ICOSLG (B7RP1, CD275, ICOSL, B7-H2) | 075144.2 | 19-129 IgV, 141-227 IgC2 | S: 1-18, E: 19-256, T: 257-277, C: 278-302 | ICOS, CD28, CTLA4 | SEQ ID NO: 5 (19-302) | SEQ ID NO: 193 | SEQ ID NO: 32 |
| CD276 (B7-H3) | Q5ZPR3.1 | 29-139 IgV, 145-238 IgC2, 243-357 IgV2, 363-456, 367-453 IgC2 | S: 1-28, E: 29-466, T: 467-487, C: 488-534 | | SEQ ID NO: 6 (29-534) | SEQ ID NO: 194 | SEQ ID NO: 33 |
| VTCN1 (B7-H4) | Q7Z7D3.1 | 35-146 IgV, 153-241 IgV | S: 1-24, E: 25-259, T: 260-280, C: 281-282 | | SEQ ID NO: 7 (25-282) | SEQ ID NO: 195 | SEQ ID NO: 34 |
| CD28 | P10747.1 | 28-137 IgV | S: 1-18, E: 19-152, T: 153-179, C: 180-220 | B7-1, B7-2, B7RP1 | SEQ ID NO: 8 (19-220) | SEQ ID NO: 196 | SEQ ID NO: 35 |
| **IgSF Member** | **NCBI Protein** | **IgSF Region &** | **Other Domains** | **Cognate Cell Surface** | **IgSF Member Amino Acid Sequence (SEQ ID** | | |

**TABLE 2. IgSF members according to the present disclosure.**

| IgSF Member (Synonyms) | NCBI Protein Accession Number/ UniProtKB Protein Identifier | IgSF Region & Domain Class | Other Domains | Cognate Cell Surface Binding Partners | IgSF Member Amino Acid Sequence (SEQ ID NO) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Precursor (mature residues) | Mature | ECD |
| (Synonyms) | Accession Number/ UniProtKB Protein Identifier | Domain Class | | Binding Partners | NO) | | |
| | | | | | Precursor (mature residues) | Mature | ECD |
| CTLA-4 | P16410.3 | 39-140 IgV | S: 1-35, E: 36-161, T: 162-182, C: 183-223 | B7-1, B7-2, B7RP1 | SEQ ID NO: 9 (36-223) | SEQ ID NO: 197 | SEQ ID NO: 36 |
| PDCD1 (PD-1) | Q15116.3 | 35-145 IgV | S: 1-20, E: 21-170, T: 171-191, C: 192-288 | PD-L1, PD-L2 | SEQ ID NO: 10 (21-288) | SEQ ID NO: 198 | SEQ ID NO: 37 |
| ICOS | Q9Y6W8.1 | 30-132 IgV | S: 1-20, E: 21-140, T: 141-161, C: 162-199 | B7RP1 | SEQ ID NO: 11 (21-199) | SEQ ID NO: 199 | SEQ ID NO: 38 |
| BTLA (CD272) | Q7Z6A9.3 | 31-132 IgV | S: 1-30, E: 31-157, T: 158-178, C: 179-289 | HVEM | SEQ ID NO: 12 (31-289) | SEQ ID NO: 200 | SEQ ID NO: 39 |
| CD4 | P01730.1 | 26-125 IgV, 126-203 IgC2, 204-317 IgC2, 317-389, 318-374 IgC2 | S: 1-25, E: 26-396, T: 397-418, C: 419-458 | MHC class II | SEQ ID NO: 13 (26-458) | SEQ ID NO: 201 | SEQ ID NO: 40 |
| **IgSF Member (Synonyms)** | **NCBI Protein Accession Number/ UniProtKB Protein Identifier** | **IgSF Region & Domain Class** | **Other Domains** | **Cognate Cell Surface Binding Partners** | **IgSF Member Amino Acid Sequence (SEQ ID NO)** | | |
| | | | | | **Precursor (mature residues)** | **Mature** | **ECD** |
| CD8A (CD8-alpha) | P01732.1 | 22-135 IgV | S: 1-21, E: 22-182, T: 183-203, C: 204-235 | MHC class I | SEQ ID NO: 14 (22-235) | SEQ ID NO: 202 | SEQ ID NO: 41 |
| CD8B | P10966.1 | 22-132 IgV | S: 1-21, | MHC class I | SEQ ID | SEQ | SEQ |

**TABLE 2. IgSF members according to the present disclosure.**

| IgSF Member (Synonyms) | NCBI Protein Accession Number/ UniProtKB Protein Identifier | IgSF Region & Domain Class | Other Domains | Cognate Cell Surface Binding Partners | IgSF Member Amino Acid Sequence (SEQ ID NO) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Precursor (mature residues) | Mature | ECD |
| (CD8-beta) | | | E: 22-170, T: 171-191, C: 192-210 | | NO: 15 (22-210) | ID NO: 203 | ID NO: 42 |
| LAG3 | P18627.5 | 37-167 IgV, 168-252 IgC2, 265-343 349-419 IgC2 | IgC2, S: 1-28, E: 29-450, T: 451-471, C: 472-525 | MHC class II | SEQ ID NO: 16 (29-525) | SEQ ID NO: 204 | SEQ ID NO: 43 |
| HAVCR2 (TIM-3) | Q8TDQ0.3 | 22-124 IgV | S: 1-21, E: 22-202, T: 203-223, C: 224-301 | CEACAM-1, phosphatidylserine, Galectin-9, HMGB1 | SEQ ID NO: 17 (22-301) | SEQ ID NO: 205 | SEQ ID NO: 44 |
| CEACAM1 | P13688.2 | 35-142 IgV, 145-232 IgC2, 237-317 IgC2, 323-413 IgC2 | S: 1-34, E: 35-428, T: 429-452, C: 453-526 | TIM-3 | SEQ ID NO: 18 (35-526) | SEQ ID NO: 206 | SEQ ID NO: 45 |
| **IgSF Member (Synonyms)** | **NCBI Protein Accession Number/ UniProtKB Protein Identifier** | **IgSF Region & Domain Class** | **Other Domains** | **Cognate Cell Surface Binding Partners** | **IgSF Member Amino Acid Sequence (SEQ ID NO)** | | |
| | | | | | **Precursor (mature residues)** | **Mature** | **ECD** |
| TIGIT | Q495A1.1 | 22-124 IgV | S: 1-21, E: 22-141, T: 142-162, C: 163-244 | CD155, CD112 | SEQ ID NO: 19 (22-244) | SEQ ID NO: 207 | SEQ ID NO: 46 |
| PVR (CD155) | P15151.2 | 24-139 IgV, 145-237 IgC2, 244-328 IgC2 | S: 1-20, E: 21-343, T: 344-367, C: 368-417 | TIGIT, CD226, CD96, poliovirus | SEQ ID NO: 20 (21-417) | SEQ ID NO: 208 | SEQ ID NO: 47 |
| PVRL2 (CD112) | Q92692.1 | 32-156 IgV, 162-256 IgC2, 261-345 IgC2 | S: 1-31, E: 32-360, T: 361-381, C: 382-538 | TIGIT, CD226, CD112R | SEQ ID NO: 21 (32-538) | SEQ ID NO: 209 | SEQ ID NO: 48 |
| CD226 | Q15762.2 | 19-126 IgC2, 135- | S: 1-18, E: 19-254, | CD155, CD112 | SEQ ID NO: 22 | SEQ ID NO: | SEQ ID |

**TABLE 2. IgSF members according to the present disclosure.**

| IgSF Member (Synonyms) | NCBI Protein Accession Number/ UniProtKB Protein Identifier | IgSF Region & Domain Class | Other Domains | Cognate Cell Surface Binding Partners | IgSF Member Amino Acid Sequence (SEQ ID NO) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Precursor (mature residues) | Mature | ECD |
| | | 239 IgC2 | T: 255-275, C: 276-336 | | (19-336) | 210 | NO: 49 |
| CD2 | P06729.2 | 25-128 IgV, 129-209 IgC2 | S: 1-24, E: 25-209, T: 210-235, C: 236-351 | CD58 | SEQ ID NO: 23 (25-351) | SEQ ID NO: 211 | SEQ ID NO: 50 |
| CD 160 | 095971.1 | 27-122 IgV | N/A | HVEM, MHC family of proteins | SEQ ID NO: 24 (27-159) | SEQ ID NO: 212 | SEQ ID NO: 51 |
| **IgSF Member (Synonyms)** | **NCBI Protein Accession Number/ UniProtKB Protein Identifier** | **IgSF Region & Domain Class** | **Other Domains** | **Cognate Cell Surface Binding Partners** | **IgSF Member Amino Acid Sequence (SEQ ID NO)** | | |
| | | | | | **Precursor (mature residues)** | **Mature** | **ECD** |
| CD200 | P41217.4 | 31-141 IgV, 142-232 IgC2 | S: 1-30, E: 31-232, T: 233-259, C: 260-278 | CD200R | SEQ ID NO: 25 (31-278) | SEQ ID NO: 213 | SEQ ID NO: 52 |
| CD200R1 (CD200R) | Q8TD46.2 | 53-139 IgV, 140-228 IgC2 | S: 1-28, E: 29-243, T: 244-264, C: 265-325 | CD200 | SEQ ID NO: 26 (29-325) | SEQ ID NO: 214 | SEQ ID NO: 53 |
| NCR3 (NKp30) | 014931.1 | 19-126 IgC-like | S: 1-18, E: 19-135, T: 136-156, C: 157-201 | B7-H6 | SEQ ID NO:27 (19-201) | SEQ ID NO: 215 | SEQ ID NO: 54 |
| VSIG8 | Q5VU13 | 22-141 IgV1, 146-257 IgV2 | S: 1-21 E: 22-263 T: 264-284 C: 285-414 | VISTA | SEQ ID NO: 216 (22-414) | SEQ ID NO: 217 | SEQ ID NO: 218 |

**[0216]** In some embodiments, the provided immunomodulatory proteins, in addition to containing a variant PD-L1 polypeptide, also contains at least 1, 2, 3, 4, 5 or 6 additional immunoglobulin superfamily (IgSF) domains, such as an IgD domain of an IgSF family member set forth in Table 2. In some embodiments, the provided immunomodulatory protein contains at least one additional IgSF domain (e.g. second IgSF

domain). In some embodiments, the provided immunomodulatory protein contains at least two additional IgSF domains (e.g. second and third IgSF domain). In some embodiments, the provided immunomodulatory protein contains at least three additional IgSF domains (e.g. second, third and fourth). In some embodiments, the provided immunomodulatory protein contains at least four additional IgSF domains (e.g. second, third, fourth and fifth). In some embodiments, the provided immunomodulatory protein contains at least five additional IgSF domains (e.g. second, third, fourth, fifth and sixth). In some embodiments, the provided immunomodulatory protein contains at least six additional IgSF domains (e.g. second, third, fourth, fifth, sixth and seventh). In some embodiments, each of the IgSF domains in the immunomodulatory protein are different. In some embodiments, at least one of the additional IgSF domain is the same as at least one other IgSF domain in the immunomodulatory protein. In some embodiments, each of the IgSF domains is from or derived from a different IgSF family member. In some embodiments, at least two of the IgSF domains is from or derived from the same IgSF family member.

**[0217]** In some embodiments, the additional IgSF domain comprises an IgV domain or an IgC (e.g., IgC2) domain or domains, or a specific binding fragment of the IgV domain or a specific binding fragment of the IgC (e.g., IgC2) domain or domains. In some embodiments, the additional IgSF domain is or comprises a full-length IgV domain. In some embodiments, the additional IgSF domain is or comprises a full-length IgC (e.g., IgC2) domain or domains. In some embodiments, the additional IgSF domain is or comprises a specific binding fragment of the IgV domain. In some embodiments, the additional IgSF domain is or comprises a specific binding fragment of the IgC (e.g., IgC2) domain or domains. In some embodiments, the immunomodulatory protein contains at least two additional IgSF domains from a single (same) IgSF member. For example, in some aspects, the immunomodulatory protein contains an ECD or portion thereof of an IgSF member containing a full-length IgV domain and a full-length IgC (e.g., IgC2) domain or domains or specific binding fragments thereof.

**[0218]** In some embodiments, the provided immunomodulatory proteins contains at least one additional IgSF domain (e.g. a second or, in some cases, also a third IgSF domain and so on) in which at least one additional, e.g. a second or third IgSF domain, is an IgSF domain set forth in a wild-type or unmodified IgSF domain or a specific binding fragment thereof contained in the sequence of amino acids set forth in any of SEQ ID NOS: 1-27 and 216. In some embodiments, the wild-type or unmodified IgSF domain is an IgV domain or an IgC domain, such as an IgC1 or IgC2 domain.

**[0219]** In some embodiments, the provided immunomodulatory proteins, in addition to containing a variant PD-L1 polypeptide, also contains at least one additional affinity-modified IgSF domain (e.g. a second or, in some cases, also a third affinity-modified IgSF domain and so on) in which at least one additional IgSF domain is a vIgD that contains one or more amino acid modifications (e.g. substitution, deletion or mutation) compared to an IgSF domain in a wild-type or unmodified IgSF domain, such as an IgSF domain in an IgSF family member set forth in Table 2. In some embodiments, the additional, e.g., second or third, affinity-modified IgSF domain comprises at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to a wild-type or unmodified IgSF domain or a specific binding fragment thereof contained in the sequence of amino acids set forth in any of SEQ ID NOS: 1-27 and 216. In some embodiments, the wild-type or unmodified IgSF domain is an IgV domain or an IgC domain, such as an IgC1 or IgC2 domain. In some embodiments, the additional, e.g., or second or third, IgSF domain is an affinity-modified IgV domain and/or IgC domain. In some embodiments, the one or more additional IgSF domain is an affinity-modified IgSF domain that contains an IgV domain and/or an IgC (e.g., IgC2) domain or domains, or a specific binding fragment of the IgV domain and/or a specific binding fragment of the IgC (e.g., IgC2) domain or domains, in which the IgV and/or IgC domain contains the amino acid modification(s) (e.g., substitution(s)). In some embodiments, the one or more additional affinity-modified IgSF domain contains an IgV domain containing the amino acid modification(s) (e.g. substitution(s)). In some embodiments, the one or more additional affinity-modified IgSF domain include

IgSF domains present in the ECD or a portion of the ECD of the corresponding unmodified IgSF family member, such as a full-length IgV domain and a full-length IgC (e.g., IgC2) domain or domains, or specific binding fragments thereof, in which one or both of the IgV and IgC contain the amino acid modification(s) (e.g. substitution(s)).

**[0220]** In some embodiments, the immunomodulatory polypeptide comprising a variant PD-L1 can include one or more vIgD of PD-L1 provided herein. In some embodiments, a variant PD-L1 immunomodulatory protein provided herein will comprise exactly 1, 2, 3, 4, 5 or more variant PD-L1 sequences. In some embodiments, at least two of variant PD-L1 sequences are identical variant IgSF domains.

**[0221]** In some embodiments, the provided immunomodulatory polypeptide comprises two or more vIgD sequences of PD-L1. Multiple variant PD-L1 within the polypeptide chain can be identical (i.e., the same species) to each other or be non-identical (i.e., different species) variant PD-L1 sequences. In addition to single polypeptide chain embodiments, in some embodiments two, three, four, or more of the polypeptides of the invention can be covalently or non-covalently attached to each other. Thus, monomeric, dimeric, and higher order (e.g., 3, 4, 5, or more) multimeric proteins are provided herein. For example, in some embodiments exactly two polypeptides of the invention can be covalently or non-covalently attached to each other to form a dimer. In some embodiments, attachment is made via interchain cysteine disulfide bonds. Compositions comprising two or more polypeptides of the invention can be of an identical species or substantially identical species of polypeptide (e.g., a homodimer) or of non-identical species of polypeptides (e.g., a heterodimer). A composition having a plurality of linked polypeptides of the invention can, as noted above, have one or more identical or non-identical variant PD-L1 of the invention in each polypeptide chain. In some specific embodiments, identical or substantially identical species (allowing for 3 or fewer N-terminus or C-terminus amino acid sequence differences) of PD-L1-Fc variant fusion polypeptides will be dimerized to create a homodimer. Alternatively, different species of PD-L1-Fc variant fusion polypeptides can be dimerized to yield a heterodimer.

**[0222]** In some embodiments, the provided immunomodulatory protein contains at least one additional (e.g. or second or, in some cases, also a third IgSF domain and so on)IgSF domain that is a vIgD that contains one or more amino acid substitutions compared to an IgSF domain (e.g. IgV) of a wild-type or unmodified IgSF domain other than PD-L1.

**[0223]** In some embodiments, the one or more additional IgSF domain (e.g. second or third IgSF) domain is an IgSF domain (e.g. ECD or IgV) of another IgSF family member that itself also binds to an inhibitory receptor. In some aspects, the one or more additional IgSF domain (e.g. second or third IgSF) domain is an affinity-modified IgSF domain that is a variant IgSF domain (vIgD) of an IgSF family member that bind to an inhibitory receptor and that contains one or more amino acid substitutions in an IgSF domain (e.g. ECD or IgV), in which, in some cases, the one or more amino acid modifications result in increased binding to the inhibitory receptor. In some embodiments, the vIgD contains one or more amino acid modifications (e.g. substitutions, deletions or additions) in a wild-type or unmodified IgSF domain (e.g. ECD or IgV) of an IgSF family member that binds to an inhibitory receptor. In addition to PD-1, exemplary of such inhibitory receptors are CTLA-4, LAG3, TIGIT, TIM-3, or BTLA. In some embodiments, the one or more additional IgSF domain is from an IgSF family member selected from CD155, CD112, PD-L2, CD80 or CEACAM1. Thus, in some aspects, provided are multi-target checkpoint antagonists that target or block activity of more than one inhibitory receptor.

**[0224]** In some embodiments, the immunomodulatory protein is a multi-target checkpoint antagonist that targets or blocks activity of at least two, three, four or more inhibitory receptors. In some embodiments, there is provided an immunomodulatory protein containing any one of the variant PD-L1 polypeptides and one or more IgSF domain of an inhibitory receptor, such as a wild-type or unmodified inhibitory receptor. In

some embodiments, there is provided an immunomodulatory protein containing any one of the variant PD-L1 polypeptides and one or more IgSF domain of CD80, e.g. wild-type or unmodified CD80, such as an IgV domain set forth in SEQ ID NO:1005, 1079 or 2030 or an ECD or a portion thereof (containing the IgV and IgC domain or specific binding fragments thereof) set forth in SEQ ID NO:28 or a portion thereof. In some embodiments, there is provided an immunomodulatory protein containing any one of the variant PD-L1 polypeptides and one or more IgSF domain of CD112, e.g. wild-type or unmodified CD112, such as an IgV domain set forth in SEQ ID NO:666 or 761 or an ECD or a portion thereof (containing the IgV and IgC domain or specific binding fragments thereof) set forth in SEQ ID NO:48 or a portion thereof. In some embodiments, there is provided an immunomodulatory protein containing any one of the variant PD-L1 polypeptides and one or more IgSF domain of PD-L2, e.g. wild-type or unmodified PD-L2, such as an IgV domain set forth in SEQ ID NO:1203 or 1263 or an ECD or a portion thereof (containing the IgV and IgC domain or specific binding fragments thereof) set forth in SEQ ID NO:31 or a portion thereof. In some embodiments, there is provided an an immunomodulatory protein containing any one of the variant PD-L1 polypeptides and one or more IgSF domain of CD155, e.g. wild-type or unmodified CD155, such as an IgV domain set forth in SEQ ID NO 310 or 353 or an ECD or a portion thereof (containing the IgV and IgC domain or specific binding fragments thereof) set forth in SEQ ID NO:47 or a portion thereof.

**[0225]** In some embodiments, there is provided an immunomodulatory protein containing one or more additional IgSF domain (e.g., second or third IgSF) that is a vIgD of an IgSF family member that binds to an inhibitory receptor in which the one or more amino acid modifications in an IgSF domain (e.g. IgV) results in increased binding affinity of the vIgD, or a fusion or immunomodulatory protein containing the vIgD, for its inhibitory receptor cognate binding partner compared to the unmodified IgSF domain, such as binding affinity that is increased more than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold. In some embodiments, the one or more amino acid modifications in an IgSF domain (e.g. IgV) results in increased selectivity of the vIgD, or a fusion or immunomodulatory protein containing the vIgD for its inhibitory receptor compared to the unmodified IgSF domain. In some embodiments, the increased selectivity is a greater ratio of binding of the vIgD for the inhibitory receptor versus another cognate binding partner, such as a cognate binding partner that is not an inhibitory receptor, compared to the ratio of binding of the unmodified IgSF for the inhibitory receptor versus the another cognate binding partner. In some embodiments, the ratio is greater by at least or at least about 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold.

**[0226]** In some embodiments, the at least one additional (e.g. second or third) vIgD is an IgSF domain (e.g. IgV) of a variant CD80 polypeptide that contains one or more amino acid modifications (e.g. substitutions, deletions or additions) in the IgSF domain (e.g. IgV) of CD155 or CD112, which are IgSF family members that bind to the inhibitory receptor TIGIT. In some embodiments, the at least one additional (e.g. second) vIgD contains one or more amino acid modifications (e.g. substitutions, deletions or additions) in an IgSF domain (e.g. IgV) of CD80, which is an IgSF family member that bind to the inhibitory receptor CTLA-4. Exemplary amino acid modifications, such as substitutions, deletions or additions, in an IgSF domain (e.g. ECD or IgV containing IgV and IgC) of a variant CD80 polypeptide are set forth in Table 3. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD80 polypeptide containing an IgV domain including any of the amino acid modifications set forth in Table 3, such as the IgV domain set forth in any of SEQ ID NOS:1006-1078, 1080-1112, 1114-1152 or an IgV domain that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 1006-1078, 1080-1112, 1114-1152 and contains the one or more amino acid modifications. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD80 polypeptide containing an ECD or a portion thereof containing the IgV and/or IgC domains, in which is

contained any of the amino acid modifications set forth in Table 3, such as the ECD set forth in any of SEQ ID NOS: 932-964, 966-1004 or an ECD that contains at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 932-964, 966-1004 and contains the one or more amino acid modifications.

**[0227]** In some embodiments, the at least one additional (e.g., second or third) vIgD is an IgSF domain (e.g. IgV) of a variant CD155 polypeptide that contains one or more amino acid modifications (e.g., substitutions, deletions or additions) in the IgSF domain (e.g., IgV) compared to unmodified or wild-type CD155, which, in some aspects, result in increased binding to the inhibitory receptor TIGIT. Exemplary amino acid modifications, such as substitutions, deletions or additions, in an IgSF domain (e.g. IgV or ECD containing IgV and IgC) of a variant CD155 polypeptide are set forth in Table 5. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD155 polypeptide containing an IgV domain including any of the amino acid modifications set forth in Table 5, such as the IgV domain set forth in any of SEQ ID NOS: 332-352, 354-374, 472-665, 1505-1550, 1570-1714, or an IgV domain that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 332-352, 354-374, 472-665, 1505-1550, 1570-1714 and contains the one more amino acid modifications. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD155 polypeptide containing an ECD or a portion thereof containing the IgV and/or IgC domains, in which is contained any of the amino acid modifications set forth in Table 5, such as the ECD set forth in any of SEQ ID NOS: 311-331, 375-471, 1551-1622 or an ECD that contains at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 311-331, 375-471, 1551-1622 and contains the one or more amino acid modifications.

**[0228]** In some embodiments, the at least one additional (e.g., second or third) vIgD is an IgSF domain (e.g. IgV) of a variant CD112 polypeptide that contains one or more amino acid modifications (e.g., substitutions, deletions or additions) in the IgSF domain (e.g., IgV) compared to unmodified or wild-type CD112, which, in some aspects, result in increased binding to the inhibitory receptor TIGIT. Exemplary amino acid modifications, such as substitutions, deletions or additions, in an IgSF domain (e.g. IgV or ECD containing IgV and IgC) of a variant CD112 polypeptide are set forth in Table 4. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD112 polypeptide containing an IgV domain including any of the amino acid modifications set forth in Table 4, such as the IgV domain set forth in any of SEQ ID NOS: 714-760, 762-808, 850-931, or an IgV domain that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 714-760, 762-808, 850-931 and contains the one more amino acid modifications. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1polypeptides and a variant CD112 polypeptide containing an ECD or a portion thereof containing the IgV and/or IgC domains, in which is contained any of the amino acid modifications set forth in Table 4, such as the ECD set forth in any of SEQ ID NOS: 667-713, 809-849, 1433-1456 or an ECD that contains at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 667-713, 809-849, 1433-1456 and contains the one or more amino acid modifications.

**[0229]** In some embodiments, the at least one additional (e.g., second or third) vIgD is an IgSF domain (e.g. IgV) of a variant PD-L2 polypeptide that contains one or more amino acid modifications (e.g., substitutions, deletions or additions) in the IgSF domain (e.g., ECD or IgV) compared to unmodified or wild-type PD-L2, which, in some aspects, result in increased binding to the inhibitory receptor PD-1. Exemplary amino acid modifications, such as substitutions, deletions or additions, in an IgSF domain (e.g. IgV or ECD containing IgV and IgC) of a variant PD-L2 polypeptide are set forth in Table 8. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides

and a variant PD-L2 polypeptide containing an IgV domain including any of the amino acid modifications set forth in Table 8, such as the IgV domain set forth in any of SEQ ID NOS: 1281-1331, 1333-1407, 1409-1432 or an IgV domain that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 1281-1331, 1333-1407, 1409-1432 and contains the one more more amino acid modifications. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant PD-L2 polypeptide containing an ECD or a portion thereof containing the IgV and/or IgC domains, in which is contained any of the amino acid modifications set forth in Table 8, such as the ECD set forth in any of SEQ ID NOS:1204-1254, 1256-1280 or an ECD that contains at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 1204-1254, 1256-1280 and contains the one or more amino acid modifications.

**[0230]** In some embodiments, the one or more additional IgSF domain (e.g. second IgSF) domain is an IgSF domain (e.g. IgV) of another IgSF family member that binds or recognizes a tumor antigen. In such embodiments, the IgSF family member serves as a tumor-localizing moiety, thereby bringing the vIgD of PD-L1 in close proximity to immune cells in the tumor microenvironment. In some embodiments, the additional IgSF domain (e.g. second IgSF) domain is an IgSF domain of NKp30, which binds or recognizes B7-H6 expressed on a tumor cell. In some embodiments, the at least one additional (e.g. second) IgSF domain, e.g. NKp30, is an affinity-modified IgSF domain or vIgD that contains one or more amino acid modifications (e.g. substitutions, deletions or additions). In some embodiments, the one or more amino acid modifications increase binding affinity and/or selectivity to B7-H6 compared to unmodified IgSF domain, e.g. NKp30, such as by at least or at least about 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold 40-fold or 50-fold. Exemplary amino acid modifications, such as substitutions, deletions or additions, in an IgSF domain (e.g. IgC-like or full ECD) of a variant NKp30 polypeptide are set forth in Table 6. Among the exemplary polypeptides is an NKp30 variant that contains the mutations L30V/A60V/S64P/S86G with reference to positions in the NKp30 extracellular domain corresponding to positions set forth in SEQ ID NO: 54. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant NKp30 polypeptide containing an IgC-like domain including any of the amino acid modifications set forth in Table 6, such as the IgC-like domain set forth in any of SEQ ID NOS: 1184-1188 or an IgC-like domain that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 1184-1188 and contains the one more amino acid modifications. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant NKp30 polypeptide containing an ECD or a portion thereof containing an IgSF domain or domains, in which is contained any of the amino acid modifications set forth in Table 6, such as the ECD set forth in any of SEQ ID NOS: 1178-1182 or an ECD that contains at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 1178-1182 and contains the one or more amino acid modifications.

**[0231]** In some embodiments, the at least one additional (e.g., second or third) vIgD is an IgSF domain (e.g. IgV) of a variant CD86 polypeptide that contains one or more amino acid modifications (e.g., substitutions, deletions or additions) in the IgSF domain (e.g., IgV) compared to unmodified or wild-type CD86, which, in some aspects, result in increased binding to its cognate binding partner. Exemplary amino acid modifications, such as subtitutions, deletions or additions, in an IgSF domain (e.g. IgV or ECD containing IgV and IgC) of a variant CD86 polypeptide are set forth in Table 7. Among exemplary polypeptides include CD86 variants that contain the mutations Q35H/H90L/Q102H with reference to positions in the CD86 extracellular domain corresponding to positions set forth in SEQ ID NO: 29. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD86 polypeptide containing an IgV domain including any of the amino acid modifications set forth in Table 7, such as the IgV domain set forth in any of SEQ ID NOS: 1196-1199 or an

IgV domain that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS: 1196-1199 and contains the one more amino acid modifications. In some embodiments, there is provided an immunomodulatory protein containing any of the provided variant PD-L1 polypeptides and a variant CD86 polypeptide containing an ECD or a portion thereof containing the IgV and/or IgC domains, in which is contained any of the amino acid modifications set forth in Table 7, such as the ECD set forth in any of SEQ ID NOS: 1191-1194 or an ECD that contains at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any of SEQ ID NOS:1191-1194 and contains the one or more amino acid modifications.

**[0232]** Tables 3-8 provide exemplary polypeptides containing one or more affinity-modified IgSF domains that can be used in stack constructs provided herein.

**TABLE 3: Exemplary variant CD80 polypeptides**

| Mutation(s) | ECD SEQ ID NO | IgV SEQ ID NO |
|---|---|---|
| Wild-type | 28 | 1005, 2030 |
| L70P | 932 | 1006, 1080 |
| I30F/L70P | 933 | 1007, 1081 |
| Q27H/T41S/A71D | 934 | 1008, 1082 |
| I30T/L70R | 935 | 1009, 1083 |
| T13R/C16R/L70Q/A71D | 936 | 1010, 1084 |
| T57I | 937 | 1011, 1085 |
| M43I/C82R | 938 | 1012, 1086 |
| V22L/M38V/M47T/A71D/L85M | 939 | 1013, 1087 |
| I30V/T57I/L70P/A71D/A91T | 940 | 1014, 1088 |
| V22I/L70M/A71D | 941 | 1015, 1089 |
| N55D/L70P/E77G | 942 | 1016, 1090 |
| T57A/I69T | 943 | 1017, 1091 |
| N55D/K86M | 944 | 1018, 1092 |
| L72P/T79I | 945 | 1019, 1093 |
| L70P/F92S | 946 | 1020, 1094 |
| T79P | 947 | 1021, 1095 |
| E35D/M47I/L65P/D90N | 948 | 1022, 1096 |
| L25S/E35D/M47I/D90N | 949 | 1023, 1097 |
| A71D | 951 | 1025, 1099 |
| E81K/A91S | 953 | 1027, 1101 |
| A12V/M47V/L70M | 954 | 1028, 1102 |
| K34E/T41A/L72V | 955 | 1029, 1103 |
| T41S/A71D/V84A | 956 | 1030, 1104 |
| E35D/A71D | 957 | 1031, 1105 |
| E35D/M47I | 958 | 1032, 1106 |
| K36R/G78A | 959 | 1033, 1107 |
| Q33E/T41A | 960 | 1034, 1108 |
| M47V/N48H | 961 | 1035, 1109 |

| TABLE 3: Exemplary variant CD80 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| M47L/V68A | 962 | 1036, 1110 |
| S44P/A71D | 963 | 1037, 1111 |
| Q27H/M43I/A71D/R73S | 964 | 1038, 1112 |
| E35D/T57I/L70Q/A71D | 966 | 1040, 1114 |
| M47I/E88D | 967 | 1041, 1115 |
| M42I/I61V/A71D | 968 | 1042, 1116 |
| P51A/A71D | 969 | 1043, 1117 |
| H18Y/M47I/T57I/A71G | 970 | 1044, 1118 |
| V20I/M47V/T57I/V84I | 971 | 1045, 1119 |
| V20I/M47V/A71D | 972 | 1046, 1120 |
| A71D/L72V/E95K | 973 | 1047, 1121 |
| V22L/E35G/A71D/L72P | 974 | 1048, 1122 |
| E35D/A71D | 975 | 1049, 1123 |
| E35D/I67L/A71D | 976 | 1050, 1124 |
| Q27H/E35G/A71D/L72P/T79I | 977 | 1051, 1125 |
| T13R/M42V/M47I/A71D | 978 | 1052, 1126 |
| E35D | 979 | 1053, 1127 |
| E35D/M47I/L70M | 980 | 1054, 1128 |
| E35D/A71D/L72V | 981 | 1055, 1129 |
| E35D/M43L/L70M | 982 | 1056, 1130 |
| A26P/E35D/M43I/L85Q/E88D | 983 | 1057, 1131 |
| E35D/D46V/L85Q | 984 | 1058, 1132 |
| Q27L/E3 5 D/M47I/T5 7I/L70Q/E8 8D | 985 | 1059, 1133 |
| M47V/I69F/A71D/V83I | 986 | 1060, 1134 |
| E35D/T57A/A71D/L85Q | 987 | 1061, 1135 |
| H18Y/A26T/E35D/A71D/L85Q | 988 | 1062, 1136 |
| E35D/M47L | 989 | 1063, 1137 |
| E23D/M42V/M43I/I58V/L70R | 990 | 1064, 1138 |
| V68M/L70M/A71D/E95K | 991 | 1065, 1139 |
| N55I/T57I/I69F | 992 | 1066, 1140 |
| E35D/M43I/A71D | 993 | 1067, 1141 |
| T41S/T57I/L70R | 994 | 1068, 1142 |
| H18Y/A71D/L72P/E88V | 995 | 1069, 1143 |
| V20I/A71D | 996 | 1070, 1144 |
| E23G/A26S/E35D/T62N/A71D/L72V/L85M | 997 | 1071, 1145 |
| A12T/E24D/E35D/D46V/I61V/L72P/E95V | 998 | 1072, 1146 |
| V22L/E35D/M43L/A71G/D76H | 999 | 1073, 1147 |
| E35G/K54E/A71D/L72P | 1000 | 1074, 1148 |

| TABLE 3: Exemplary variant CD80 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| L70Q/A71D | 1001 | 1075, 1149 |
| A26E/E35D/M47L/L85Q | 1002 | 1076, 1150 |
| D46E/A71D | 1003 | 1077, 1151 |
| Y31H/E35D/T41S/V68L/K93R/R94W | 1004 | 1078, 1152 |

| TABLE 4: Exemplary variant CD112 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| Wild-type | 48 | 666, 761 |
| Y33H, A112V, G117D | 667 | 714, 762 |
| V19A, Y33H, S64G, S80G, G98S, N106Y, A112V | 668 | 715, 763 |
| L32P, A112V | 669 | 716, 764 |
| A95V, A112I | 670 | 717, 765 |
| P28S, A112V | 671 | 718, 766 |
| P27A, T38N, V101A, A112V | 672 | 719, 767 |
| S118F | 673 | 720, 768 |
| R12W, H48Y, F54S, S118F | 674 | 721, 769 |
| R12W, Q79R, S118F | 675 | 722, 770 |
| T113S, S118Y | 676 | 723, 771 |
| S118Y | 677 | 724, 772 |
| N106I, S118Y | 678 | 725, 773 |
| N106I, S118F | 679 | 726, 774 |
| A95T, L96P, S118Y | 680 | 727, 775 |
| Y33H, P67S, N106Y, A112V | 681 | 728, 776 |
| N106Y, A112V | 682 | 729, 777 |
| T18S, Y33H, A112V | 683 | 730, 778 |
| P9S, Y33H, N47S, A112V | 684 | 731, 779 |
| P42S, P67H, A112V | 685 | 732, 780 |
| P27L, L32P, P42S, A112V | 686 | 733, 781 |
| G98D, A112V | 687 | 734, 782 |
| Y33H, S35P, N106Y, A112V | 688 | 735, 783 |
| L32P, P42S, T100A, A112V | 689 | 736, 784 |
| P27S, P45S, N106I, A112V | 690 | 737, 785 |
| Y33H, N47K, A112V | 691 | 738, 786 |
| Y33H, N106Y, A112V | 692 | 739, 787 |
| K78R, D84G, A112V, F114S | 693 | 740, 788 |
| Y33H, N47K, F54L, A112V | 694 | 741, 789 |
| Y33H, A112V | 695 | 742, 790 |
| A95V, A112V | 696 | 743, 791 |
| R12W, A112V | 697 | 744, 792 |

| TABLE 4: Exemplary variant CD112 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| R12W, P27S, A112V | 698 | 745, 793 |
| Y33H, V51M, A112V | 699 | 746, 794 |
| Y33H, A112V, S118T | 700 | 747, 795 |
| Y33H, V101A, A112V, P115S | 701 | 748, 796 |
| H24R, T38N, D43G, A112V | 702 | 749, 797 |
| A112V | 703 | 750, 798 |
| P27A, A112V | 704 | 751, 799 |
| A112V, S118T | 705 | 752, 800 |
| R12W, A112V, M122I | 706 | 753, 801 |
| Q83K, N106Y, A112V | 707 | 754, 802 |
| R12W, P27S, A112V, S118T | 708 | 755, 803 |
| P28S, Y33H, A112V | 709 | 756, 804 |
| P27S, Q90R, A112V | 710 | 757, 805 |
| L15V, P27A, A112V, S118T | 711 | 758, 806 |
| Y33H, N106Y, T108I, A112V | 712 | 759, 807 |
| Y33H, P56L, V75M, V101M, A112V | 713 | 760, 808 |
| N47K, Q79R, S118F | 809 | 850, 891 |
| Q40R, P60T, A112V, S118T | 810 | 851, 892 |
| F114Y, S118F | 811 | 852, 893 |
| Y33H, K78R, S118Y | 812 | 853, 894 |
| R12W, A46T, K66M, Q79R, N106I, T113A, S118F | 813 | 854, 895 |
| Y33H, A112V, S118F | 814 | 855, 896 |
| R12W, Y33H, N106I, S118F | 815 | 856, 897 |
| L15V, Q90R, S118F | 816 | 857, 898 |
| N47K, D84G, N106I, S118Y | 817 | 858, 899 |
| L32P, S118F | 818 | 859, 900 |
| Y33H, Q79R, A112V, S118Y | 819 | 860, 901 |
| T18A, N106I, S118T | 820 | 861, 902 |
| L15V, Y33H, N106Y, A112V, S118F | 821 | 862, 903 |
| V37M, S118F | 822 | 863, 904 |
| N47K, A112V, S118Y | 823 | 864, 905 |
| A46T, A112V | 824 | 865, 906 |
| P28S, Y33H, N106I, S118Y | 825 | 866, 907 |
| P30S, Y33H, N47K, V75M, Q79R, N106I, S118Y | 826 | 867, 908 |
| V19A, N47K, N106Y, K116E, S118Y | 827 | 868, 909 |
| Q79R, T85A, A112V, S118Y | 828 | 869, 910 |
| V101M, N106I, S118Y | 829 | 870, 911 |
| Y33H, Q79R, N106I, A112V, S118T | 830 | 871, 912 |

| TABLE 4: Exemplary variant CD112 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| Q79R, A112V | 831 | 872, 913 |
| Y33H, A46T, Q79R, N106I, S118F | 832 | 873, 914 |
| A112V, G121S | 833 | 874, 915 |
| Y33H, Q79R, N106I, S118Y | 834 | 875, 916 |
| Y33H, N106I, A112V | 835 | 876, 917 |
| Y33H, A46T, V101M, A112V, S118T | 836 | 877, 918 |
| L32P, L99M, N106I, S118F | 837 | 878, 919 |
| L32P, T108A, S118F | 838 | 879, 920 |
| R12W, Q79R, A112V | 839 | 880, 921 |
| Y33H, N106Y, E110G, A112V | 840 | 881, 922 |
| Y33H, N106I, S118Y | 841 | 882, 923 |
| Q79R, S118F | 842 | 883, 924 |
| Y33H, Q79R, G98D, V101M, A112V | 843 | 884, 925 |
| N47K, T81S, V101M, A112V, S118F | 844 | 885, 926 |
| G82S, S118Y | 845 | 886, 927 |
| Y33H, A112V, S118Y | 846 | 887, 928 |
| Y33H, N47K, Q79R, N106Y, A112V | 847 | 888, 929 |
| Y33H, S118T | 848 | 889, 930 |
| R12W, Y33H, Q79R, V101M, A112V | 849 | 890, 931 |
| Y33H, Q83K, A112V, S118T | 1433 | 1457, 1481 |
| V29M, Y33H, N106I, S118F | 1434 | 1458, 1482 |
| Y33H, A46T, A112V | 1435 | 1459, 1483 |
| Y33H, Q79R, S118F | 1436 | 1460, 1484 |
| Y33H, N47K, F74L, S118F | 1437 | 1461, 1485 |
| R12W, V101M, N106I, S118Y | 1438 | 1462, 1486 |
| A46T, V101A, N106I, S118Y | 1439 | 1463, 1487 |
| N106Y, A112V, S118T | 1440 | 1464, 1488 |
| S76P, T81I, V101M, N106Y, A112V, S118F | 1441 | 1465, 1489 |
| P9R, L21V, P22L, I34M, S69F, F74L, A87V, A112V, L125A | 1442 | 1466, 1490 |
| Y33H, V101M, A112V | 1443 | 1467, 1491 |
| V29A, L32P, S118F | 1444 | 1468, 1492 |
| Y33H, V101M, N106I, A112V | 1445 | 1469, 1493 |
| R12W, Y33H, N47K, Q79R, S118Y | 1446 | 1470, 1494 |
| Y33H, A46T, A112V, S118T | 1447 | 1471, 1495 |
| Y33H, A112V, F114L, S118T | 1448 | 1472, 1496 |
| Y33H, T38A, A46T, V101M, A112V | 1449 | 1473, 1497 |
| P28S, Y33H, S69P, N106I, A112V, S118Y | 1450 | 1474, 1498 |

| TABLE 4: Exemplary variant CD112 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| Y33H, P42L, N47K, V101M, A112V | 1451 | 1475, 1499 |
| Y33H, N47K, F74S, Q83K, N106I, F111L, A112V, S118T | 1452 | 1476, 1500 |
| Y33H, A112V, S118T, V119A | 1453 | 1477, 1501 |
| Y33H, N106I, A112V, S118F | 1454 | 1478, 1502 |
| Y33H, K66M, S118F, W124L | 1455 | 1479, 1503 |
| N106I, A112V | 1456 | 1480, 1504 |

| TABLE 5: Exemplary variant CD155 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| Wild-type | 47 | 310, 353 |
| P18S, P64S, F91S | 311 | 332, 354 |
| P18S, F91S, L104P | 312 | 333, 355 |
| L44P | 313 | 334, 356 |
| A56V | 314 | 335, 357 |
| P18L, L79V, F91S | 315 | 336, 358 |
| P18S, F91S | 316 | 337, 359 |
| P18T, F91S | 317 | 338, 360 |
| P18T, S42P, F91S | 318 | 339, 361 |
| G7E, P18T, Y30C, F91S | 319 | 340, 362 |
| P18T, F91S, G111D | 320 | 341, 363 |
| P18S, F91P | 321 | 342, 364 |
| P18T, F91S, F108L | 322 | 343, 365 |
| P18T, T45A, F91S | 323 | 344, 366 |
| P18T, F91S, R94H | 324 | 345, 367 |
| P18S, Y30C, F91S | 325 | 346, 368 |
| A81V, L83P | 326 | 347, 369 |
| L88P | 327 | 348, 370 |
| R94H | 328 | 349, 371 |
| A13E, P18S, A56V, F91S | 329 | 350, 372 |
| P18T, F91S, V115A | 330 | 351, 373 |
| P18T, Q60K | 331 | 352, 374 |
| S52M | 375 | 472, 569 |
| T45Q, S52L, L104E, G111R | 376 | 473, 570 |
| S42G | 377 | 474, 571 |
| Q62F | 378 | 475, 572 |
| S52Q | 379 | 476, 573 |
| S42A, L104Q, G111R | 380 | 477, 574 |
| S42A, S52Q, L104Q, G111R | 381 | 478, 575 |

| TABLE 5: Exemplary variant CD155 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| S52W, L104E | 382 | 479, 576 |
| S42C | 383 | 480, 577 |
| S52W | 384 | 481, 578 |
| S52M, L104Q | 385 | 482, 579 |
| S42L, S52L, Q62F, L104Q | 386 | 483, 580 |
| S42W | 387 | 484, 581 |
| S42Q | 388 | 485, 582 |
| S52L | 389 | 486, 583 |
| S52R | 390 | 487, 584 |
| L104E | 391 | 488, 585 |
| G111R | 392 | 489, 586 |
| S52E | 393 | 490, 587 |
| Q62Y | 394 | 491, 588 |
| T45Q, S52M, L104E | 395 | 492, 589 |
| S42N, L104Q, G111R | 396 | 493, 590 |
| S52M, V57L | 397 | 494, 591 |
| S42N, S52Q, Q62F | 398 | 495, 592 |
| S42A, S52L, L104E, G111R | 399 | 496, 593 |
| S42W, S52Q, V57L, Q62Y | 400 | 497, 594 |
| L104Q | 401 | 498, 595 |
| S42L, S52Q, L104E | 402 | 499, 596 |
| S42C, S52L | 403 | 500, 597 |
| S42W, S52R, Q62Y, L104Q | 404 | 501, 598 |
| T45Q, S52R, L104E | 405 | 502, 599 |
| S52R, Q62F, L104Q, G111R | 406 | 503, 600 |
| T45Q, S52L, V57L, L104E | 407 | 504, 601 |
| S52M, Q62Y | 408 | 505, 602 |
| Q62F, L104E, G111R | 409 | 506, 603 |
| T45Q, S52Q | 410 | 507, 604 |
| S52L, L104E | 411 | 508, 605 |
| S42V, S52E | 412 | 509, 606 |
| T45Q, S52R, G111R | 413 | 510, 607 |
| S42G, S52Q, L104E, G111R | 414 | 511, 608 |
| S42N, S52E, V57L, L104E | 415 | 512, 609 |
| S42C, S52M, Q62F | 416 | 513, 610 |
| S42L | 417 | 514, 611 |
| S42A | 418 | 515, 612 |

| TABLE 5: Exemplary variant CD155 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| S42G, S52L, Q62F, L104Q | 419 | 516, 613 |
| S42N | 420 | 517, 614 |
| P18T, S65A, S67V, F91S | 421 | 518, 615 |
| P18F, T39A, T45Q, T61R, S65N, S67L, E73G, R78G | 422 | 519, 616 |
| P18T, T45Q, T61R, S65N, S67L | 423 | 520, 617 |
| P18F, S65A, S67V, F91S | 424 | 521, 618 |
| P18F, T45Q, T61R, S65N, S67L, F91S, L104P | 425 | 522, 619 |
| P18S, L79P, L104M | 426 | 523, 620 |
| P18S, L104M | 427 | 524, 621 |
| L79P, L104M | 428 | 525, 622 |
| P18T, T45Q, L79P | 429 | 526, 623 |
| P18T, T45Q, T61R, S65H, S67H | 430 | 527, 624 |
| P18T, A81E | 431 | 528, 625 |
| P18S, D23Y, E37P, S52G, Q62M, G80S, A81P, G99Y, S112N | 432 | 529, 626 |
| A13R, D23Y, E37P, S42P, Q62Y, A81E | 433 | 530, 627 |
| A13R, D23Y, E37P, G99Y, S112N | 434 | 531, 628 |
| A13R, D23Y, E37P, Q62M, A77V, G80S, A81P, G99Y | 435 | 532, 629 |
| P18L, E37S, Q62M, G80S, A81P, G99Y, S112N | 436 | 533, 630 |
| P18S, L104T | 437 | 534, 631 |
| P18S, Q62H, L79Q, F91S | 438 | 535, 632 |
| T45Q, S52K, Q62F, L104Q, G111R | 439 | 536, 633 |
| T45Q, S52Q, Q62Y, L104Q, G111R | 440 | 537, 634 |
| T45Q, S52Q, Q62Y, L104E, G111R | 441 | 538, 635 |
| V57A, T61M, S65W, S67A, E96D, L104T | 442 | 539, 636 |
| P18L, V57T, T61S, S65Y, S67A, L104T | 443 | 540, 637 |
| P18T, T45Q | 444 | 541, 638 |
| P18L, V57A, T61M, S65W, S67A, L104T | 445 | 542, 639 |
| T61M, S65W, S67A, L104T | 446 | 543, 640 |
| P18S, V41A, S42G, T45G, L104N | 447 | 544, 641 |
| P18H, S42G, T45I, S52T, G53R, S54H, V57L, H59E, T61S, S65D, E68G, L104N | 448 | 545, 642 |
| P18S, S42G, T45V, F58L, S67W, L104N | 449 | 546, 643 |
| P18S, T45I, L104N | 450 | 547, 644 |
| P18S, S42G, T45G, L104N, V106A | 451 | 548, 645 |
| P18H, H40R, S42G, T45I, S52T, G53R, S54H, V57L, H59E, T61S, S65D, E68G, L104Y, V106L, F108H | 452 | 549, 646 |
| E37V, S42G, T45G, L104N | 453 | 550, 647 |
| P18S, T45Q, L79P, L104T | 454 | 551, 648 |

| TABLE 5: Exemplary variant CD155 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| P18L, Q62R | 455 | 552, 649 |
| A13R, D23Y, E37P, S42L, S52G, Q62Y, A81E | 456 | 553, 650 |
| P18L, H49R, L104T, D116N | 457 | 554, 651 |
| A13R, D23Y, E37P, Q62M, G80S, A81P, L104T | 458 | 555, 652 |
| S65T, L104T | 459 | 556, 653 |
| A13R, D23Y, E37P, S52G, V57A, Q62M, K70E, L104T | 460 | 557, 654 |
| P18L, A47V, Q62Y, E73D, L104T | 461 | 558, 655 |
| H40T, V41M, A47V, S52Q, Q62L, S65T, E73R, D97G, E98S, L104T, D116N | 462 | 559, 656 |
| P18L, S42P, T45Q, T61G, S65H, S67E, L104T, D116N | 463 | 560, 657 |
| P18S, H40T, V41M, A47V, S52Q, Q62L, S65T, E73R, L104M, V106A | 464 | 561, 658 |
| H40T, V41M, A47V, S52Q, Q62L, S65T, E68G, E73R, D97G, E98S, L104T | 465 | 562, 659 |
| T45Q, S52E, L104E | 466 | 563, 660 |
| T45Q, S52E, Q62F, L104E | 467 | 564, 661 |
| P18F, T26M, L44V, Q62K, L79P, F91S, L104M, G111D | 468 | 565, 662 |
| P18S, T45S, T61K, S65W, S67A, F91S, G111R | 469 | 566, 663 |
| P18S, L79P, L104M, T107M | 470 | 567, 664 |
| P18S, S65W, S67A, M90V, V95A, L104Q, G111R | 471 | 568, 665 |
| P18S, A47G, L79P, F91S, L104M, T107A, R113W | 1551 | 1505, 1528 |
| P18T, D23G, S24A, N35D, H49L, L79P, F91S, L104M, G111R | 1552 | 1506, 1529 |
| V9L, P18S, Q60R, V75L, L79P, R89K, F91S, L104E, G111R | 1553 | 1507, 1530 |
| P18S, H49R, E73D, L79P, N85D, F91S, V95A, L104M, G111R | 1554 | 1508, 1531 |
| V11A, P18S, L79P, F91S, L104M, G111R | 1555 | 1509, 1532 |
| V11A, P18S, S54R, Q60P, Q62K, L79P, N85D, F91S, T107M | 1556 | 1510, 1533 |
| P18T, S52P, S65A, S67V, L79P, F91S, L104M, G111R | 1557 | 1511, 1534 |
| P18T, M36T, L79P, F91S, G111R | 1558 | 1512, 1535 |
| D8G, P18S, M36I, V38A, H49Q, A76E, F91S, L104M, T107A, R113W | 1559 | 1513, 1536 |
| P18S, S52P, S65A, S67V, L79P, F91S, L104M, T107S, R113W | 1560 | 1514, 1537 |
| T15I, P18T, L79P, F91S, L104M, G111R | 1561 | 1515, 1538 |
| P18F, T26M, L44V, Q62K, L79P, E82D, F91S, L104M, G111D | 1562 | 1516, 1539 |
| P18T, E37G, G53R, Q62K, L79P, F91S, E98D, L104M, T107M | 1563 | 1517, 1540 |
| P18L, K70E, L79P, F91S, V95A, G111R | 1564 | 1518, 1541 |
| V9I, Q12K, P18F, S65A, S67V, L79P, L104T, G111R, S112I | 1565 | 1519, 1542 |
| P18F, S65A, S67V, F91S, L104M, G111R | 1566 | 1520, 1543 |
| V9I, V10I, P18S, F20S, T45A, L79P, F91S, L104M, F108Y, G111R, S112V | 1567 | 1521, 1544 |

| TABLE 5: Exemplary variant CD155 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| V9L, P18L, L79P, M90I, F91S, T102S, L104M, G111R | 1568 | 1522, 1545 |
| P18C, T26M, L44V, M55I, Q62K, L79P, F91S, L104M, T107M | 1569 | 1523, 1546 |
| V9I, P18T, D23G, L79P, F91S, G111R | 1570 | 1524, 1547 |
| P18F, L79P, M90L, F91S, V95A, L104M, G111R | 1571 | 1525, 1548 |
| P18T, M36T, S65A, S67E, L79Q, A81T, F91S, G111R | 1572 | 1526, 1549 |
| V9L, P18T, Q62R, L79P, F91S, L104M, G111R | 1573 | 1527, 1550 |
| P18S, S65W, S67A, L104Q, G111R | 1574 | 1575, 1576 |
| P18T, G19D, M36T, S54N, L79P, L83Q, F91S, T107M, F108Y | 1577 | 1623, 1669 |
| V9L, P18L, M55V, S69L, L79P, A81E, F91S, T107M | 1578 | 1624, 1670 |
| P18F, H40Q, T61K, Q62K, L79P, F91S, L104M, T107V | 1579 | 1625, 1671 |
| P18S, Q32R, Q62K, R78G, L79P, F91S, T107A, R113W | 1580 | 1626, 1672 |
| Q12H, P18T, L21S, G22S, V57A, Q62R, L79P, F91S, T107M | 1581 | 1627, 1673 |
| V9I, P18S, S24P, H49Q, F58Y, Q60R, Q62K, L79P, F91S, T107M | 1582 | 1628, 1674 |
| P18T, W46C, H49R, S65A, S67V, A76T, L79P, S87T, L104M | 1583 | 1629, 1675 |
| P18S, S42T, E51G, L79P, F91S, G92W, T107M | 1584 | 1630, 1676 |
| V10F, T15S, P18L, R48Q, L79P, F91S, T107M, V115M | 1585 | 1631, 1677 |
| P18S, L21M, Y30F, N35D, R84W, F91S, T107M, D116G | 1586 | 1632, 1678 |
| P18F, E51V, S54G, Q60R, L79Q, E82G, S87T, M90I, F91S, G92R, T107M | 1587 | 1633, 1679 |
| Q16H, P18F, F91S, T107M | 1588 | 1634, 1680 |
| P18T, D23G, Q60R, S67L, L79P, F91S, T107M, V115A | 1589 | 1635, 1681 |
| D8G, V9I, V11A, P18T, T26M, S52P, L79P, F91S, G92A, T107L, V115A | 1590 | 1636, 1682 |
| V9I, P18F, A47E, G50S, E68G, L79P, F91S, T107M | 1591 | 1637, 1683 |
| P18S, M55I, Q62K, S69P, L79P, F91S, T107M | 1592 | 1638, 1684 |
| P18T, T39S, S52P, S54R, L79P, F91S, T107M | 1593 | 1639, 1685 |
| P18S, D23N, L79P, F91S, T107M, S114N | 1594 | 1640, 1686 |
| P18S, P34S, E51V, L79P, F91S, G111R | 1595 | 1641, 1687 |
| P18S, H59N, V75A, L79P, A81T, F91S, L104M, T107M | 1596 | 1642, 1688 |
| P18S, W46R, E68D, L79P, F91S, T107M, R113G | 1597 | 1643, 1689 |
| V9L, P18F, T45A, S65A, S67V, R78K, L79V, F91S, T107M, S114T | 1598 | 1644, 1690 |
| P18T, M55L, T61R, L79P, F91S, V106I, T107M | 1599 | 1645, 1691 |
| T15I, P18S, V33M, N35F, T39S, M55L, R78S, L79P, F91S, T107M | 1600 | 1646, 1692 |
| P18S, Q62K, K70E, L79P, F91S, G92E, R113W | 1601 | 1647, 1693 |
| P18F, F20I, T26M, A47V, E51K, L79P, F91S | 1602 | 1648, 1694 |
| P18T, D23A, Q60H, L79P, M90V, F91S, T107M | 1603 | 1649, 1695 |

| TABLE 5: Exemplary variant CD155 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| P18S, D23G, C29R, N35D, E37G, M55I, Q62K, S65A, S67G, R78G, L79P, F91S, L104M, T107M, Q110R | 1604 | 1650, 1696 |
| A13E, P18S, M36R, Q62K, S67T, L79P, N85D, F91S, T107M | 1605 | 1651, 1697 |
| V9I, P18T, H49R, L79P, N85D, F91S, L104T, T107M | 1606 | 1652, 1698 |
| V9A, P18F, T61S, Q62L, L79P, F91S, G111R | 1607 | 1653, 1699 |
| D8E, P18T, T61A, L79P, F91S, T107M | 1608 | 1654, 1700 |
| P18S, V41A, H49R, S54C, L79S, N85Y, L88P, F91S, L104M, T107M | 1609 | 1655, 1701 |
| V11E, P18H, F20Y, V25E, N35S, H49R, L79P, F91S, T107M, G111R | 1610 | 1656, 1702 |
| V11A, P18F, D23A, L79P, G80D, V95A, T107M | 1611 | 1657, 1703 |
| P18S, K70R, L79P, F91S, G111R | 1612 | 1658, 1704 |
| V9L, V11M, P18S, N35S, S54G, Q62K, L79P, L104M, T107M, V115M | 1613 | 1659, 1705 |
| V9L, P18Y, V25A, V38G, M55V, A77T, L79P, M90I, F91S, L104M | 1614 | 1660, 1706 |
| V10G, P18T, L72Q, L79P, F91S, T107M | 1615 | 1661, 1707 |
| P18S, H59R, A76G, R78S, L79P | 1616 | 1662, 1708 |
| V9A, P18S, M36T, S65G, L79P, F91S, L104T, G111R, S112I | 1617 | 1663, 1709 |
| P18T, S52A, V57A, Q60R, Q62K, S65C, L79P, F91T, N100Y, T107M | 1618 | 1664, 1710 |
| V11A, P18F, N35D, A47E, Q62K, L79P, F91S, G99D, T107M, S114N | 1619 | 1665, 1711 |
| V11A, P18T, N35S, L79P, S87T, F91S | 1620 | 1666, 1712 |
| V9D, V11M, Q12L, P18S, E37V, M55I, Q60R, K70Q, L79P, F91S, L104M, T 107M | 1621 | 1667, 1713 |
| T15S, P18S, Y30H, Q32L, Q62R, L79P, F91S, T107M | 1622 | 1668, 1714 |

| TABLE 6: Exemplary variant NKp30 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgC-like domain SEQ ID NO** |
| Wild-type | 54 | 1183 |
| L30V/A60V/S64P/S86G | 1178 | 1184 |
| L30V | 1179 | 1185 |
| A60V | 1180 | 1186 |
| S64P | 1181 | 1187 |
| S86G | 1182 | 1188 |

| TABLE 7: Exemplary variant CD86 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| Wild-type | 29 | 1195 |
| Q35H/H90L/Q102H | 1191 | 1196 |
| Q35H | 1192 | 1197 |

| TABLE 7: Exemplary variant CD86 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| H90L | 1193 | 1198 |
| Q102H | 1194 | 1199 |

| TABLE 8: Exemplary variant PD-L2 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| Wild-type | 31 | 1203, 1263 |
| H15Q | 1204 | 1281, 1357 |
| N24D | 1205 | 1282, 1358 |
| E44D | 1206 | 1283, 1359 |
| V89D | 1207 | 1284, 1360 |
| Q82R/V89D | 1208 | 1285, 1361 |
| E59G/Q82R | 1209 | 1286, 1362 |
| S39I/V89D | 1210 | 1287, 1363 |
| S67L/V89D | 1211 | 1288, 1364 |
| S67L/I85F | 1212 | 1289, 1365 |
| S67L/I86T | 1213 | 1290, 1366 |
| H15Q/K65R | 1214 | 1291, 1367 |
| H15Q/Q72H/V89D | 1215 | 1292, 1368 |
| H15Q/S67L/R76G | 1216 | 1293, 1369 |
| H15Q/R76G/I85F | 1217 | 1294, 1370 |
| H15Q/T47A/Q82R | 1218 | 1295, 1371 |
| H15Q/Q82R/V89D | 1219 | 1296, 1372 |
| H15Q/C23S/I86T | 1220 | 1297, 1373 |
| H15Q/S39I/I86T | 1221 | 1298, 1374 |
| H15Q/R76G/I85F | 1222 | 1299, 1375 |
| E44D/V89D/W91R | 1223 | 1300, 1376 |
| I13V/S67L/V89D | 1224 | 1301, 1377 |
| H15Q/S67L/I86T | 1225 | 1302, 1378 |
| I13V/H15Q/S67L/I86T | 1226 | 1303, 1379 |
| I13V/H15Q/E44D/V89D | 1227 | 1304, 1380 |
| I13V/S39I/E44D/Q82R/V89D | 1228 | 1305, 1381 |
| I13 V/E44D/Q82R/V89D | 1229 | 1306, 1382 |
| I13V/Q72H/R76G/I86T | 1230 | 1307, 1383 |
| I13V/H15Q/R76G/I85F | 1231 | 1308, 1384 |
| H15Q/S39I/R76G/V89D | 1232 | 1309, 1385 |
| H15Q/S67L/R76G/I85F | 1233 | 1310, 1386 |
| H15Q/T47A/Q72H/R76G/I86T | 1234 | 1311, 1387 |
| H15Q/T47A/Q72H/R76G | 1235 | 1312, 1388 |
| I13V/H15Q/T47A/Q72H/R76G | 1236 | 1313, 1389 |

| TABLE 8: Exemplary variant PD-L2 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| H15Q/E44D/R76G/I85F | 1237 | 1314, 1390 |
| H15Q/S39I/S67L/V89D | 1238 | 1315, 1391 |
| H15Q/N32D/S67L/V89D | 1239 | 1316, 1392 |
| N32D/S67L/V89D | 1240 | 1317, 1393 |
| H15Q/S67L/Q72H/R76G/V89D | 1241 | 1318, 1394 |
| H15Q/Q72H/Q74R/R76G/186T | 1242 | 1319, 1395 |
| G28V/Q72H/R76G/I86T | 1243 | 1320, 1396 |
| I13V/H15Q/S39I/E44D/S67L | 1244 | 1321, 1397 |
| E44D/S67L/Q72H/Q82R/V89D | 1245 | 1322, 1398 |
| H15Q/V89D | 1246 | 1323, 1399 |
| H15Q/T47A | 1247 | 1324, 1400 |
| I13V/H15Q/Q82R | 1248 | 1325, 1401 |
| I13V/H15Q/V89D | 1249 | 1326, 1402 |
| I13V/S67L/Q82R/V89D | 1250 | 1327, 1403 |
| I13V/H15Q/Q82R/V89D | 1251 | 1328, 1404 |
| H15Q/V31M/S67L/Q82R/V89D | 1252 | 1329, 1405 |
| I13V/H15Q/T47A/Q82R | 1253 | 1330, 1406 |
| I13V/H15Q/V31A/N45S/Q82R/V89D | 1254 | 1331, 1407 |
| H15Q/T47A/H69L/Q82R/V89D | 1256 | 1333, 1409 |
| I13V/H15Q/T47A/H69L/R76G/V89D | 1257 | 1334, 1410 |
| I12V/I13V/H15Q/T47A/Q82RN89D | 1258 | 1335, 1411 |
| I13V/H15Q/R76G/D77N/Q82R/V89D | 1259 | 1336, 1412 |
| I13V/H15Q/T47A/R76G/V89D | 1260 | 1337, 1413 |
| I13V/H15Q/T47A/Q82R/V89D | 1261 | 1338, 1414 |
| I13V/H15Q/N24D/Q82R/V89D | 1262 | 1339, 1415 |
| I13V/H15Q/I36V/T47A/S67L/V89D | 1264 | 1340, 1416 |
| H15Q/T47A/K65R/S67L/Q82R/V89D | 1265 | 1341, 1417 |
| H15Q/L33P/T47A/S67L/P71S/V89D | 1266 | 1342, 1418 |
| I13V/H15Q/Q72H/R76G/I86T | 1267 | 1343, 1419 |
| H15Q/T47A/S67L/Q82R/V89D | 1268 | 1344, 1420 |
| F2L/H15Q/D46E/T47A/Q72H/R76G/Q82R/V89D | 1269 | 1345, 1421 |
| I13V/H15Q/L33F/T47A/Q82R/V89D | 1270 | 1346, 1422 |
| I13V/H15Q/T47A/E58G/S67L/Q82R/V89D | 1271 | 1347, 1423 |
| H15Q/N24S/T47A/Q72H/R76G/V89D | 1272 | 1348, 1424 |
| I13V/H15Q/E44V/T47A/Q82R/V89D | 1273 | 1349, 1425 |
| H15Q/N18D/T47A/Q72H/V73A/R76G/I86T/V89D | 1274 | 1350, 1426 |
| I13V/H15Q/T37A/E44D/S48C/S67L/Q82R/V89D | 1275 | 1351, 1427 |
| H15Q/L33H/S67L/R76G/Q82R/V89D | 1276 | 1352, 1428 |

| TABLE 8: Exemplary variant PD-L2 polypeptides | | |
|---|---|---|
| **Mutation(s)** | **ECD SEQ ID NO** | **IgV SEQ ID NO** |
| I13V/H15Q/T47A/Q72H/R76G/I86T | 1277 | 1353, 1429 |
| H15Q/S39I/E44D/Q72H/V75G/R76G/Q82R/V89D | 1278 | 1354, 1430 |
| H15Q/T47A/S67L/R76G/Q82R/V89D | 1279 | 1355, 1431 |
| I13V/H15Q/T47A/S67L/Q72H/R76G/Q82R/V89D | 1280 | 1356, 1432 |

**[0233]** The number of such non-affinity modified or affinity modified IgSF domains present in a "stacked" immunomodulatory protein construct (whether non-wild type combinations or non-wild type arrangements) is at least 2, 3, 4, or 5 and in some embodiments exactly 2, 3, 4, or 5 IgSF domains (whereby determination of the number of affinity modified IgSF domains disregards any non-specific binding fractional sequences thereof and/or substantially immunologically inactive fractional sequences thereof).

**[0234]** In some embodiments of a stacked immunomodulatory protein provided herein, the number of IgSF domains is at least 2 wherein the number of affinity modified and the number of non-affinity modified IgSF domains is each independently at least: 0, 1, 2, 3, 4, 5, or 6. Thus, the number of affinity modified IgSF domains and the number of non-affinity modified IgSF domains, respectively, (affinity modified IgSF domain: non-affinity modified IgSF domain), can be exactly or at least: 2:0 (affinity modified: wild-type), 0:2, 2:1, 1:2, 2:2, 2:3, 3:2, 2:4, 4:2, 1:1, 1:3, 3:1, 1:4, 4:1, 1:5, or 5:1.

**[0235]** In some embodiments of a stacked immunomodulatory protein, at least two of the non-affinity modified and/or affinity modified IgSF domains are identical IgSF domains.

**[0236]** In some embodiments, a stacked immunomodulatory protein provided herein comprises at least two affinity modified and/or non-affinity modified IgSF domains from a single IgSF member but in a non-wild-type arrangement (alternatively, "permutation"). One illustrative example of a non-wild type arrangement or permutation is an immunomodulatory protein comprising a non-wild-type order of affinity modified and/or non-affinity modified IgSF domain sequences relative to those found in the wild-type PD-L1 whose IgSF domain sequences served as the source of the variant IgSF domains as provided herein. Thus, in one example, the immunomodulatory protein can comprise an IgV proximal and an IgC distal to the transmembrane domain albeit in a non-affinity modified and/or affinity modified form. The presence, in an immunomodulatory protein provided herein, of both non-wild-type combinations and non-wild-type arrangements of non-affinity modified and/or affinity modified IgSF domain is also within the scope of the provided subject matter.

**[0237]** In some embodiments of a stacked immunomodulatory protein, the non-affinity modified and/or affinity modified IgSF domains are non-identical (i.e., different) IgSF domains. Non-identical affinity modified IgSF domains specifically bind, under specific binding conditions, different cognate binding partners and are "non-identical" irrespective of whether or not the wild-type or unmodified IgSF domains from which they are engineered was the same. Thus, for example, a non-wild-type combination of at least two non-identical IgSF domains in an immunomodulatory protein can comprise at least one IgSF domain sequence whose origin is from and unique to one PD-L1, and at least one of a second IgSF domain sequence whose origin is from and unique to another IgSF family member that is not PD-L1, wherein the IgSF domains of the immunomodulatory protein are in non-affinity modified and/or affinity modified form. However, in alternative embodiments, the two non-identical IgSF domains originate from the same IgSF domain sequence but at least one is affinity modified such that they specifically bind to different cognate binding partners.

**[0238]** A plurality of non-affinity modified and/or affinity modified IgSF domains in a stacked immunomodulatory protein polypeptide chain need not be covalently linked directly to one another. In some embodiments, an intervening span of one or more amino acid residues indirectly covalently bonds the non-affinity modified and/or affinity modified IgSF domains to each other. The linkage can be via the N-terminal to C-terminal residues.

**[0239]** In some embodiments, the two or more IgSF domain, including a vIgD of PD-L1 and one or more additional IgSF domain (e.g. second or third variant IgSF domain) from another IgSF family member, are covalently or non-covalently linked. In some embodiments, the two or more IgSF domains are linked directly or indirectly, such as via a linker. In some embodiments, an intervening span of one or more amino acid residues indirectly covalently bonds IgSF domains to each other. The linkage can be via the N-terminal to C-terminal residues. In some embodiments, the linkage can be made via side chains of amino acid residues that are not located at the N-terminus or C-terminus of the IgSF domain(s). Thus, linkages can be made via terminal or internal amino acid residues or combinations thereof.

**[0240]** In some embodiments, the immunomodulatory protein contains at least two IgSF domains, each linked directly or indirectly via a linker. In some embodiments, the immunomodulatory protein contains at least three immunomodulatory proteins, each linked directly or indirectly via a linker. Various configurations are shown in FIG. 5A and 5B.

**[0241]** In some embodiments, one or more "peptide linkers" link the vIgD of PD-L1 and an one or more additional IgSF domain (e.g. second or third variant IgSF domain). In some embodiments, a peptide linker can be a single amino acid residue or greater in length. In some embodiments, the peptide linker has at least one amino acid residue but is no more than 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid residues in length. In some embodiments, the linker is a flexible linker. In some embodiments, the linker is (in one-letter amino acid code): GGGGS ("4GS"; SEQ ID NO: 1942) or multimers of the 4GS linker, such as repeats of 2, 3, 4, or 5 4GS linkers. In some embodiments, the peptide linker is $(GGGGS)_2$ (SEQ ID NO: 240) or $(GGGGS)_3$ (SEQ ID NO: 239) . In some embodiments, the linker (in one-letter amino acid code) is GSGGGGS (SEQ ID NO:1941). In some embodiments, the linker also can include a series of alanine residues alone or in addition to another peptide linker (such as a 4GS linker or multimer thereof). In some embodiments, the number of alanine residues in each series is: 2, 3, 4, 5, or 6 alanines. In some embodiments, the linker also can include a series of alanine residues alone or in addition to another peptide linker (such as a 4GS linker or multimer thereof). In some embodiments, the number of alanine residues in each series is: 2, 3, 4, 5, or 6 alanines. In some embodiments, the linker is a rigid linker. For example, the linker is an α-helical linker. In some embodiments, the linker is (in one-letter amino acid code): EAAAK or multimers of the EAAAK linker, such as repeats of 2, 3, 4, or 5 EAAAK linkers, such as set forth in SEQ ID NO: 2022 (1xEAAAK), SEQ ID NO: 2023 (3xEAAAK) or SEQ ID NO: 2024 (5xEAAAK). In some embodiments, the linker can further include amino acids introduced by cloning and/or from a restriction site, for example the linker can include the amino acids GS (in one-letter amino acid code) as introduced by use of the restriction site BAMHI. For example, in some embodiments, the linker (in one-letter amino acid code) is GSGGGGS (SEQ ID NO:1941), $GS(G_4S)_3$ (SEQ ID NO: 2031), or $GS(G_4S)_5$ (SEQ ID NO: 2032). In some examples, the linker is a 2xGGGGS followed by three alanines (GGGGSGGGGSAAA; SEQ ID NO: 241). In some cases, various combinations of peptide is used linkers.

**[0242]** In some embodiments, the non-affinity modified and/or affinity modified IgSF domains are linked by "wild-type peptide linkers" inserted at the N-terminus and/or C-terminus of a non-affinity modified and/or affinity modified IgSF domains. These linkers are also called leading sequences (N-terminal to non-affinity modified or affinity modified IgSF domain) or trailing sequences (C-terminal to non-affinity modified or affinity modified IgSF domain), and sequences that exist in the wild-type protein that span immediately

outside the structural prediction of the Ig fold of the IgSF. In some embodiments, the "wild-type linker" is an amino acid sequence that exists after the signal sequence, but before in the IgSF domain, such as the defined IgV domain, in the amino acid sequence of the wild-type protein. In some embodiments, the "wild-type" linker is an amino acid sequence that exists immediately after the IgSF domain, such as immediately after the defined IgV domain but before the IgC domain, in the amino acid sequence of the wild-type protein. These linker sequences can contribute to the proper folding and function of the neighboring IgSF domain(s). In some embodiments, there is present a leading peptide linker inserted at the N-terminus of the first IgSF domain and/or a trailing sequence inserted at the C-terminus of the first non-affinity modified and/or affinity modified IgSF domain. In some embodiments, there is present a second leading peptide linker inserted at the N-terminus of the second IgSF domain and/or a second trailing sequence inserted at the C-terminus of the second non-affinity modified and/or affinity modified IgSF domain. When the first and second non-affinity modified and/or affinity modified IgSF domains are derived from the same parental protein and are connected in the same orientation, wild-type peptide linkers between the first and second non-affinity modified and/or affinity modified IgSF domains are not duplicated. For example, when the first trailing wild-type peptide linker and the second leading wild-type peptide linker are the same, the Type II immunomodulatory protein does not comprise either the first trailing wild-type peptide linker or the second leading wild-type peptide linker.

**[0243]** In some embodiments, the Type II immunomodulatory protein comprises a first leading wild-type peptide linker inserted at the N-terminus of the first non-affinity modified and/or affinity modified IgSF domain, wherein the first leading wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain). In some embodiments, the first leading wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain).

**[0244]** In some embodiments, the Type II immunomodulatory protein further comprises a first trailing wild-type peptide linker inserted at the C-terminus of the first non-affinity modified and/or affinity modified IgSF domain, wherein the first trailing wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain). In some embodiments, the first trailing wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the first non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain).

**[0245]** In some embodiments, the Type II immunomodulatory protein further comprises a second leading wild-type peptide linker inserted at the N-terminus of the second non-affinity modified and/or affinity modified IgSF domain, wherein the second leading wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain). In some embodiments, the second leading wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately preceding domain (such as a signal peptide or an IgSF domain).

**[0246]** In some embodiments, the Type II immunomodulatory protein further comprises a second trailing wild-type peptide linker inserted at the C-terminus of the second non-affinity modified and/or affinity modified IgSF domain, wherein the second trailing wild-type peptide linker comprises at least 5 (such as at least about any of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more) consecutive amino acids from the intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain). In some embodiments, the second trailing wild-type peptide linker comprises the entire intervening sequence in the wild-type protein from which the second non-affinity modified and/or affinity modified IgSF domain is derived between the parental IgSF domain and the immediately following domain (such as an IgSF domain or a transmembrane domain).

**[0247]** In some embodiments, the two or more IgSF domain, including a vIgD of PD-L1 and one or more additional IgSF domain (e.g. second and/or third variant IgSF domain) from another IgSF family member, are linked or attached to a multimerization domain, such as to an Fc to form an Fc fusion, which, upon expression in a cell can, in some aspects, produce a dimeric multi-domain stack immunomodulatory protein. Thus, also provided are dimeric multi-domain immunomodulatory proteins.

**[0248]** In some embodiments, the variant PD-L1 polypeptide and one or more additional IgSF domain are independently linked, directly or indirectly, to the N- or C-terminus of a multimerization domain, such as an Fc region. In some embodiments, the variant PD-L1 polypeptide and at least one of the one or more additional IgSF domain are linked, directly or indirectly, and one of the variant PD-L1 and one of the one or more additional IgSF domain is also linked, directly or indirectly, to the N- or C-terminus of a multimerization domain, such as an Fc region. In some embodiments, the N- or C-terminus of a multimerization domain, such as an Fc region is linked to the variant PD-L1 polypeptide or the one or more additional IgSF domain and the other of the N- or C-terminus of the Fc region is linked to the other of the PD-L1 variant or another of the one or more additional IgSF domain. In some embodiments, linkage to the multimerization domain, such as to an Fc is via a peptide linker, e.g. a peptide linker, such as described above. In some embodiments, linkage between the variant PD-L1 and the one or more additional IgSF domain is via a peptide linker, e.g. a peptide linker, such as described above. In some embodiments, the vIgD of PD-L1, the one or more additional IgSF domains, and the multimerization domain, such as an Fc domain can be linked together in any of numerous configurations as depicted in FIG. 5A and 5B. Exemplary configurations are described in the Examples.

**[0249]** In some embodiments, the stacked immunomodulatory protein is a dimer formed by two immunomodulatory Fc fusion polypeptides. Also provided are nucleic acid molecules encoding any of the stacked immunomodulatory proteins. In some embodiments, the dimeric multi-domain stack immunomodulatory protein can be produced in cells by expression, or in some cases co-expression, of stack immunomodulatory Fc fusion polypeptides, such as described above in accord with generating dimeric Fc fusion proteins.

**[0250]** In some embodiments, the dimeric multi-domain stack immunomodulatory protein is divalent for each Fc region, monovalent for each subunit, or divalent for one subunit and tetravalent for the other.

**[0251]** In some embodiments, the dimeric multi-domain stack immunomodulatory protein is a homodimeric multi-domain stack Fc protein. In some embodiments, the dimeric multi-domain stack immunomodulatory protein comprises a first stack immunomodulatory Fc fusion polypeptide and a second stack immunomodulatory Fc fusion polypeptide in which the first and second polypeptide are the same. In some embodiments, the multi-domain stack molecule contains a first Fc fusion polypeptide containing a variant PD-L1 and a second IgSF domain and a second Fc fusion polypeptide containing the variant PD-L1 and

the second IgSF domain. In some embodiments, the multi-domain stack molecule contains a first Fc fusion polypeptide containing a variant PD-L1, a second IgSF domain, and a third IgSF domain and a second Fc fusion polypeptide containing the variant PD-L1, the second IgSF domain, and the third IgSF domain. In some embodiments, the Fc portion of the first and/or second fusion polypeptide can be any Fc as described above. In some embodiments, the Fc portion or region of the first and second fusion polypeptide is the same.

**[0252]** In some embodiments, the multi-domain stack molecule is heterodimeric, comprising two different Fc fusion polypeptides, e.g. a first and a second Fc fusion polypeptide, wherein at least one is an Fc fusion polypeptide containing at least one is an Fc fusion polypeptide containing a variant PD-L1 polypeptide and/or at least one second IgSF domain (e.g. second variant IgSF domain). In some embodiments, the first or second Fc fusion polypeptide further contains a third IgSF domain (e.g. third variant IgSF domain).

**[0253]** In some embodiments, the multi-domain stack molecule contains a first Fc fusion polypeptide containing a variant PD-L1 and a second Fc fusion polypeptide containing at a second IgSF domain, in which, in some cases, the first or second Fc fusion polypeptide additionally contains a third IgSF domain. In some embodiments, the multi-domain stack molecule contains a first Fc fusion polypeptide containing a variant PD-L1, a second IgSF domain, and in some cases, a third IgSF domain and a second Fc fusion polypeptide that is not linked to either a variant PD-L1 polypeptide or an additional IgSF domain. In some embodiments, the Fc portion or region of the first and second fusion polypeptide is the same. In some embodiments, the Fc portion or region of the first and second fusion polypeptide is different.

**[0254]** In some embodiments, the multi-domain stack molecule contains a first Fc fusion polypeptide containing 1, 2, 3, 4 or more variant PD-L1 polypeptides and 1, 2, 3, 4 or more additional IgSF domains, wherein the total number of IgSF domains in the first stack Fc fusion polypeptide is greater than 2, 3, 4, 5, 6 or more. In one example of such an embodiment, the second stack Fc fusion polypeptide contains 1, 2, 3, 4 or more variant PD-L1 polypeptides and 1, 2, 3, 4 or more additional IgSF domains, wherein the total number of IgSF domains in the second stack Fc fusion polypeptide is greater than 2, 3, 4, 5, 6 or more. In another example of such an embodiment, the second Fc fusion polypeptide is not linked to either a variant PD-L1 polypeptide or additional IgSF domain.

**[0255]** In some embodiments, the heterodimeric stack molecule contains a first stack immunomodulatory Fc fusion polypeptide and a second stack immunomodulatory Fc fusion polypeptide in which the first and second polypeptide are different. In some embodiments, a heterodimeric stack molecule contains a first Fc polypeptide fusion containing an Fc region and a a first variant PD-L1 polypeptide and/or second IgSF domain (e.g. second variant IgSF domain) and a second polypeptide fusion containing an Fc region and the other of the first variant PD-L1 polypeptide or the second IgSF domain. In some embodiments, a heterodimeric stack molecule contains a first Fc polypeptide fusion containing an Fc region and a first variant PD-L1 polypeptide and/or second IgSF domain (e.g. second variant IgSF domain) and a second Fc polypeptide fusion containing an Fc region and the first variant PD-L1 polypeptide and second IgSF domain (e.g. second variant IgSF domain) but in a different orientation or configuration from the first Fc region. In some embodiments, the first and/or second Fc fusion polypeptide also contains a third IgSF domain (e.g. third variant IgSF domain).

**[0256]** In some embodiments, the Fc domain of one or both of the first and second stacked immunomodulatory Fc fusion polypeptide comprises a modification (e.g. substitution) such that the interface of the Fc molecule is modified to facilitate and/or promote heterodimerization. In some embodiments, modifications include introduction of a protuberance (knob) into a first Fc polypeptide and a cavity (hole) into a second Fc polypeptide such that the protuberance is positionable in the cavity to promote complexing of the first and second Fc-containing polypeptides. Amino acids targeted for

replacement and/or modification to create protuberances or cavities in a polypeptide are typically interface amino acids that interact or contact with one or more amino acids in the interface of a second polypeptide.

**[0257]** In some embodiments, a sequence of amino acids is added preceding the Fc sequence for constructs in which the Fc sequence was the N-terminal portion of the sequence. In some cases, the sequence of amino acids HMSSVSAQ (SEQ ID NO:1156) is added immediately preceding the Fc sequence for constructs in which the Fc sequence was the N-terminal portion of the sequence. In some embodiments, a heterodimeric stack molecule contains a first Fc polypeptide fusion containing an Fc region (knob) and a first variant polypeptide and/or second IgSF domain (e.g. second variant IgSF domain) and a second Fc polypeptide fusion containing an Fc region (hole) contains a stuffer sequence HMSSVSAQ (SEQ ID NO:1156) immediately preceding both Fc regions of the first and second Fc polypeptide fusion.

**[0258]** In some embodiments, a first polypeptide that is modified to contain protuberance (hole) amino acids include replacement of a native or original amino acid with an amino acid that has at least one side chain which projects from the interface of the first polypeptide and is therefore positionable in a compensatory cavity (hole) in an adjacent interface of a second polypeptide. Most often, the replacement amino acid is one which has a larger side chain volume than the original amino acid residue. One of skill in the art knows how to determine and/or assess the properties of amino acid residues to identify those that are ideal replacement amino acids to create a protuberance. In some embodiments, the replacement residues for the formation of a protuberance are naturally occurring amino acid residues and include, for example, arginine (R), phenylalanine (F), tyrosine (Y), or tryptophan (W). In some examples, the original residue identified for replacement is an amino acid residue that has a small side chain such as, for example, alanine, asparagine, aspartic acid, glycine, serine, threonine, or valine.

**[0259]** In some embodiments, a second polypeptide that is modified to contain a cavity (hole) is one that includes replacement of a native or original amino acid with an amino acid that has at least one side chain that is recessed from the interface of the second polypeptide and thus is able to accommodate a corresponding protuberance from the interface of a first polypeptide. Most often, the replacement amino acid is one which has a smaller side chain volume than the original amino acid residue. One of skill in the art knows how to determine and/or assess the properties of amino acid residues to identify those that are ideal replacement residues for the formation of a cavity. Generally, the replacement residues for the formation of a cavity are naturally occurring amino acids and include, for example, alanine (A), serine (S), threonine (T) and valine (V). In some examples, the original amino acid identified for replacement is an amino acid that has a large side chain such as, for example, tyrosine, arginine, phenylalanine, or tryptophan.

**[0260]** The CH3 interface of human IgG1, for example, involves sixteen residues on each domain located on four anti-parallel β-strands which buries 1090 Å2 from each surface (see e.g., Deisenhofer et al. (1981) Biochemistry, 20:2361-2370; Miller et al., (1990) J Mol. Biol., 216, 965-973; Ridgway et al., (1996) Prot. Engin., 9: 617-621; U.S. Pat. No. 5,731,168). Modifications of a CH3 domain to create protuberances or cavities are described, for example, in U.S. Pat. No. 5,731,168; International Patent Applications WO98/50431 and WO 2005/063816; and Ridgway et al., (1996) Prot. Engin., 9: 617-621. In some examples, modifications of a CH3 domain to create protuberances or cavities are typically targeted to residues located on the two central anti-parallel β-strands. The aim is to minimize the risk that the protuberances which are created can be accommodated by protruding into the surrounding solvent rather than being accommodated by a compensatory cavity in the partner CH3 domain.

**[0261]** In some embodiments, the heterodimeric molecule contains a T366W mutation in the CH3 domain of the "knobs chain" and T366S, L368A, Y407V mutations in the CH3 domain of the "hole chain". In some

cases, an additional interchain disulfide bridge between the CH3 domains can also be used (Merchant, A. M., et al., Nature Biotech. 16 (1998) 677-681) e.g. by introducing a Y349C mutation into the CH3 domain of the "knobs" or "hole" chain and a E356C mutation or a S354C mutation into the CH3 domain of the other chain. In some embodiments, the heterodimeric molecule contains S354C, T366W mutations in one of the two CH3 domains and Y349C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In some embodiments, the heterodimeric molecule comprises E356C, T366W mutations in one of the two CH3 domains and Y349C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In some embodiments, the heterodimeric molecule comprises Y349C, T366W mutations in one of the two CH3 domains and E356C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. In some embodiments, the heterodimeric molecule comprises Y349C, T366W mutations in one of the two CH3 domains and S354C, T366S, L368A, Y407V mutations in the other of the two CH3 domains. Examples of other knobs-in-holes technologies are known in the art, e.g. as described by EP 1 870 459 A1.

**[0262]** In some embodiments, the Fc region of the heterodimeric molecule additionally can contain one or more other Fc mutation, such as any described above. In some embodiments, the heterodimer molecule contains an Fc region with a mutation that reduces effector function.

**[0263]** In some embodiments, an Fc variant containing CH3 protuberance(knob) or cavity(hole) modifications can be joined to a stacked immunomodulatory polypeptide anywhere, but typically via its N- or C-terminus, to the N- or C-terminus of a first and/or second stacked immunomodulatory polypeptide, such as to form a fusion polypeptide. The linkage can be direct or indirect via a linker. Typically, a knob and hole molecule is generated by co-expression of a first stacked immunomodulatory polypeptide linked to an Fc variant containing CH3 protuberance modification(s) with a second stacked immunomodulatory polypeptide linked to an Fc variant containing CH3 cavity modification(s).

**[0264]** There is provided herein a homodimeric multi-domain stack molecule produced from a stack immunomodulatory Fc fusion polypeptide containing an IgSF domain, e.g. IgV domain, of a variant PD-L1 polypeptide and a second IgSF domain, e.g. IgV, of a variant CD155 polypeptide. In some embodiments, the first and second immunomodulatory Fc fusion polypeptide of the multi-domain stack molecule has the sequence set forth in any of SEQ ID NOS: 1716, 1717, 1718, 1719, 1720 or 1721 or a sequence of amino acids that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 1716, 1717, 1718, 1719, 1720 or 1721 and contains the one more amino acid modifications in the variant PD-L1 and/or CD155 IgSF domain. In some embodiments, the resulting multi-domain stack molecules bind to both TIGIT and PD-1. In some aspects, the binding to TIGIT is to the same or similar degree or, in some cases, is increased, compared to the binding to TIGIT of the corresponding IgSF domain of unmodified or wild-type PD-L1 or CD155. In some aspects, the binding to PD-1 is to the same or similar degree, or, in some cases, is increased, compared to the binding to PD-1 of the corresponding IgSF domain of unmodified or wild-type PD-L1. In some embodiments, the binding to TIGIT or PD-1 is at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the binding to TIGIT or PD-1 of the non-stacked form of the variant PD-L1 IgSF-Fc. In some embodiments, the binding to TIGIT is at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the binding to TIGIT of the non-stacked form of the variant CD155 IgSF-Fc. In some embodiments, the resulting multi-domain stack molecule increases T cell immune responses compared to the non-stack variant PD-L1 IgSF-Fc and/or variant CD155-IgSF-Fc, such as determined in a reporter assay. In some embodiments, the increase is greater than 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5.0-fold or more.

**[0265]** There is provided herein a homodimeric multi-domain stack molecule produced from a stack immunomodulatory Fc fusion polypeptide containing an IgSF domain, e.g. IgV domain, of a variant PD-L1 polypeptide, a second IgSF domain, e.g. IgV, of a variant CD155 polypeptide and a third IgSF domain, e.g. IgV, of a variant CD112 polypeptide. In some embodiments, the first and second immunomodulatory Fc

fusion polypeptide of the multi-domain stack molecule has the sequence set forth in any of SEQ ID NOS: 1722, 1723 and 1724 or a sequence of amino acids that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 1722, 1723 and 1724 and contains the one more amino acid modifications in the variant CD112, CD155 and/or PD-L1 IgSF domain. In some embodiments, the resulting multi-domain stack molecules bind to both TIGIT, CD112R and PD-1. In some aspects, the binding to TIGIT is to the same or similar degree or, in some cases, is increased, compared to the binding to TIGIT of the corresponding IgSF domain of unmodified or wild-type CD112 or CD155. In some aspects, the binding to CD112R is to the same or similar degree, or, in some cases, is increased, compared to the binding to CD112R of the corresponding IgSF domain of unmodified or wild-type CD112. In some embodiments, the binding to TIGIT or CD112R is at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the binding to TIGIT or CD112R of the non-stacked form of the variant CD112 IgSF-Fc. In some embodiments, the binding to TIGIT is at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the binding to TIGIT of the non-stacked form of the variant CD155 IgSF-Fc. In some embodiments, the resulting multi-domain stack molecule increases T cell immune responses compared to the non-stack variant CD112 IgSF-Fc, variant CD155-IgSF-Fc and/or variant PD-L1-IgSF-Fc, such as determined in a reporter assay. In some embodiments, the increase is greater than 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 2.0-fold, 3.0-fold, 4.0-fold, 5.0-fold or more.

**C. Conjugates and Fusions of Variant Polypeptides and Immunomodulatory Proteins**

**[0266]** In some embodiments, the variant polypeptides provided herein, which are immunomodulatory proteins comprising variants of an Ig domain of the IgSF family (vIgD), can be conjugated with or fused with a moiety, such as an effector moiety, such as another protein, directly or indirectly, to form a conjugate ("IgSF conjugate"). In some embodiments, the attachment can be covalent or non-covalent, e.g., via a biotin-streptavidin non-covalent interaction. In some embodiments, the moiety can be a targeting moiety, a small molecule drug (non-polypeptide drug of less than 500 daltons molar mass), a toxin, a cytostatic agent, a cytotoxic agent, an immunosuppressive agent, a radioactive agent suitable for diagnostic purposes, a radioactive metal ion for therapeutic purposes, a prodrug-activating enzyme, an agent that increases biological half-life, or a diagnostic or detectable agent.

**[0267]** In some embodiments the effector moiety is a therapeutic agent, such as a cancer therapeutic agent, which is either cytotoxic, cytostatic or otherwise provides some therapeutic benefit. In some embodiments, the effector moiety is a targeting moiety or agent, such as an agent that targets a cell surface antigen, e.g., an antigen on the surface of a tumor cell. In some embodiments, the effector moiety is a label, which can generate a detectable signal, either directly or indirectly. In some embodiments, the effector moiety is a toxin. In some embodiments, the effector moiety is a protein, peptide, nucleic acid, small molecule or nanoparticle.

**[0268]** In some embodiments, 1, 2, 3, 4, 5 or more effector moieties, which can be the same or different, are conjugated, linked or fused to the variant polypeptide or protein to form an IgSF conjugate. In some embodiments, such effector moieties can be attached to the variant polypeptide or immunomodulatory protein using various molecular biological or chemical conjugation and linkage methods known in the art and described below. In some embodiments, linkers such as peptide linkers, cleavable linkers, non-cleavable linkers or linkers that aid in the conjugation reaction, can be used to link or conjugate the effector moieties to the variant polypeptide or immunomodulatory protein.

**[0269]** In some embodiments, the IgSF conjugate comprises the following components: (protein or polypeptide), $(L)_q$ and $(\text{effector moiety})_m$, wherein the protein or polypeptide is any of the described variant

polypeptides or immunomodulatory proteins capable of binding one or more cognate counter structure ligands as described; L is a linker for linking the protein or polypeptide to the moiety; m is at least 1; q is 0 or more; and the resulting IgSF conjugate binds to the one or more counter structure ligands. In particular embodiments, m is 1 to 4 and q is 0 to 8.

**[0270]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a targeting agent that binds to a cell surface molecule, for example, for targeted delivery of the variant polypeptide or immunomodulatory protein to a specific cell. In some embodiments, the targeting agent is a molecule(s) that has the ability to localize and bind to a molecule present on a normal cell/tissue and/or tumor cell/tumor in a subject. In other words, IgSF conjugates comprising a targeting agent can bind to a ligand (directly or indirectly), which is present on a cell, such as a tumor cell. The targeting agents of the invention contemplated for use include antibodies, polypeptides, peptides, aptamers, other ligands, or any combination thereof, that can bind a component of a target cell or molecule.

**[0271]** In some embodiments, the targeting agent binds a tumor cell(s) or can bind in the vicinity of a tumor cell(s) (e.g., tumor vasculature or tumor microenvironment) following administration to the subject. The targeting agent may bind to a receptor or ligand on the surface of the cancer cell. In another aspect of the invention, a targeting agent is selected which is specific for a noncancerous cells or tissue. For example, a targeting agent can be specific for a molecule present normally on a particular cell or tissue. Furthermore, in some embodiments, the same molecule can be present on normal and cancer cells. Various cellular components and molecules are known. For example, if a targeting agent is specific for EGFR, the resulting IgSF conjugate can target cancer cells expressing EGFR as well as normal skin epidermal cells expressing EGFR. Therefore, in some embodiments, an IgSF conjugate of the invention can operate by two separate mechanisms (targeting cancer and non-cancer cells).

**[0272]** In various aspects of the invention disclosed herein an IgSF conjugate of the invention comprises a targeting agent which can bind/target a cellular component, such as a tumor antigen, a bacterial antigen, a viral antigen, a mycoplasm antigen, a fungal antigen, a prion antigen, an antigen from a parasite. In some aspects, a cellular component, antigen or molecule can each be used to mean, a desired target for a targeting agent. For example, in various embodiments, a targeting agent is specific for or binds to a component, which includes but is not limited to, epidermal growth factor receptor (EGFR, ErbB-1, HER1), ErbB-2 (HER2/neu), ErbB-3/HER3, ErbB-4/HER4, EGFR ligand family; insulin-like growth factor receptor (IGFR) family, IGF-binding proteins (IGFBPs), IGFR ligand family; platelet derived growth factor receptor (PDGFR) family, PDGFR ligand family; fibroblast growth factor receptor (FGFR) family, FGFR ligand family, vascular endothelial growth factor receptor (VEGFR) family, VEGF family; HGF receptor family; TRK receptor family; ephrin (EPH) receptor family; AXL receptor family; leukocyte tyrosine kinase (LTK) receptor family; TIE receptor family, angiopoietin 1,2; receptor tyrosine kinase-like orphan receptor (ROR) receptor family, e.g. ROR1; CD171 (L1CAM); B7-H6 (NCR3LG1); PD-L1, tumor glycosylation antigen, e.g. sTn or Tn, such as sTn Ag of MUC1; LHR (LHCGR); phosphatidylserine, discoidin domain receptor (DDR) family; RET receptor family; KLG receptor family; RYK receptor family; MuSK receptor family; Transforming growth factor-α (TGF-α) receptors, TGF-β; Cytokine receptors, Class I (hematopoietin family) and Class II (interferon/IL-10 family) receptors, tumor necrosis factor (TNF) receptor superfamily (TNFRSF), death receptor family; cancer-testis (CT) antigens, lineage-specific antigens, differentiation antigens, alpha-actinin-4, ARTCl, breakpoint cluster region-Abelson (Bcr-abl) fusion products, B-RAF, caspase-5 (CASP-5), caspase-8 (CASP-8), β-catenin (CTNNB1), cell division cycle 27 (CDC27), cyclin-dependent kinase 4 (CDK4), CDKN2A, COA-I, dek-can fusion protein, EFTUD-2, Elongation factor 2 (ELF2), Ets variant gene 6/acute myeloid leukemia 1 gene ETS (ETC6-AML1) fusion protein, fibronectin (FN), e.g. the extradomain A (EDA) of fibronectin, GPNMB, low density lipid receptor/GDP-L fucose: β-D-galactose 2-α-L-fucosyltransferase (LDLR/FUT) fusion protein, HLA-A2. arginine to isoleucine exchange at residue 170 of

the α-helix of the α2-domain in the HLA-A2gene (HLA-A*201-R170I), HLA-Al 1, heat shock protein 70-2 mutated (HSP70-2M), K1AA0205, MART2, melanoma ubiquitous mutated 1, 2, 3 (MUM-I, 2, 3), prostatic acid phosphatase (PAP), neo-PAP, Myosin class I, NFYC, OGT, OS-9, pml-RARα fusion protein, PRDX5, PTPRK, K-ras (KRAS2), N-ras (NRAS), HRAS, RBAF600, SIRT2, SNRPDI, SYT-SSXI or -SSX2 fusion protein, Triosephosphate Isomerase, BAGE, BAGK- 1, BAGE-2,3,4,5, GAGE-1,2,3,4,5,6,7,8, GnT-V (aberrant N-acetyl glucosaminyl transferase V, MGAT5), HERV-K-MEL, KK-LC, KM-HN-I, LAGE, LAGE-I, CTL-recognized antigen on melanoma (CAMEL), MAGE-Al (MAGE-I), MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A8, MAGE-A9, MAGE-AIO, MAGE-Al I, MAGE-A12, MAGE-3, MAGE-BI, MAGE-B2, MAGE-B5, MAGE-B6, MAGE- CI, MAGE-C2, mucin 1 (MUCI), MART-1/Melan-A (MLANA), gpIOO, gpIOO/ Pmell7 (SILV), tyrosinase (TYR), TRP-I, HAGE, NA-88, NY-ESO-I, NY-ESO-I/LAGE-2, SAGE, SpI7, SSX-1,2,3,4, TRP2-INT2, carcino-embryonic antigen (CEA), Kallikrein 4, mammaglobin-A, OAI, prostate specific antigen (PSA), TRP- 1/ gp75, TRP-2, adipophilin, interferon inducible protein absent in melanoma 2 (AIM-2), BING-4, CPSF, cyclin DI, epithelial cell adhesion molecule (Ep-CAM), EphA3, fibroblast growth factor-5 (FGF-5), glycoprotein 250 (gp250), EGFR (ERBB1), HER-2/neu (ERBB2), interleukin 13 receptor α2 chain (IL13Rα2), IL-6 receptor, intestinal carboxyl esterase (iCE), alpha-feto protein (AFP), M-CSF, mdm-2, MUCI, p53 (TP53), PBF, PRAME, PSMA, RAGE-I, RNF43, RU2AS, SOXIO, STEAPI, survivin (BIRC5), human telomerase reverse transcriptase (hTERT), telomerase, Wilms' tumor gene (WTI), SYCPI, BRDT, SPANX, XAGE, ADAM2, PAGE-5, LIP1, CTAGE-I, CSAGE, MMAI, CAGE, BORIS, HOM-TES-85, AF15qI4, HCA661, LDHC, MORC, SGY-1, SPOI 1, TPXI, NY-SAR-35, FTHL17, NXF2, TDRDI, TEX15, FATE, TPTE, immunoglobulin idiotypes, Bence-Jones protein, estrogen receptors (ER), androgen receptors (AR), CD40, CD30, CD20, CD 19, CD33, cancer antigen 72-4 (CA 72-4), cancer antigen 15-3 (CA 15-3), cancer antigen 27- 29 (CA 27-29), cancer antigen 125 (CA 125), cancer antigen 19-9 (CA 19-9), β-human chorionic gonadotropin, β-2 microglobulin, squamous cell carcinoma antigen, neuron-specific enolase, heat shock protein gp96, GM2, sargramostim, CTLA-4, 707 alanine proline (707-AP), adenocarcinoma antigen recognized by T cells 4 (ART- 4), carcinoembryogenic antigen peptide-1 (CAP-I), calcium-activated chloride channel-2 (CLCA2), cyclophilin B (Cyp-B), human signet ring tumor-2 (HST-2), Human papilloma virus (HPV) proteins (HPV-E6, HPV-E7, major or minor capsid antigens, others), Epstein-Barr virus (EBV) proteins (EBV latent membrane proteins - LMPI, LMP2; others), Hepatitis B or C virus proteins, and HIV proteins.

**[0273]** In some embodiments, an IgSF conjugate, through its targeting agent, will bind a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby promoting killing of targeted cells via modulation of the immune response, (e.g., by activation of co-stimulatory molecules or inhibition of negative regulatory molecules of immune cell activation), inhibition of survival signals (e.g., growth factor or cytokine or hormone receptor antagonists), activation of death signals, and/or immune-mediated cytotoxicity, such as through antibody dependent cellular cytotoxicity. Such IgSF conjugates can function through several mechanisms to prevent, reduce or eliminate tumor cells, such as to facilitate delivery of conjugated effector moieties to the tumor target, such as through receptor-mediated endocytosis of the IgSF conjugate; or such conjugates can recruit, bind, and/or activate immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells). Moreover, in some instances one or more of the foregoing pathways may operate upon administration of one or more IgSF conjugates of the invention.

**[0274]** In some embodiments, an IgSF conjugate, through its targeting agent, will be localized to, such as bind to, a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby modulating cells of the immune response in the vicinity of the tumor. In some embodiments, the targeting agent facilitates delivery of the conjugated IgSF (e.g. vIgD) to the tumor target, such as to interact with its cognate binding partner to alter signaling of immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells) bearing the cognate binding partner. In some embodiments, localized delivery mediates an antagonizing or blocking activity of the PD-1 inhibitory receptor. In some

embodiments, localized delivery agonizes the PD-1 inhibitory receptor, which, in some cases, can occur where there is proximal clustering of an activating receptor.

**[0275]** In some embodiments, the targeting agent is an immunoglobulin. As used herein, the term "immunoglobulin" includes natural or artificial mono- or polyvalent antibodies including, but not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, signle chain Fv (scFv); anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, e.g., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgGl, IgG2, IgG3, IgG4, IgAl , and IgA2) or subclass of immunoglobulin molecule.

**[0276]** In some embodiments, an IgSF conjugate, through its antibody targeting moiety, will bind a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby promoting apoptosis of targeted cells via modulation of the immune response, (e.g., by activation of co-stimulatory molecules or inhibition of negative regulatory molecules of immune cell activation), inhibition of survival signals (e.g., growth factor or cytokine or hormone receptor antagonists), activation of death signals, and/or immune-mediated cytotoxicity, such as through antibody dependent cellular cytotoxicity. Such IgSF conjugates can function through several mechanisms to prevent, reduce or eliminate tumor cells, such as to facilitate delivery of conjugated effector moieties to the tumor target, such as through receptor-mediated endocytosis of the IgSF conjugate; or such conjugates can recruit, bind, and/or activate immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells).

**[0277]** In some embodiments, an IgSF conjugate, through its antibody targeting moiety, will bind a cellular component of a tumor cell, tumor vasculature or tumor microenvironment, thereby modulating the immune response (e.g., by activation of co-stimulatory molecules or inhibition of negative regulatory molecules of immune cell activation). In some embodiments, such conjugates can recognize, bind, and/or modulate (e.g. inhibit or activate) immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells, T cells, B cells).

**[0278]** Antibody targeting moieties of the invention include antibody fragments that include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. Also included in the invention are Fc fragments, antigen-Fc fusion proteins, and Fc-targeting moiety conjugates or fusion products (Fc-peptide, Fc-aptamer). The antibody targeting moieties of the invention may be from any animal origin including birds and mammals. In one aspect, the antibody targeting moieties are human, murine (e.g., mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken. Further, such antibodies may be humanized versions of animal antibodies. The antibody targeting moieties of the invention may be monospecific, bispecific, trispecific, or of greater multi specificity.

**[0279]** In various embodiments, an antibody/targeting moiety recruits, binds, and/or activates immune cells (e.g. NK cells, monocytes/macrophages, dendritic cells) via interactions between Fc (in antibodies) and Fc receptors (on immune cells) and via the conjugated variant polypeptides or immunomodulatory proteins provided herein. In some embodiments, an antibody/targeting moiety recognizes or binds a tumor agent via and localizes to the tumor cell the conjugated variant polypeptides or immunomodulatory proteins

provided herein to facilitate modulation of immune cells in the vicinity of the tumor.

**[0280]** Examples of antibodies which can be incorporated into IgSF conjugates include but are not limited to antibodies such as Cetuximab (IMC-C225; Erbitux®), Trastuzumab (Herceptin®), Rituximab (Rituxan®; MabThera®), Bevacizumab (Avastin®), Alemtuzumab (Campath®; Campath-1H®; Mabcampath®), Panitumumab (ABX-EGF; Vectibix®), Ranibizumab (Lucentis®), Ibritumomab, Ibritumomab tiuxetan, (Zevalin ®), Tositumomab, Iodine I 131 Tositumomab (BEXXAR®), Catumaxomab (Removab®), Gemtuzumab, Gemtuzumab ozogamicine (Mylotarg®), Abatacept (CTLA4-Ig; Orencia®), Belatacept (L104EA29YIg; LEA29Y; LEA), Ipilimumab (MDX-010; MDX-101), Tremelimumab (ticilimumab; CP-675,206), PRS-010, PRS-050, Aflibercept (VEGF Trap, AVE005), Volociximab (M200), F200, MORAb-009, SS1P (CAT-5001), Cixutumumab (IMC-A12), Matuzumab (EMD72000), Nimotuzumab (h-R3), Zalutumumab (HuMax-EGFR), Necitumumab IMC-11F8, mAb806 / ch806, Sym004, mAb-425, Panorex @ (17-1A) (murine monoclonal antibody); Panorex @ (17-1A) (chimeric murine monoclonal antibody); IDEC-Y2B8 (murine, anti- CD2O MAb); BEC2 (anti-idiotypic MAb, mimics the GD epitope) (with BCG); Oncolym (Lym-1 monoclonal antibody); SMART MI95 Ab, humanized 13' ILYM-I (Oncolym), Ovarex (B43.13, anti-idiotypic mouse MAb); MDX-210 (humanized anti-HER-2 bispecific antibody); 3622W94 MAb that binds to EGP40 (17-1A) pancarcinoma antigen on adenocarcinomas; Anti-VEGF, Zenapax (SMART Anti-Tac (IL-2 receptor); SMART MI95 Ab, humanized Ab, humanized); MDX-210 (humanized anti- HER-2 bispecific antibody); MDX-447 (humanized anti-EGF receptor bispecific antibody); NovoMAb-G2 (pancarcinoma specific Ab); TNT (chimeric MAb to histone antigens); TNT (chimeric MAb to histone antigens); Gliomab-H (Monoclon s - Humanized Abs); GNI-250 Mab; EMD-72000 (chimeric-EGF antagonist); LymphoCide (humanized LL2 antibody); and MDX-260 bispecific, targets GD-2, ANA Ab, SMART IDIO Ab, SMART ABL 364 Ab or ImmuRAIT-CEA. As illustrated by the forgoing list, it is conventional to make antibodies to a particular target epitope.

**[0281]** In some embodiments, the antibody targeting moiety is a full length antibody, or antigen-binding fragment thereof, containing an Fc domain. In some embodiments, the variant polypeptide or immunomodulatory protein is conjugated to the Fc portion of the antibody targeting moiety, such as by conjugation to the N-terminus of the Fc portion of the antibody.

**[0282]** In some embodiments, the vIgD is linked, directly or indirectly, to the N- or C-terminus of the light and/or heavy chain of the antibody. In some embodiments, linkage can be via a peptide linker, such as any described above. Various configurations can be constructed. Fig. 7A-7C depict exemplary configurations. In some embodiments, the antibody conjugate can be produced by co-expression of the heavy and light chain of the antibody in a cell.

**[0283]** In one aspect of the invention, the targeting agent is an aptamer molecule. For example, in some embodiments, the aptamer is comprised of nucleic acids that function as a targeting agent. In various embodiments, an IgSF conjugate of the invention comprises an aptamer that is specific for a molecule on a tumor cell, tumor vasculature, and/or a tumor microenvironment. In some embodiments, the aptamer itself can comprise a biologically active sequence, in addition to the targeting module (sequence), wherein the biologically active sequence can induce an immune response to the target cell. In other words, such an aptamer molecule is a dual use agent. In some embodiments, an IgSF conjugate of the invention comprises conjugation of an aptamer to an antibody, wherein the aptamer and the antibody are specific for binding to separate molecules on a tumor cell, tumor vasculature, tumor microenvironment, and/or immune cells.

**[0284]** The term "aptamer" includes DNA, RNA or peptides that are selected based on specific binding

properties to a particular molecule. For example, an aptamer(s) can be selected for binding a particular gene or gene product in a tumor cell, tumor vasculature, tumor microenvironment, and/or an immune cell, as disclosed herein, where selection is made by methods known in the art and familiar to one of skill in the art.

**[0285]** In some aspects of the invention the targeting agent is a peptide. For example, the variant polypeptides or immunomodulatory proteins provided herein can be conjugated to a peptide which can bind with a component of a cancer or tumor cells. Therefore, such IgSF conjugates of the invention comprise peptide targeting agents which binds to a cellular component of a tumor cell, tumor vasculature, and/or a component of a tumor microenvironment. In some embodiments, targeting agent peptides can be an antagonist or agonist of an integrin. Integrins, which comprise an alpha and a beta subunit, include numerous types well known to a skilled artisan.

**[0286]** In one embodiment, the targeting agent is Vvβ3. Integrin Vvβ3 is expressed on a variety of cells and has been shown to mediate several biologically relevant processes, including adhesion of osteoclasts to bone matrix, migration of vascular smooth muscle cells, and angiogenesis. Suitable targeting molecules for integrins include RGD peptides or peptidomimetics as well as non-RGD peptides or peptidomimetics (see, e.g., U.S. Pat. Nos. 5,767,071 and 5,780,426) for other integrins such as V4.βi (VLA-4), V4-P7 (see, e.g., U.S. Pat. No. 6,365,619; Chang et al, Bioorganic & Medicinal Chem Lett, 12:159-163 (2002); Lin et al., Bioorganic & Medicinal Chem Lett, 12:133-136 (2002)), and the like.

**[0287]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a therapeutic agent. In some embodiments, the therapeutic agent includes, for example, daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., Cancer Immunol. Immunother. 21:183-187, 1986). In some embodiments, the therapeutic agent has an intracellular activity. In some embodiments, the IgSF conjugate is internalized and the therapeutic agent is a cytotoxin that blocks the protein synthesis of the cell, therein leading to cell death. In some embodiments, the therapeutic agent is a cytotoxin comprising a polypeptide having ribosome-inactivating activity including, for example, gelonin, bouganin, saporin, ricin, ricin A chain, bryodin, diphtheria toxin, restrictocin, Pseudomonas exotoxin A and variants thereof. In some embodiments, where the therapeutic agent is a cytotoxin comprising a polypeptide having a ribosome-inactivating activity, the IgSF conjugate must be internalized upon binding to the target cell in order for the protein to be cytotoxic to the cells.

**[0288]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a toxin. In some embodiments, the toxin includes, for example, bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al., J.Nat. Cancer Inst. 92(19):1573-1581 (2000); Mandler et al., Bioorganic & Med. Chem. Letters 10:1025- 1028 (2000); Mandler et al., Bioconjugate Chem. 13:786-791 (2002)), maytansinoids (EP 1391213; Liu et al., Proc. Natl. Acad. Sci. USA 93:8618-8623 (1996)), and calicheamicin (Lode et al., Cancer Res. 58:2928 (1998); Hinman et al., Cancer Res. 53:3336-3342 (1993)). The toxins may exert their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition.

**[0289]** In some embodiments, there is provided an IgSF conjugate comprising a variant polypeptide or immunomodulatory protein provided herein conjugated with a label, which can generate a detectable signal, indirectly or directly. These IgSF conjugates can be used for research or diagnostic applications, such as for the in vivo detection of cancer. The label is preferably capable of producing, either directly or indirectly, a detectable signal. For example, the label may be radio-opaque or a radioisotope, such as 3H, 14C, 32P, 35S, 123I, 125I, 131I; a fluorescent (fluorophore) or chemiluminescent (chromophore)

compound, such as fluorescein isothiocyanate, rhodamine or luciferin; an enzyme, such as alkaline phosphatase, β-galactosidase or horseradish peroxidase; an imaging agent; or a metal ion. In some embodiments, the label is a radioactive atom for scintigraphic studies, for example 99Tc or 123I, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as zirconium-89, iodine-123, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron. Zirconium-89 may be complexed to various metal chelating agents and conjugated to antibodies, e.g., for PET imaging (WO 2011/056983). In some embodiments, the IgSF conjugate is detectable indirectly. For example, a secondary antibody that is specific for the IgSF conjugate and contains a detectable label can be used to detect the IgSF conjugate.

**[0290]** The IgSF conjugates may be prepared using any methods known in the art. See, e.g., WO 2009/067800, WO 2011/133886, and U.S. Patent Application Publication No. 2014322129.

**[0291]** The variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be "attached to" the effector moiety by any means by which the variant polypeptides or immunomodulatory proteins can be associated with, or linked to, the effector moiety. For example, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be attached to the effector moiety by chemical or recombinant means. Chemical means for preparing fusions or conjugates are known in the art and can be used to prepare the IgSF conjugate. The method used to conjugate the variant polypeptides or immunomodulatory proteins and effector moiety must be capable of joining the variant polypeptides or immunomodulatory proteins with the effector moiety without interfering with the ability of the variant polypeptides or immunomodulatory proteins to bind to their one or more counter structure ligands.

**[0292]** The variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be linked indirectly to the effector moiety. For example, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be directly linked to a liposome containing the effector moiety of one of several types. The effector moiety(s) and/or the variant polypeptides or immunomodulatory proteins may also be bound to a solid surface.

**[0293]** In some embodiments, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate and the effector moiety are both proteins and can be conjugated using techniques well known in the art. There are several hundred crosslinkers available that can conjugate two proteins. (See for example "Chemistry of Protein Conjugation and Crosslinking," 1991, Shans Wong, CRC Press, Ann Arbor). The crosslinker is generally chosen based on the reactive functional groups available or inserted on the variant polypeptides or immunomodulatory proteins and/or effector moiety. In addition, if there are no reactive groups, a photoactivatible crosslinker can be used. In certain instances, it may be desirable to include a spacer between the variant polypeptides or immunomodulatory proteins and the effector moiety. Crosslinking agents known to the art include the homobifunctional agents: glutaraldehyde, dimethyladipimidate and Bis(diazobenzidine) and the heterobifunctional agents: m Maleimidobenzoyl-N-Hydroxysuccinimide and Sulfo-m Maleimidobenzoyl-N-Hydroxysuccinimide.

**[0294]** In some embodiments, the variant polypeptides or immunomodulatory proteins of an IgSF conjugate may be engineered with specific residues for chemical attachment of the effector moiety. Specific residues used for chemical attachment of molecule known to the art include lysine and cysteine. The crosslinker is chosen based on the reactive functional groups inserted on the variant polypeptides or immunomodulatory proteins, and available on the effector moiety.

**[0295]** An IgSF conjugate may also be prepared using recombinant DNA techniques. In such a case a DNA sequence encoding the variant polypeptides or immunomodulatory proteins is fused to a DNA sequence encoding the effector moiety, resulting in a chimeric DNA molecule. The chimeric DNA sequence is

transfected into a host cell that expresses the fusion protein. The fusion protein can be recovered from the cell culture and purified using techniques known in the art.

**[0296]** Examples of attaching an effector moiety, which is a label, to the variant polypeptides or immunomodulatory proteins include the methods described in Hunter, et al., Nature 144:945 (1962); David, et al., Biochemistry 13:1014 (1974); Pain, et al., J. Immunol. Meth. 40:219 (1981); Nygren, J. Histochem. and Cytochem. 30:407 (1982); Wensel and Meares, Radioimmunoimaging And Radioimmunotherapy, Elsevier, N.Y. (1983); and Colcher et al., "Use Of Monoclonal Antibodies As Radiopharmaceuticals For The Localization Of Human Carcinoma Xenografts In Athymic Mice", Meth. Enzymol., 121:802-16 (1986).

**[0297]** The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as 99Tc or 123I, 186Re, 188Re and 111In can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al., Biochem. Biophys. Res. Commun. 80:49-57 (1978)) can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describes other methods in detail.

**[0298]** Conjugates of the variant polypeptides or immunomodulatory proteins and a cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1 -carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1 ,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-p-isothiocyanatobenzyl-3-methyldiethylenetriaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See, e.g., WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Research 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

**[0299]** The IgSF conjugates of the invention expressly contemplate, but are not limited to, drug conjugates prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL, U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

**D. Transmembrane and Secretable Immunomodulatory Proteins and Engineered Cells**

**[0300]** Provided herein are engineered cells which express the immunomodulatory variant PD-L1 polypeptides (alternatively, "engineered cells"). In some embodiments, the expressed immunomodulatory variant PD-L1 polypeptide is a transmembrane protein and is surface expressed. In some embodiments, the expressed immunomodulatory variant PD-L1 polypeptide is expressed and secreted from the cell.

**1. Transmembrane Immunomodulatory Proteins**

**[0301]** In some embodiments, an immunomodulatory polypeptide comprising a variant PD-L1 can be a membrane bound protein. As described in more detail below, the immunomodulatory polypeptide can be a transmembrane immunomodulatory polypeptide comprising a variant PD-L1 in which is contained: an ectodomain containing at least one affinity modified IgSF domain (IgV and optionally IgC), a transmembrane domain and, optionally, a cytoplasmic domain. In some embodiments, the transmembrane immunomodulatory protein can be expressed on the surface of an immune cell, such as a mammalian cell, including on the surface of a lymphocyte (e.g. T cell or NK cell) or antigen presenting cell. In some embodiments, the transmembrane immunomodulatory protein is expressed on the surface of a mammalian T-cell, including such T-cells as: a T helper cell, a cytotoxic T-cell (alternatively, cytotoxic T lymphocyte or CTL), a natural killer T-cell, a regulatory T-cell, a memory T-cell, or a gamma delta T-cell. In some embodiments, the mammalian cell is an antigen presenting cell (APC). Typically, but not exclusively, the ectodomain (alternatively, "extracellular domain") comprises one or more amino acid variations (e.g. amino acid substitutions) of the variant PD-L1 of the invention. Thus, for example, in some embodiments a transmembrane protein will comprise an ectodomain that comprises one or more amino acid substitutions of a variant PD-L1 of the invention.

**[0302]** In some embodiments, the engineered cells express a variant PD-L1 polypeptides are transmembrane immunomodulatory polypeptides (TIPs) that can be a membrane protein such as a transmembrane protein. In typical embodiments, the ectodomain of a membrane protein comprises an extracellular domain or IgSF domain thereof of a variant PD-L1 provided herein in which is contained one or more amino acid substitutions in at least one IgSF domain as described. The transmembrane immunomodulatory proteins provided herein further contain a transmembrane domain linked to the ectodomain. In some embodiments, the transmembrane domain results in an encoded protein for cell surface expression on a cell. In some embodiments, the transmembrane domain is linked directly to the ectodomain. In some embodiments, the transmembrane domain is linked indirectly to the ectodomain via one or more linkers or spacers. In some embodiments, the transmembrane domain contains predominantly hydrophobic amino acid residues, such as leucine and valine.

**[0303]** In some embodiments, a full length transmembrane anchor domain can be used to ensure that the TIPs will be expressed on the surface of the engineered cell, such as engineered T cell. Conveniently, this could be from a particular native protein that is being affinity modified (e.g. PD-L1 or other native IgSF protein), and simply fused to the sequence of the first membrane proximal domain in a similar fashion as the native IgSF protein (e.g. PD-L1). In some embodiments, the transmembrane immunomodulatory protein comprises a transmembrane domain of the corresponding wild-type or unmodified IgSF member, such as a transmembrane domain contained in the sequence of amino acids set forth in SEQ ID NO:3 (Table 2). In some embodiments, the membrane bound form comprises a transmembrane domain of the corresponding wild-type or unmodified polypeptide, such as corresponding to residues 239-259 of SEQ ID NO:3.

**[0304]** In some embodiments, the transmembrane domain is a non-native transmembrane domain that is not the transmembrane domain of native PD-L1. In some embodiments, the transmembrane domain is derived from a transmembrane domain from another non- PD-L1 family member polypeptide that is a membrane-bound or is a transmembrane protein. In some embodiments, a transmembrane anchor domain from another protein on T cells can be used. In some embodiments, the transmembrane domain is derived from CD8. In some embodiments, the transmembrane domain can further contain an extracellular portion of CD8 that serves as a spacer domain. An exemplary CD8 derived transmembrane domain is set forth in SEQ ID NO: 242 or 1164 or a portion thereof containing the CD8 transmembrane domain. In some embodiments, the transmembrane domain is a synthetic transmembrane domain.

**[0305]** In some embodiments, the transmembrane immunomodulatory protein further contains an endodomain, such as a cytoplasmic signaling domain, linked to the transmembrane domain. In some embodiments, the cytoplasmic signaling domain induces cell signaling. In some embodiments, the endodomain of the transmembrane immunomodulatory protein comprises the cytoplasmic domain of the corresponding wild-type or unmodified polypeptide, such as a cytoplasmic domain contained in the sequence of amino acids set forth in SEQ ID NO:3 (see Table 2).

**[0306]** In some embodiments, a provided transmembrane immunomodulatory protein that is or comprises a variant PD-L1 comprises a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 191 and contains an ectodomain comprising at least one affinity-modified PD-L1 IgSF domain as described and a transmembrane domain. In some embodiments, the transmembrane immunomodulatory protein contains any one or more amino acid substitutions in an IgSF domain (e.g. IgV domain) as described, including any set forth in Table 1. In some embodiments, the transmembrane immunomodulatory protein can further comprise a cytoplasmic domain as described. In some embodiments, the transmembrane immunomodulatory protein can further contain a signal peptide. In some embodiments, the signal peptide is the native signal peptide of wild-type IgSF member, such as contained in the sequence of amino acids set forth in SEQ ID NO:3 (see e.g. Table 2).

**[0307]** Also provided is a nucleic acid molecule encoding such transmembrane immunomodulatory proteins. In some embodiments, a nucleic acid molecule encoding a transmembrane immunomodulatory protein comprises a nucleotide sequence that encodes a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NOS: 191 and contains an ectodomain comprising at least one affinity-modified IgSF domain as described, a transmembrane domain and, optionally, a cytoplasmic domain. In some embodiments, the nucleic acid molecule can further comprise a sequence of nucleotides encoding a signal peptide. In some embodiments, the signal peptide is the native signal peptide of the corresponding wild-type IgSF member (see e.g. Table 2).

**[0308]** In some embodiments, provided are CAR-related transmembrane immunomodulatory proteins in which the endodomain of a transmembrane immunomodulatory protein comprises a cytoplasmic signaling domain that comprises at least one ITAM (immunoreceptor tyrosine-based activation motif)-containing signaling domain. ITAM is a conserved motif found in a number of protein signaling domains involved in signal transduction of immune cells, including in the CD3-zeta chain ("CD3-z") involved in T-cell receptor signal transduction. In some embodiments, the endodomain comprises at CD3-zeta signaling domain. In some embodiments, the CD3-zeta signaling domain comprises the sequence of amino acids set forth in SEQ ID NO: 243 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% to SEQ ID NO:243 and retains the activity of T cell signaling. In some embodiments, the endodomain of a CAR-related transmembrane immunomodulatory protein can further comprise a costimulatory signaling domain to further modulate immunomodulatory responses of the T-cell. In some embodiments, the costimulatory signaling domain is CD28, ICOS, 41BB or OX40. In some embodiments, the costimulatory signaling domain is a derived from CD28 or 4-1BB and comprises the sequence of amino acids set forth in any of SEQ ID NOS: 1165-1168 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% to SEQ ID NO:1165-1168 and retains the activity of T cell costimulatory signaling. In some embodiments, the provided CAR-related transmembrane immunomodulatory proteins have features of CARs to stimulate T cell signaling upon binding of an affinity modified IgSF domain to a cognate binding partner or counter structure. In some embodiments, upon specific binding by the affinity-modified IgSF domain to its counter structure can lead to changes in the immunological activity of the T-cell activity as reflected by changes in

cytotoxicity, proliferation or cytokine production.

**[0309]** In some embodiments, the transmembrane immunomodulatory protein does not contain an endodomain capable of mediating cytoplasmic signaling. In some embodiments, the transmembrane immunomodulatory protein lacks the signal transduction mechanism of the wild-type or unmodified polypeptide and therefore does not itself induce cell signaling. In some embodiments, the transmembrane immunomodulatory protein lacks an intracellular (cytoplasmic) domain or a portion of the intracellular domain of the corresponding wild-type or unmodified polypeptide, such as a cytoplasmic signaling domain contained in the sequence of amino acids set forth in SEQ ID NO:3 (see Table 2). In some embodiments, the transmembrane immunomodulatory protein does not contain an ITIM (immunoreceptor tyrosine-based inhibition motif), such as contained in certain inhibitory receptors, including inhibitory receptors of the IgSF family (e.g. PD-1 or TIGIT). Thus, in some embodiments, the transmembrane immunomodulatory protein only contains the ectodomain and the transmembrane domain, such as any as described.

**2. Secreted Immunomodulatory Proteins and Engineered Cells**

**[0310]** In some embodiments, the PD-L1 variant immunomodulatory polypeptide containing any one or more of the amino acid mutations as described herein, is secretable, such as when expressed from a cell. Such a variant PD-L1 immunomodulatory protein does not comprise a transmembrane domain. In some embodiments, the variant PD-L1 immunomodulatory protein is not conjugated to a half-life extending moiety (such as an Fc domain or a multimerization domain). In some embodiments, the variant PD-L1 immunomodulatory protein comprises a signal peptide, e.g. an antibody signal peptide or other efficient signal sequence to get domains outside of cell. When the immunomodulatory protein comprises a signal peptide and is expressed by an engineered cell, the signal peptide causes the immunomodulatory protein to be secreted by the engineered cell. Generally, the signal peptide, or a portion of the signal peptide, is cleaved from the immunomodulatory protein with secretion. The immunomodulatory protein can be encoded by a nucleic acid (which can be part of an expression vector). In some embodiments, the immunomodulatory protein is expressed and secreted by a cell (such as an immune cell, for example a primary immune cell).

**[0311]** Thus, in some embodiments, there are provided variant PD-L1 immunomodulatory proteins that further comprises a signal peptide. In some embodiments, provided herein is a nucleic acid molecule encoding the variant PD-L1 immunomodulatory protein operably connected to a secretion sequence encoding the signal peptide.

**[0312]** A signal peptide is a sequence on the N-terminus of an immunomodulatory protein that signals secretion of the immunomodulatory protein from a cell. In some embodiments, the signal peptide is about 5 to about 40 amino acids in length (such as about 5 to about 7, about 7 to about 10, about 10 to about 15, about 15 to about 20, about 20 to about 25, or about 25 to about 30, about 30 to about 35, or about 35 to about 40 amino acids in length).

**[0313]** In some embodiments, the signal peptide is a native signal peptide from the corresponding wild-type PD-L1 (see Table 2). In some embodiments, the signal peptide is a non-native signal peptide. For example, in some embodiments, the non-native signal peptide is a mutant native signal peptide from the corresponding wild-type PD-L1, and can include one or more (such as 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more) substitutions insertions or deletions. In some embodiments, the non-native signal peptide is a signal peptide or mutant thereof of a family member from the same IgSF family as the wild-type IgSF family member. In some embodiments, the non-native signal peptide is a signal peptide or mutant thereof from an

IgSF family member from a different IgSF family that the wild-type IgSF family member. In some embodiments, the signal peptide is a signal peptide or mutant thereof from a non-IgSF protein family, such as a signal peptide from an immunoglobulin (such as IgG heavy chain or IgG-kappa light chain), a cytokine (such as interleukin-2 (IL-2), or CD33), a serum albumin protein (e.g. HSA or albumin), a human azurocidin preprotein signal sequence, a luciferase, a trypsinogen (e.g. chymotrypsinogen or trypsinogen) or other signal peptide able to efficiently secrete a protein from a cell. Exemplary signal peptides include any described in the Table 9.

**TABLE 9. Exemplary Signal Peptides**

| SEQ ID NO | Signal Peptide | Peptide Sequence |
|---|---|---|
| SEQ ID NO: 221 | HSA signal peptide | MKWVTFISLLFLFSSAYS |
| SEQ ID NO: 222 | Ig kappa light chain | MDMRAPAGIFGFLLVLFPGYRS |
| SEQ ID NO: 223 | human azurocidin preprotein signal sequence | MTRLTVLALLAGLLAS SRA |
| SEQ ID NO: 224 | IgG heavy chain signal peptide | MELGLSWIFLLAILKGVQC |
| SEQ ID NO: 225 | IgG heavy chain signal peptide | MELGLRWVFLVAILEGVQC |
| SEQ ID NO: 226 | IgG heavy chain signal peptide | MKHLWFFLLLVAAPRWVLS |
| SEQ ID NO: 227 | IgG heavy chain signal peptide | MDWTWRILFLVAAATGAHS |
| SEQ ID NO: 228 | IgG heavy chain signal peptide | MDWTWRFLFVVAAATGVQS |
| SEQ ID NO: 229 | IgG heavy chain signal peptide | MEFGLSWLFLVAILKGVQC |
| SEQ ID NO: 230 | IgG heavy chain signal peptide | MEFGLSWVFLVALFRGVQC |
| SEQ ID NO: 231 | IgG heavy chain signal peptide | MDLLHKNMKHLWFFLLLVAAPRWVLS |
| SEQ ID NO: 232 | IgG Kappa light chain signal sequences: | MDMRVPAQLLGLLLLWLSGARC |
| SEQ ID NO: 233 | IgG Kappa light chain signal sequences: | MKYLLPTAAAGLLLLAAQPAMA |
| SEQ ID NO: 234 | Gaussia luciferase | MGVKVLFALICIAVAEA |
| SEQ ID NO: 235 | Human albumin | MKWVTFISLLFLFSSAYS |
| SEQ ID NO: 236 | Human chymotrypsinogen | MAFLWLLS CWALLGTTFG |
| SEQ ID NO: 237 | Human interleukin-2 | MQLLSCIALILALV |
| SEQ ID NO: 238 | Human trypsinogen-2 | MNLLLILTFVAAAVA |

**[0314]** In some embodiments of a secretable variant PD-L1 immunomodulatory protein, the immunomodulatory protein comprises a signal peptide when expressed, and the signal peptide (or a portion thereof) is cleaved from the immunomodulatory protein upon secretion.

**[0315]** In some embodiments, the engineered cells express a variant PD-L1 polypeptides that are secreted from the cell. In some embodiments, such a variant PD-L1 polypeptide is encoded by a nucleic acid molecule encoding an immunomodulatory protein under the operable control of a signal sequence for secretion. In some embodiments, the encoded immunomodulatory protein is secreted when expressed from a cell. In some embodiments, the immunomodulatory protein encoded by the nucleic acid molecule does not comprise a transmembrane domain. In some embodiments, the immunomodulatory protein encoded by the nucleic acid molecule does not comprise a half-life extending moiety (such as an Fc domain or a multimerization domain). In some embodiments, the immunomodulatory protein encoded by the nucleic acid molecule comprises a signal peptide. In some embodiments, a nucleic acid of the invention further comprises nucleotide sequence that encodes a secretory or signal peptide operably linked to the nucleic acid encoding the immunomodulatory protein, thereby allowing for secretion of the immunomodulatory protein

**3. Cells and Engineering Cells**

**[0316]** Provided herein are engineered cells expressing any of the provided immunomodulatory polypeptide. In some embodiments, the engineered cells express on their surface any of the provided transmembrane immunomodulatory polypeptides. In some embodiments, the engineered cells express and are capable of or are able to secrete the immunomodulatory protein from the cells under conditions suitable for secretion of the protein. In some embodiments, the immunomodulatory protein is expressed on a lymphocyte such as a tumor infiltrating lymphocyte (TIL), T-cell or NK cell, or on a myeloid cell. In some embodiments, the engineered cells are antigen presenting cells (APCs). In some embodiments, the engineered cells are engineered mammalian T-cells or engineered mammalian antigen presenting cells (APCs). In some embodiments, the engineered T-cells or APCs are human or murine cells.

**[0317]** In some embodiments, engineered T-cells include, but are not limited to, T helper cell, cytotoxic T-cell (alternatively, cytotoxic T lymphocyte or CTL), natural killer T-cell, regulatory T-cell, memory T-cell, or gamma delta T-cell. In some embodiments, the engineered T cells are CD4+ or CD8+.

**[0318]** In some embodiments, the engineered APCs include, for example, MHC II expressing APCs such as macrophages, B cells, and dendritic cells, as well as artificial APCs (aAPCs) including both cellular and acellular (e.g., biodegradable polymeric microparticles) aAPCs. Artificial APCs (aAPCs) are synthetic versions of APCs that can act in a similar manner to APCs in that they present antigens to T-cells as well as activate them. Antigen presentation is performed by the MHC (Class I or Class II). In some embodiments, in engineered APCs such as aAPCs, the antigen that is loaded onto the MHC is, in some embodiments, a tumor specific antigen or a tumor associated antigen. The antigen loaded onto the MHC is recognized by a T-cell receptor (TCR) of a T cell, which, in some cases, can express PD-1 and optionallyCD80 or other molecule recognized by the variant PD-L1 polypeptides provided herein. Materials which can be used to engineer an aAPC include: poly (glycolic acid), poly(lactic-co-glycolic acid), iron-oxide, liposomes, lipid bilayers, sepharose, and polystyrene.

**[0319]** In some embodiments a cellular aAPC can be engineered to contain a TIP and TCR agonist which is used in adoptive cellular therapy. In some embodiments, a cellular aAPC can be engineered to contain a TIP and TCR agonist which is used in ex vivo expansion of human T cells, such as prior to administration,

e.g., for reintroduction into the patient. In some aspects, the aAPC may include expression of at least one anti-CD3 antibody clone, e.g. such as, for example, OKT3 and/or UCHT1. In some aspects, the aAPCs may be inactivated (e.g. irradiated). In some embodiment, the TIP can include any variant IgSF domain that exhibits binding affinity for a cognate binding partner on a T cell.

**[0320]** In some embodiments, an immunomodulatory protein provided herein, such as a transmembrane immunomodulatory protein or a secretable immunomodulatory protein, is co-expressed or engineered into a cell that expresses an antigen-binding receptor, such as a recombinant receptor, such as a chimeric antigen receptor (CAR) or T cell receptor (TCR). In some embodiments, the engineered cell, such as an engineered T cell, recognizes a desired antigen associated with cancer, inflammatory and autoimmune disorders, or a viral infection. In specific embodiments, the antigen-binding receptor contains an antigen-binding moiety that specifically binds a tumor specific antigen or a tumor associated antigen. In some embodiments, the engineered T-cell is a CAR (chimeric antigen receptor) T-cell that contains an antigen-binding domain (e.g. scFv) that specifically binds to an antigen, such as a tumor specific antigen or tumor associated antigen. Iin some embodiments, the TIP protein is expressed in an engineered T-cell receptor cell or and engineered chimeric antigen receptor cell. In such embodiments, the engineered cell co-expresses the TIP and the CAR or TCR. In some embodiments, the SIP protein is expressed in an engineered T-cell receptor cell or an engineered chimeric antigen receptor cell. In such embodiments, the engineered cell co-expresses the SIP and the CAR or TCR.

**[0321]** Chimeric antigen receptors (CARs) are recombinant receptors that include an antigen-binding domain (ectodomain), a transmembrane domain and an intracellular signaling region (endodomain) that is capable of inducing or mediating an activation signal to the T cell after the antigen is bound. In some example, CAR-expressing cells are engineered to express an extracellular single chain variable fragment (scFv) with specificity for a particular tumor antigen linked to an intracellular signaling part comprising an activating domain and, in some cases, a costimulatory domain. The costimulatory domain can be derived from, e.g., CD28, OX-40, 4-1BB/CD137, inducible T cell costimulator (ICOS). The activating domain can be derived from, e.g., CD3, such as CD3 zeta, epsilon, delta, gamma, or the like. In certain embodiments, the CAR is designed to have two, three, four, or more costimulatory domains. The CAR scFv can be designed to target an antigen expressed on a cell associated with a disease or condition, e.g. a tumor antigen, such as, for example, CD19, which is a transmembrane protein expressed by cells in the B cell lineage, including all normal B cells and B cell malignances, including but not limited to NHL, CLL, and non-T cell ALL. Example CAR+ T cell therapies and constructs are described in U.S. Patent Publication Nos. 2013/0287748, 2014/0227237, 2014/0099309, and 2014/0050708.

**[0322]** In some aspects, the antigen-binding domain is an antibody or antigen-binding fragment thereof, such as a single chain fragment (scFv). In some embodiments, the antigen is expressed on a tumor or cancer cell. Exemplary of an antigen is CD19. Exemplary of a CAR is an anti-CD19 CAR, such as a CAR containing an anti-CD19 scFv set forth in SEQ ID NO:1163 or SEQ ID NO:1174. In some embodiments, the CAR further contains a spacer, a transmembrane domain, and an intracellular signaling domain or region comprising an ITAM signaling domain, such as a CD3zeta signaling domain. In some embodiments, the CAR further includes a costimulatory signaling domain.

**[0323]** In some embodiments, the spacer and transmembrane domain are the hinge and transmembrane domain derived from CD8, such as having an exemplary sequence set forth in SEQ ID NO: 242, 1164, 2014 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 242, 1164, or 2014. In some embodiments, the endodomain comprises at CD3-zeta signaling domain. In some embodiments, the CD3-zeta signaling domain comprises the sequence of amino acids set forth in SEQ ID NO: 243 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,

97%, 98% or 99% or more sequence identity to SEQ ID NO:243 and retains the activity of T cell signaling. In some embodiments, the endodomain of a CAR can further comprise a costimulatory signaling region to further modulate immunomodulatory responses of the T-cell. In some embodiments, the costimulatory signaling domain is or comprises a costimulatory region, or is derived from a costimulatory region, of CD28, ICOS, 41BB or OX40. In some embodiments, the costimulatory signaling domain is a derived from CD28 or 4-1BB and comprises the sequence of amino acids set forth in any of SEQ ID NOS: 1165-1168 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to SEQ ID NO: 1165-1168 and retains the activity of T cell costimulatory signaling.

**[0324]** In some embodiments, the construct encoding the CAR further encodes a second protein, such as a marker, e.g. detectable protein, separated from the CAR by a self-cleaving peptide sequence. In some embodiments, the self-cleaving peptide sequence is an F2A, T2A, E2A or P2A self-cleaving peptide. Exemplary sequences of a T2A self-cleaving peptide are set for the in any one of SEQ ID NOS: 1167, 1177 or 2021 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to any of SEQ ID NOS: 1167, 1177 or 2021. In some embodiments, the T2A is encoded by the sequence of nucleotides set forth in SEQ ID NO: 1176 or a sequence that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to any of SEQ ID NO: 1176. An exemplary sequence of a P2A self-cleaving peptide is set in SEQ ID NO: 2026 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to SEQ ID NOS: 2026. In some cases, a nucleic acid construct that encodes more than one P2A self-cleaving peptide (such as a P2A1 and P2A2), in which the nucleotide sequence P2A1 and P2A2 each encode the P2A set forth in SEQ ID NO:2026, the nucleotide sequence may be different to avoid recombination between sequences.

**[0325]** In some embodiments, the marker is a detectable protein, such as a fluorescent protein, e.g. a green fluorescent protein (GFP) or blue fluorescent protein (BFP). Exemplary sequences of a fluorescent protein marker are set forth in SEQ ID NO: 1170, 2020, 2027-2029 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to SEQ ID NO: 1170 or 2020.

**[0326]** In some embodiments, the CAR has the sequence of amino acids set forth in any of SEQ ID NOS: 1160, 1171, 1172, 1173, 2015, 2016, 2018 or 2019 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to any one of SEQ ID NOS: 1160, 1171, 1172, 1173, 2015, 2016, 2018 or 2019. In some embodiments, the CAR is encoded by a sequence of nucleotides set forth in SEQ ID NO: 1175 or 2017 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more sequence identity to any one of SEQ ID NO: 1175 or 2017.

**[0327]** In another embodiment, the engineered T-cell possesses a TCR, including a recombinant or engineered TCR. In some embodiments, the TCR can be a native TCR. Those of skill in the art will recognize that generally native mammalian T-cell receptors comprise an alpha and a beta chain (or a gamma and a delta chain) involved in antigen specific recognition and binding. In some embodiments, the TCR is an engineered TCR that is modified. In some embodiments, the TCR of an engineered T-cell specifically binds to a tumor associated or tumor specific antigen presented by an APC.

**[0328]** In some embodiments, the immunomodulatory polypeptides, such as transmembrane immunomodulatory polypeptides or secretable immunomodulatory polypeptides, can be incorporated into engineered cells, such as engineered T cells or engineered APCs, by a variety of strategies such as those

employed for recombinant host cells. A variety of methods to introduce a DNA construct into primary T cells are known in the art. In some embodiments, viral transduction or plasmid electroporation are employed. In typical embodiments, the nucleic acid molecule encoding the immunomodulatory protein, or the expression vector, comprises a signal peptide that localizes the expressed transmembrane immunomodulatory proteins to the cellular membrane or for secretion. In some embodiments, a nucleic acid encoding a transmembrane immunomodulatory proteins of the invention is sub-cloned into a viral vector, such as a retroviral vector, which allows expression in the host mammalian cell. The expression vector can be introduced into a mammalian host cell and, under host cell culture conditions, the immunomodulatory protein is expressed on the surface or is secreted.

**[0329]** In an exemplary example, primary T-cells can be purified ex vivo (CD4 cells or CD8 cells or both) and stimulated with an activation protocol consisting of various TCR/CD28 agonists, such as anti-CD3/anti-CD28 coated beads. After a 2 or 3 day activation process, a recombinant expression vector containing an immunomodulatory polypeptide can be stably introduced into the primary T cells through art standard lentiviral or retroviral transduction protocols or plasmid electroporation strategies. Cells can be monitored for immunomodulatory polypeptide expression by, for example, flow cytometry using anti-epitope tag or antibodies that cross-react with native parental molecule and polypeptides comprising variant PD-L1. T-cells that express the immunomodulatory polypeptide can be enriched through sorting with anti-epitope tag antibodies or enriched for high or low expression depending on the application.

**[0330]** Upon immunomodulatory polypeptide expression the engineered T-cell can be assayed for appropriate function by a variety of means. The engineered CAR or TCR co-expression can be validated to show that this part of the engineered T cell was not significantly impacted by the expression of the immunomodulatory protein. Once validated, standard in vitro cytotoxicity, proliferation, or cytokine assays (e.g., IFN-gamma expression) can be used to assess the function of engineered T-cells. Exemplary standard endpoints are percent lysis of the tumor line, proliferation of the engineered T-cell, or IFN-gamma protein expression in culture supernatants. An engineered construct which results in statistically significant increased lysis of tumor line, increased proliferation of the engineered T-cell, or increased IFN-gamma expression over the control construct can be selected for. Additionally, non-engineered, such as native primary or endogenous T-cells could also be incorporated into the same in vitro assay to measure the ability of the immunomodulatory polypeptide construct expressed on the engineered cells, such as engineered T-cells, to modulate activity, including, in some cases, to activate and generate effector function in bystander, native T-cells. Increased expression of activation markers such as CD69, CD44, or CD62L could be monitored on endogenous T cells, and increased proliferation and/or cytokine production could indicate desired activity of the immunomodulatory protein expressed on the engineered T cells.

**[0331]** In some embodiments, the similar assays can be used to compare the function of engineered T cells containing the CAR or TCR alone to those containing the CAR or TCR and a TIP construct. Typically, these in vitro assays are performed by plating various ratios of the engineered T cell and a "tumor" cell line containing the cognate CAR or TCR antigen together in culture. Standard endpoints are percent lysis of the tumor line, proliferation of the engineered T cell, or IFN-gamma production in culture supernatants. An engineered immunomodulatory protein which resulted in statistically significant increased lysis of tumor line, increased proliferation of the engineered T cell, or increased IFN-gamma production over the same TCR or CAR construct alone can be selected for. Engineered human T cells can be analyzed in immunocompromised mice, like the NSG strain, which lacks mouse T, NK and B cells. Engineered human T cells in which the CAR or TCR binds a target counter-structure on the xenograft and is co-expressed with the TIP affinity modified IgSF domain can be adoptively transferred in vivo at different cell numbers and ratios compared to the xenograft. For example, engraftment of CD19+ leukemia tumor lines containing a luciferase/GFP vector can be monitored through bioluminescence or ex vivo by flow cytometry. In a common embodiment, the xenograft is introduced into the murine model, followed by the engineered T

cells several days later. Engineered T cells containing the immunomodulatory protein can be assayed for increased survival, tumor clearance, or expanded engineered T cells numbers relative to engineered T cells containing the CAR or TCR alone. As in the in vitro assay, endogenous, native (i.e., non-engineered) human T cells could be co-adoptively transferred to look for successful epitope spreading in that population, resulting in better survival or tumor clearance.

**E. Infectious Agents Expressing Variant Polypeptides and Immunomodulatory Proteins**

**[0332]** Also provided are infectious agents that contain nucleic acids encoding any of the variant polypeptides, such as PD-L1 vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins described herein. In some embodiments, such infectious agents can deliver the nucleic acids encoding the variant immunomodulatory polypeptides described herein, such as PD-L1 vIgD polypeptides, to a target cell in a subject, e.g., immune cell and/or antigen-presenting cell (APC) or tumor cell in a subject. Also provided are nucleic acids contained in such infectious agents, and/or nucleic acids for generation or modification of such infectious agents, such as vectors and/or plasmids, and compositions containing such infectious agents.

**[0333]** In some embodiments, the infectious agent is a microorganism or a microbe. In some embodiments, the infectious agent is a virus or a bacterium. In some embodiments, the infectious agent is a virus. In some embodiments, the infectious agent is a bacterium. In some embodiments, such infectious agents can deliver nucleic acid sequences encoding any of the variant polypeptides, such as PD-L1 vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, described herein. Thus, in some embodiments, the cell in a subject that is infected or contacted by the infectious agents can be rendered to express on the cell surface or secrete, the variant immunomodulatory polypeptides. In some embodiments, the infectious agent can also deliver one or more other therapeutics or nucleic acids encoding other therapeutics to the cell and/or to an environment within the subject. In some embodiments, other therapeutics that can be delivered by the infectious agents include cytokines or other immunomodulatory molecules.

**[0334]** In some embodiments, the infectious agent, e.g., virus or bacteria, contains nucleic acid sequences that encode any of the variant polypeptides, such as PD-L1 vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, described herein, and by virtue of contact and/or infection of a cell in the subject, the cell expresses the variant polypeptides, such as PD-L1 vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, encoded by the nucleic acid sequences contained in the infectious agent. In some embodiments, the infectious agent can be administered to the subject. In some embodiments, the infectious agent can be contacted with cells from the subject *ex vivo.*

**[0335]** In some embodiments, the variant polypeptides, such as PD-L1 vIgD polypeptides, including transmembrane immunomodulatory proteins, expressed by the cell infected by the infectious agent is a transmembrane protein and is surface expressed. In some embodiments, the variant polypeptides, such as PD-L1 vIgD polypeptides, including secretable immunomodulatory proteins, expressed by the cell infected by the infectious agent is expressed and secreted from the cell. The transmembrane immunomodulatory protein or secreted immunomodulatory protein can be any described herein.

**[0336]** In some embodiments, the cells in the subject that are targeted by the infectious agent include a tumor cell, an immune cell, and/or an antigen-presenting cell (APC). In some embodiments, the infectious agent targets a cell in the tumor microenvironment (TME). In some embodiments, the infectious agent

delivers the nucleic acids encoding the variant polypeptides, such as PD-L1 vIgD polypeptides, including secretable or transmembrane immunomodulatory proteins, to an appropriate cell (for example, an APC, such as a cell that displays a peptide/MHC complex on its cell surface, such as a dendritic cell) or tissue (e.g., lymphoid tissue) that will induce and/or augment the desired effect, e.g., immunomodulation and/or a specific cell-medicated immune response, e.g., CD4 and/or CD8 T cell response, which CD8 T cell response may include a cytotoxic T cell (CTL) response. In some embodiments, the infectious agent targets an APC, such as a dendritic cell (DC). In some embodiments, the nucleic acid molecule delivered by the infectious agents described herein include appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequences encoding the variant immunomodulatory polypeptides, in a particular target cell, e.g., regulatory elements such as promoters.

**[0337]** In some embodiments, the infectious agent that contains nucleic acid sequences encoding the immunomodulatory polypeptides can also contain nucleic acid sequences that encode one or more additional gene products, e.g., cytokines, prodrug converting enzymes, cytotoxins and/or detectable gene products. For example, in some embodiments, the infectious agent is an oncolytic virus and the virus can include nucleic acid sequences encoding additional therapeutic gene products (see, e.g., Kirn et al., (2009) Nat Rev Cancer 9:64-71; Garcia-Aragoncillo et al., (2010) Curr Opin Mol Ther 12:403-411; see U.S. Pat. Nos. 7,588,767, 7,588,771, 7,662,398 and 7,754,221 and U.S. Pat. Publ. Nos. 2007/0202572, 2007/0212727, 2010/0062016, 2009/0098529, 2009/0053244, 2009/0155287, 2009/0117034, 2010/0233078, 2009/0162288, 2010/0196325, 2009/0136917 and 2011/0064650. In some embodiments, the additional gene product can be a therapeutic gene product that can result in death of the target cell (e.g., tumor cell) or gene products that can augment or boost or regulate an immune response (e.g., cytokine). Exemplary gene products also include among an anticancer agent, an anti-metastatic agent, an antiangiogenic agent, an immunomodulatory molecule, an immune checkpoint inhibitor, an antibody, a cytokine, a growth factor, an antigen, a cytotoxic gene product, a pro-apoptotic gene product, an anti-apoptotic gene product, a cell matrix degradative gene, genes for tissue regeneration or reprogramming human somatic cells to pluripotency, and other genes described herein or known to one of skill in the art. In some embodiments, the additional gene product is Granulocyte-macrophage colony-stimulating factor (GM-CSF).

**1. Viruses**

**[0338]** In some embodiments, the infectious agent is a virus. In some embodiments, the infectious agent is an oncolytic virus, or a virus that targets particular cells, e.g., immune cells. In some embodiments, the infectious agent targets a tumor cell and/or cancer cell in the subject. In some embodiments, the infectious agent targets an immune cell or an antigen-presenting cell (APC).

**[0339]** In some embodiments, the infectious agent is an oncolytic virus. Oncolytic viruses are viruses that accumulate in tumor cells and replicate in tumor cells. By virtue of replication in the cells, and optional delivery of nucleic acids encoding variant PD-L1 polypeptide or immunomodulatory polypeptides described herein, tumor cells are lysed, and the tumor shrinks and can be eliminated. Oncolytic viruses can also have a broad host and cell type range. For example, oncolytic viruses can accumulate in immunoprivileged cells or immunoprivileged tissues, including tumors and/or metastases, and also including wounded tissues and cells, thus allowing the delivery and expression of nucleic acids encoding the variant immunomodulatory polypeptides described herein in a broad range of cell types. Oncolytic viruses can also replicate in a tumor cell specific manner, resulting in tumor cell lysis and efficient tumor regression.

**[0340]** Exemplary oncolytic viruses include adenoviruses, adeno-associated viruses, herpes viruses,

Herpes Simplex Virus, Vesicular Stomatic virus, Reovirus, Newcastle Disease virus, parvovirus, measles virus, vesicular stomatitis virus (VSV), Coxsackie virus and Vaccinia virus. In some embodiments, oncolytic viruses can specifically colonize solid tumors, while not infecting other organs, and can be used as an infectious agent to deliver the nucleic acids encoding the variant immunomodulatory polypeptides described herein to such solid tumors.

**[0341]** Oncolytic viruses for use in delivering the nucleic acids encoding variant PD-L1 polypeptides or immunomodulatory polypeptides described herein, can be any of those known to one of skill in the art and include, for example, vesicular stomatitis virus, see, e.g., U.S. Pat. Nos. 7,731,974, 7,153,510, 6,653,103 and U.S. Pat. Pub. Nos. 2010/0178684, 2010/0172877, 2010/0113567, 2007/0098743, 20050260601, 20050220818 and EP Pat. Nos. 1385466, 1606411 and 1520175; herpes simplex virus, see, e.g., U.S. Pat. Nos. 7,897,146, 7,731,952, 7,550,296, 7,537,924, 6,723,316, 6,428,968 and U.S. Pat. Pub. Nos., 2014/0154216, 2011/0177032, 2011/0158948, 2010/0092515, 2009/0274728, 2009/0285860, 2009/0215147, 2009/0010889, 2007/0110720, 2006/0039894, 2004/0009604, 2004/0063094, International Patent Pub. Nos., WO 2007/052029, WO 1999/038955; retroviruses, see, e.g., U.S. Pat. Nos. 6,689,871, 6,635,472, 5,851,529, 5,716,826, 5,716,613 and U.S. Pat. Pub. No. 20110212530; vaccinia viruses, see, e.g., 2016/0339066, and adeno-associated viruses, see, e.g., U.S. Pat. Nos. 8,007,780, 7,968,340, 7,943,374, 7,906,111, 7,927,585, 7,811,814, 7,662,627, 7,241,447, 7,238,526, 7,172,893, 7,033,826, 7,001,765, 6,897,045, and 6,632,670.

**[0342]** Oncolytic viruses also include viruses that have been genetically altered to attenuate their virulence, to improve their safety profile, enhance their tumor specificity, and they have also been equipped with additional genes, for example cytotoxins, cytokines, prodrug converting enzymes to improve the overall efficacy of the viruses (see, e.g., Kirn et al., (2009) Nat Rev Cancer 9:64-71; Garcia-Aragoncillo et al., (2010) Curr Opin Mol Ther 12:403-411; see U.S. Pat. Nos. 7,588,767, 7,588,771, 7,662,398 and 7,754,221 and U.S. Pat. Publ. Nos. 2007/0202572, 2007/0212727, 2010/0062016, 2009/0098529, 2009/0053244, 2009/0155287, 2009/0117034, 2010/0233078, 2009/0162288, 2010/0196325, 2009/0136917 and 2011/0064650). In some embodiments, the oncolytic viruses can be those that have been modified so that they selectively replicate in cancerous cells, and, thus, are oncolytic. For example, the oncolytic virus is an adenovirus that has been engineered to have modified tropism for tumor therapy and also as gene therapy vectors. Exemplary of such is ONYX-015, H101 and Ad5ΔCR (Hallden and Portella (2012) Expert Opin Ther Targets, 16:945-58) and TNFerade (McLoughlin et al. (2005) Ann. Surg. Oncol., 12:825-30), or a conditionally replicative adenovirus Oncorine®.

**[0343]** In some embodiments, the infectious agent is a modified herpes simplex virus. In some embodiments, the infectious agent is a modified version of Talimogene laherparepvec (also known as T-Vec, Imlygic or OncoVex GM-CSF), that is modified to contain nucleic acids encoding any of the variant immunomodulatory polypeptides described herein, such as variant PD-L1 polypeptide described herein. In some embodiments, the infectious agent is a modified herpes simplex virus that is described, e.g., in WO 2007/052029, WO 1999/038955, US 2004/0063094, US 2014/0154216, or, variants thereof.

**[0344]** In some embodiments, the infectious agent is a virus that targets a particular type of cells in a subject that is administered the virus, e.g., a virus that targets immune cells or antigen-presenting cells (APCs). Dendritic cells (DCs) are essential APCs for the initiation and control of immune responses. DCs can capture and process antigens, migrate from the periphery to a lymphoid organ, and present the antigens to resting T cells in a major histocompatibility complex (MHC)-restricted fashion. In some embodiments, the infectious agent is a virus that specifically can target DCs to deliver nucleic acids encoding the variant PD-L1 polypeptides or immunomodulatory polypeptides for expression in DCs. In some embodiments, the virus is a lentivirus or a variant or derivative thereof, such as an integration-

deficient lentiviral vector. In some embodiments, the virus is a lentivirus that is pseudotyped to efficiently bind to and productively infect cells expressing the cell surface marker dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), such as DCs. In some embodiments, the virus is a lentivirus pseudotyped with a Sindbis virus E2 glycoprotein or modified form thereof, such as those described in WO 2013/149167. In some embodiments, the virus allows for delivery and expression of a sequence of interest (e.g., a nucleic acid encoding any of the variant PD-L1 polypeptides or immunomodulatory polypeptides described herein) to a DC. In some embodiments, the virus includes those described in WO 2008/011636 or US 2011/0064763, Tareen et al. (2014) Mol. Ther., 22:575-587, or variants thereof. Exemplary of a dendritic cell-tropic vector platform is ZVex™.

**2. Bacteria**

**[0345]** In some embodiments, the infectious agent is a bacterium. For example, in some embodiments, the bacteria can deliver nucleic acids encoding any of the variant immunomodulatory polypeptides described herein to a target cell in the subject, such as a tumor cell, an immune cell, an antigen-presenting cell and/or a phagocytic cell. In some embodiments, the bacterium can be preferentially targeted to a specific environment within a subject, such as a tumor microenvironment (TME), for expression and/or secretion of the variant immunomodulatory polypeptides and/or to target specific cells in the environment for expression of the variant immunomodulatory polypeptides.

**[0346]** In some embodiments, the bacterium delivers the nucleic acids to the cells via bacterial-mediated transfer of plasmid DNA to mammalian cells (also referred to as "bactofection"). For example, in some embodiments, delivery of genetic material is achieved through entry of the entire bacterium into target cells. In some embodiments, spontaneous or induced bacterial lysis can lead to the release of plasmid for subsequent eukaryotic cell expression. In some embodiments, the bacterium can deliver nucleic acids to non-phagocytic mammalian cells (e.g., tumor cells) and/or to phagocytic cells, e.g., certain immune cells and/or APCs. In some embodiments, the nucleic acids delivered by the bacterium can be transferred to the nucleus of the cell in the subject for expression. In some embodiments, the nucleic acids also include appropriate nucleic acid sequences necessary for the expression of the operably linked sequences encoding the variant immunomodulatory polypeptides in a particular host cell, e.g., regulatory elements such as promoters or enhancers. In some embodiments, the infectious agent that is a bacterium can deliver nucleic acids encoding the immunomodulatory proteins in the form of an RNA, such as a pre-made translation-competent RNA delivered to the cytoplasm of the target cell for translation by the target cell's machinery.

**[0347]** In some embodiments, the bacterium can replicate and lyse the target cells, e.g., tumor cells. In some embodiments, the bacterium can contain and/or release nucleic acid sequences and/or gene products in the cytoplasm of the target cells, thereby killing the target cell, e.g., tumor cell. In some embodiments, the infectious agent is bacterium that can replicate specifically in a particular environment in the subject, e.g., tumor microenvironment (TME). For example, in some embodiments, the bacterium can replicate specifically in anaerobic or hypoxic microenvironments. In some embodiments, conditions or factors present in particular environments, e.g., aspartate, serine, citrate, ribose or galactose produced by cells in the TME, can act as chemoattractants to attract the bacterium to the environment. In some embodiments, the bacterium can express and/or secrete the immunomodulatory proteins described herein in the environment, e.g., TME.

**[0348]** In some embodiments, the infectious agent is a bacterium that is a Listeria sp., a Bifidobacterium sp., an Escherichia sp., a Clostridium sp., a Salmonella sp., a Shigella sp., a Vibrio sp. or a Yersinia sp. In

some embodiments, the bacterium is selected from among one or more of *Listeria monocytogenes, Salmonella typhimurium, Salmonella choleraesuis, Escherichia coli, Vibrio cholera, Clostridium perfringens, Clostridium butyricum, Clostridium novyi, Clostridium acetobutylicum, Bifidobacterium infantis, Bifidobacterium longum* and *Bifidobacterium adolescentis.* In some embodiments, the bacterium is an engineered bacterium. In some embodiments, the bacterium is an engineered bacterium such as those described in, e.g., Seow and Wood (2009) Molecular Therapy 17(5):767-777; Baban et al. (2010) Bioengineered Bugs 1:6, 385-394; Patyar et al. (2010) J Biomed Sci 17:21; Tangney et al. (2010) Bioengineered Bugs 1:4, 284-287; van Pijkeren et al. (2010) Hum Gene Ther. 21(4):405-416; WO 2012/149364; WO 2014/198002; US 9103831; US 9453227; US 2014/0186401; US 2004/0146488; US 2011/0293705; US 2015/0359909 and EP 3020816. The bacterium can be modified to deliver nucleic acid sequences encoding any of the variant immunomodulatory polypeptides, conjugates and/or fusions provided herein, and/or to express such variant immunomodulatory polypeptides in the subject.

**F. Nucleic Acids, Vectors and Methods for Producing the Polypeptides or Cells**

**[0349]** Provided herein are isolated or recombinant nucleic acids collectively referred to as "nucleic acids" which encode any of the various provided embodiments of the variant PD-L1 polypeptides or immunomodulatory polypeptides provided herein. In some embodiments, nucleic acids provided herein, including all described below, are useful in recombinant production (e.g., expression) of variant PD-L1 polypeptides or immunomodulatory polypeptides provided herein. In some embodiments, nucleic acids provided herein, including all described below, are useful in expression of variant PD-L1 polypeptides or immunomodulatory polypeptides provided herein in cells, such as in engineered cells, e.g. immune cells, or infectious agent cells. The nucleic acids provided herein can be in the form of RNA or in the form of DNA, and include mRNA, cRNA, recombinant or synthetic RNA and DNA, and cDNA. The nucleic acids provided herein are typically DNA molecules, and usually double-stranded DNA molecules. However, single-stranded DNA, single-stranded RNA, double-stranded RNA, and hybrid DNA/RNA nucleic acids or combinations thereof comprising any of the nucleotide sequences of the invention also are provided.

**[0350]** Also provided herein are recombinant expression vectors and recombinant host cells useful in producing the variant PD-L1 polypeptides or immunomodulatory polypeptides provided herein.

**[0351]** Also provided herein are engineered cells, such as engineered immune cells, containing any of the provided nucleic acids or encoded variant PD-L1 polypeptides or immunomodulatory polypeptides, such as any of the transmembrane immunomodulatory polypeptides or secretable immunomodulatory polypeptides.

**[0352]** Also provided herein are infectious agents, such as bacterial or viral cells, containing any of the provided nucleic acids or encoded variant PD-L1 polypeptides or immunomodulatory polypeptides, such as any of the transmembrane immunomodulatory polypeptides or secretable immunomodulatory polypeptides.

**[0353]** In any of the above provided embodiments, the nucleic acids encoding the immunomodulatory polypeptides provided herein can be introduced into cells using recombinant DNA and cloning techniques. To do so, a recombinant DNA molecule encoding an immunomodulatory polypeptide is prepared. Methods of preparing such DNA molecules are well known in the art. For instance, sequences coding for the peptides could be excised from DNA using suitable restriction enzymes. Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidite method. Also, a combination of these techniques could be used. In some instances, a recombinant or synthetic nucleic acid

may be generated through polymerase chain reaction (PCR). In some embodiments, a DNA insert can be generated encoding one or more variant PD-L1 polypeptides containing at least one affinity-modified IgSF domain and, in some embodiments, a signal peptide, a transmembrane domain and/or an endodomain in accord with the provided description. This DNA insert can be cloned into an appropriate transduction/transfection vector as is known to those of skill in the art. Also provided are expression vectors containing the nucleic acid molecules.

**[0354]** In some embodiments, the expression vectors are capable of expressing the immunomodulatory proteins in an appropriate cell under conditions suited to expression of the protein. In some aspects, nucleic acid molecule or an expression vector comprises the DNA molecule that encodes the immunomodulatory protein operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the DNA molecule is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation.

**[0355]** In some embodiments, expression of the immunomodulatory protein is controlled by a promoter or enhancer to control or regulate expression. The promoter is operably linked to the portion of the nucleic acid molecule encoding the variant polypeptide or immunomodulatory protein. In some embodiments, the promotor is a constitutively active promotor (such as a tissue-specific constitutively active promotor or other constitutive promotor). In some embodiments, the promotor is an inducible promotor, which may be responsive to an inducing agent (such as a T cell activation signal).

**[0356]** In some embodiments, a constitutive promoter is operatively linked to the nucleic acid molecule encoding the variant polypeptide or immunomodulatory protein. Exemplary constitutive promoters include the Simian vacuolating virus 40 (SV40) promoter, the cytomegalovirus (CMV) promoter, the ubiquitin C (UbC) promoter, and the EF-1 alpha (EF1a) promoter. In some embodiments, the constitutive promoter is tissue specific. For example, in some embodiments, the promoter allows for constitutive expression of the immunomodulatory protein in specific tissues, such as immune cells, lymphocytes, or T cells. Exemplary tissue-specific promoters are described in U.S. Patent No. 5,998,205, including, for example, a fetoprotein, DF3, tyrosinase, CEA, surfactant protein, and ErbB2 promoters.

**[0357]** In some embodiments, an inducible promoter is operatively linked to the nucleic acid molecule encoding the variant polypeptide or immunomodulatory protein such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription. For example, the promoter can be a regulated promoter and transcription factor expression system, such as the published tetracycline-regulated systems or other regulatable systems (see, e.g. published International PCT Appl. No. WO 01/30843), to allow regulated expression of the encoded polypeptide. An exemplary regulatable promoter system is the Tet-On (and Tet-Off) system available, for example, from Clontech (Palo Alto, CA). This promoter system allows the regulated expression of the transgene controlled by tetracycline or tetracycline derivatives, such as doxycycline. Other regulatable promoter systems are known (see e.g., published U.S. Application No. 2002-0168714, entitled "Regulation of Gene Expression Using Single-Chain, Monomeric, Ligand Dependent Polypeptide Switches," which describes gene switches that contain ligand binding domains and transcriptional regulating domains, such as those from hormone receptors).

**[0358]** In some embodiments, the promotor is responsive to an element responsive to T-cell activation signaling. Solely by way of example, in some embodiments, an engineered T cell comprises an expression vector encoding the immunomodulatory protein and a promotor operatively linked to control expression of the immunomodulatory protein. The engineered T cell can be activated, for example by signaling through

an engineered T cell receptor (TCR) or a chimeric antigen rector (CAR), and thereby triggering expression and secretion of the immunomodulatory protein through the responsive promotor.

**[0359]** In some embodiments, an inducible promoter is operatively linked to the nucleic acid molecule encoding the immunomodulatory protein such that the immunomodulatory protein is expressed in response to a nuclear factor of activated T-cells (NFAT) or nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). For example, in some embodiments, the inducible promoter comprises a binding site for NFAT or NF-κB. For example, in some embodiments, the promoter is an NFAT or NF-κB promoter or a functional variant thereof. Thus, in some embodiments, the nucleic acids make it possible to control the expression of immunomodulatory protein while also reducing or eliminating the toxicity of the immunomodulatory protein. In particular, engineered immune cells comprising the nucleic acids of the invention express and secrete the immunomodulatory protein only when the cell (e.g., a T-cell receptor (TCR) or a chimeric antigen receptor (CAR) expressed by the cell) is specifically stimulated by an antigen and/or the cell (e.g., the calcium signaling pathway of the cell) is non-specifically stimulated by, e.g., phorbol myristate acetate (PMA)/Ionomycin. Accordingly, the expression and, in some cases, secretion, of immunomodulatory protein can be controlled to occur only when and where it is needed (e.g., in the presence of an infectious disease-causing agent, cancer, or at a tumor site), which can decrease or avoid undesired immunomodulatory protein interactions.

**[0360]** In some embodiments, the nucleic acid encoding an immunomodulatory protein described herein comprises a suitable nucleotide sequence that encodes a NFAT promoter, NF-κB promoter, or a functional variant thereof. "NFAT promoter" as used herein means one or more NFAT responsive elements linked to a minimal promoter. "NF-κB promoter" refers to one or more NF-κB responsive elements linked to a minimal promoter. In some embodiments, the minimal promoter of a gene is a minimal human IL-2 promoter or a CMV promoter. The NFAT responsive elements may comprise, e.g., NFAT1, NFAT2, NFAT3, and/or NFAT4 responsive elements. The NFAT promoter, NF-κB promoter, or a functional variant thereof may comprise any number of binding motifs, e.g., at least two, at least three, at least four, at least five, or at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or up to twelve binding motifs.

**[0361]** The resulting recombinant expression vector having the DNA molecule thereon is used to transform an appropriate host. This transformation can be performed using methods well known in the art. In some embodiments, a nucleic acid provided herein further comprises nucleotide sequence that encodes a secretory or signal peptide operably linked to the nucleic acid encoding an immunomodulatory polypeptide such that a resultant soluble immunomodulatory polypeptide is recovered from the culture medium, host cell, or host cell periplasm. In other embodiments, the appropriate expression control signals are chosen to allow for membrane expression of an immunomodulatory polypeptide. Furthermore, commercially available kits as well as contract manufacturing companies can also be utilized to make engineered cells or recombinant host cells provided herein.

**[0362]** In some embodiments, the resulting expression vector having the DNA molecule thereon is used to transform, such as transduce, an appropriate cell. The introduction can be performed using methods well known in the art. Exemplary methods include those for transfer of nucleic acids encoding the receptors, including via viral, e.g., retroviral or lentiviral, transduction, transposons, and electroporation. In some embodiments, the expression vector is a viral vector. In some embodiments, the nucleic acid is transferred into cells by lentiviral or retroviral transduction methods.

**[0363]** Any of a large number of publicly available and well-known mammalian host cells, including mammalian T-cells or APCs, can be used in the preparing the polypeptides or engineered cells. The selection of a cell is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA

molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all cells can be equally effective for the expression of a particular DNA sequence.

**[0364]** In some embodiments, the host cells can be a variety of eukaryotic cells, such as in yeast cells, or with mammalian cells such as Chinese hamster ovary (CHO) or HEK293 cells. In some embodiments, the host cell is a suspension cell and the polypeptide is engineered or produced in cultured suspension, such as in cultured suspension CHO cells, e.g. CHO-S cells. In some examples, the cell line is a CHO cell line that is deficient in DHFR (DHFR-), such as DG44 and DUXB11. In some embodiments, the cell is deficient in glutamine synthase (GS), e.g. CHO-S cells, CHOK1 SV cells, and CHOZN((R)) GS-/- cells. In some embodiments, the CHO cells, such as suspension CHO cells, may be CHO-S-2H2 cells, CHO-S-clone 14 cells, or ExpiCHO-S cells.

**[0365]** In some embodiments, host cells can also be prokaryotic cells, such as with *E. coli.* The transformed recombinant host is cultured under polypeptide expressing conditions, and then purified to obtain a soluble protein. Recombinant host cells can be cultured under conventional fermentation conditions so that the desired polypeptides are expressed. Such fermentation conditions are well known in the art. Finally, the polypeptides provided herein can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, and affinity chromatography. Protein refolding steps can be used, as desired, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed in the final purification steps.

**[0366]** In some embodiments, the cell is an immune cell, such as any described above in connection with preparing engineered cells. In some embodiments, such engineered cells are primary cells. In some embodiments, the engineered cells are autologous to the subject. In some embodiment, the engineered cells are allogeneic to the subject. In some embodiments, the engineered cells are obtained from a subject, such as by leukapheresis, and transformed ex vivo for expression of the immunomodulatory polypeptide, e.g. transmembrane immunomodulatory polypeptide or secretable immunomodulatory polypeptide.

**[0367]** Also provided are nucleic acids encoding any of the variant immunomodulatory polypeptides contained in infectious agents described herein. In some embodiments, the infectious agents deliver the nucleic acids to a cell in the subject, and/or permit expression of the encoded variant polypeptides in the cell. Also provided are nucleic acids that are used to generate, produce or modify such infectious agents. For example, in some embodiments, provided are vectors and/or plasmids that contain nucleic acids encoding the variant immunomodulatory polypeptides, for generation of the infectious agents, delivery to the cells in a subject and/or expression of the variant immunomodulatory polypeptides in the cells in the subject.

**[0368]** In some embodiments, the provided nucleic acids are recombinant viral or bacterial vectors containing nucleic acid sequences encoding the variant immunomodulatory polypeptides. In some embodiments, the recombinant vectors can be used to produce an infectious agent that contains nucleic acid sequences encoding the variant immunomodulatory polypeptides and/or to be delivered to a target cell in the subject for expression by the target cell. In some embodiments, the recombinant vector is an expression vector. In some embodiments, the recombinant vector includes appropriate sequences necessary for generation and/or production of the infectious agent and expression in the target cell.

**[0369]** In some embodiments, the recombinant vector is a plasmid or cosmid. Plasmid or cosmid containing nucleic acid sequences encoding the variant immunomodulatory polypeptides, as described

herein, is readily constructed using standard techniques well known in the art. For generation of the infectious agent, the vector or genome can be constructed in a plasmid form that can then be transfected into a packaging or producer cell line or a host bacterium. The recombinant vectors can be generated using any of the recombinant techniques known in the art. In some embodiments, the vectors can include a prokaryotic origin of replication and/or a gene whose expression confers a detectable or selectable marker such as a drug resistance for propagation and/or selection in prokaryotic systems.

**[0370]** In some embodiments, the recombinant vector is a viral vector. Exemplary recombinant viral vectors include a lentiviral vector genome, poxvirus vector genome, vaccinia virus vector genome, adenovirus vector genome, adenovirus-associated virus vector genome, herpes virus vector genome, and alpha virus vector genome. Viral vectors can be live, attenuated, replication conditional or replication deficient, non-pathogenic (defective), replication competent viral vector, and/or is modified to express a heterologous gene product, e.g., the variant immunomodulatory polypeptides provided herein. Vectors for generation of viruses also can be modified to alter attenuation of the virus, which includes any method of increasing or decreasing the transcriptional or translational load.

**[0371]** Exemplary viral vectors that can be used include modified vaccinia virus vectors (see, e.g., Guerra et al., J. Virol. 80:985-98 (2006); Tartaglia et al., AIDS Research and Human Retroviruses 8: 1445-47 (1992); Gheradi et al., J. Gen. Virol. 86:2925-36 (2005); Mayr et al., Infection 3:6-14 (1975); Hu et al., J. Virol. 75: 10300-308 (2001); U.S. Patent Nos. 5,698,530, 6,998,252, 5,443,964, 7,247,615 and 7,368,116); adenovirus vector or adenovirus-associated virus vectors (see., e.g., Molin et al., J. Virol. 72:8358-61 (1998); Narumi et al., Am J. Respir. Cell Mol. Biol. 19:936-41 (1998); Mercier et al., Proc. Natl. Acad. Sci. USA 101:6188-93 (2004); U.S. PatentNos. 6,143,290; 6,596,535; 6,855,317; 6,936,257; 7,125,717; 7,378,087; 7,550,296); retroviral vectors including those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), ecotropic retroviruses, simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and combinations (see, e.g., Buchscher et al., J. Virol. 66:2731-39 (1992); Johann et al., J. Virol. 66: 1635-40 (1992); Sommerfelt et al., Virology 176:58-59 (1990); Wilson et al., J. Virol. 63:2374-78 (1989); Miller et al., J. Virol. 65:2220-24 (1991); Miller et al., Mol. Cell Biol. 10:4239 (1990); Kolberg, NIH Res. 4:43 1992; Cornetta et al., Hum. Gene Ther. 2:215 (1991)); lentiviral vectors including those based upon Human Immunodeficiency Virus (HIV-1), HIV-2, feline immunodeficiency virus (FIV), equine infectious anemia virus, Simian Immunodeficiency Virus (SIV), and maedi/visna virus (see, e.g., Pfeifer et al., Annu. Rev. Genomics Hum. Genet. 2: 177-211 (2001); Zufferey et al., J. Virol. 72: 9873, 1998; Miyoshi et al., J. Virol. 72:8150, 1998; Philpott and Thrasher, Human Gene Therapy 18:483, 2007; Engelman et al., J. Virol. 69: 2729, 1995; Nightingale et al., Mol. Therapy, 13: 1121, 2006; Brown et al., J. Virol. 73:9011 (1999); WO 2009/076524; WO 2012/141984; WO 2016/011083; McWilliams et al., J. Virol. 77: 11150, 2003; Powell et al., J. Virol. 70:5288, 1996) or any, variants thereof, and/or vectors that can be used to generate any of the viruses described above. In some embodiments, the recombinant vector can include regulatory sequences, such as promoter or enhancer sequences, that can regulate the expression of the viral genome, such as in the case for RNA viruses, in the packaging cell line (see, e.g., U.S. Patent Nos.5,385,839 and 5,168,062).

**[0372]** In some embodiments, the recombinant vector is an expression vector, e.g., an expression vector that permits expression of the encoded gene product when delivered into the target cell, e.g., a cell in the subject, e.g., a tumor cell, an immune cell and/or an APC. In some embodiments, the recombinant expression vectors contained in the infectious agent are capable of expressing the immunomodulatory proteins in the target cell in the subject, under conditions suited to expression of the protein.

**[0373]** In some aspects, nucleic acids or an expression vector comprises a nucleic acid sequence that encodes the immunomodulatory protein operatively linked to appropriate expression control sequences. Methods of affecting this operative linking, either before or after the nucleic acid sequence encoding the

immunomodulatory protein is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation. The promoter can be operably linked to the portion of the nucleic acid sequence encoding the immunomodulatory protein. In some embodiments, the promotor is a constitutively active promotor in the target cell (such as a tissue-specific constitutively active promotor or other constitutive promotor). For example, the recombinant expression vector may also include, lymphoid tissue-specific transcriptional regulatory elements (TRE) such as a B lymphocyte, T lymphocyte, or dendritic cell specific TRE. Lymphoid tissue specific TRE are known in the art (see, e.g., Thompson et al., Mol. Cell. Biol. 12:1043-53 (1992); Todd et al., J. Exp. Med. 177:1663-74 (1993); Penix et al., J. Exp. Med. 178:1483-96 (1993)). In some embodiments, the promotor is an inducible promotor, which may be responsive to an inducing agent (such as a T cell activation signal). In some embodiments, nucleic acids delivered to the target cell in the subject, e.g., tumor cell, immune cell and/or APC, can be operably linked to any of the regulatory elements described above.

**[0374]** In some embodiments, the vector is a bacterial vector, e.g., a bacterial plasmid or cosmid. In some embodiments, the bacterial vector is delivered to the target cell, e.g., tumor cells, immune cells and/or APCs, via bacterial-mediated transfer of plasmid DNA to mammalian cells (also referred to as "bactofection"). In some embodiments, the delivered bacterial vector also contains appropriate expression control sequences for expression in the target cells, such as a promoter sequence and/or enhancer sequences, or any regulatory or control sequences described above. In some embodiments, the bacterial vector contains appropriate expression control sequences for expression and/or secretion of the encoded variant polypeptides in the infectious agent, e.g., the bacterium.

**[0375]** In some embodiments, polypeptides provided herein can also be made by synthetic methods. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides. For example, well known solid phase synthesis techniques include the use of protecting groups, linkers, and solid phase supports, as well as specific protection and deprotection reaction conditions, linker cleavage conditions, use of scavengers, and other aspects of solid phase peptide synthesis. Peptides can then be assembled into the polypeptides as provided herein.

## IV. METHODS OF ASSESSING ACTIVITY IMMUNE MODULATION OF VARIANT PD-L1 POLYPEPTIDES AND IMMUNOMODULATORY PROTEINS

**[0376]** In some embodiments, the variant PD-L1 polypeptides provided herein (e.g. full-length and/or specific binding fragments or conjugates, stack constructs or fusion thereof, engineered cells or infectious agents) exhibit immunomodulatory activity to modulate T cell activation. In some embodiments, PD-L1 polypeptides modulate IFN-gamma expression in a T cell assay relative to a wild-type or unmodified PD-L1 control. In some cases, modulation of IFN-gamma expression can increase or decrease IFN-gamma expression relative to the control. Assays to determine specific binding and IFN-gamma expression are well-known in the art and include the MLR (mixed lymphocyte reaction) assays measuring interferon-gamma cytokine levels in culture supernatants (Wang et al., Cancer Immunol Res. 2014 Sep: 2(9):846-56), SEB (staphylococcal enterotoxin B) T cell stimulation assay (Wang et al., Cancer Immunol Res. 2014 Sep: 2(9):846-56), and anti-CD3 T cell stimulation assays (Li and Kurlander, J Transl Med. 2010: 8: 104).

**[0377]** In some embodiments, a variant PD-L1 polypeptide can in some embodiments increase or, in alternative embodiments, decrease IFN-gamma (interferon-gamma) expression in a primary T-cell assay relative to a wild-type PD-L1 control. In some embodiments, such activity may depend on whether the

variant PD-L1 polypeptide is provided in a form for antagonist activity or in a form for agonist activity. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein is an antagonist of the inhibitory receptor, such as blocks an inhibitory signal in the cell that may occur to decrease response to an activating stimuli, e.g. CD3 and/or CD28 costimulatory signal or a mitogenic signal. Those of skill will recognize that different formats of the primary T-cell assay used to determine an increase or decrease in IFN-gamma expression exist.

**[0378]** In assaying for the ability of a variant PD-L1 to increase or decrease IFN-gamma expression in a primary T-cell assay, a Mixed Lymphocyte Reaction (MLR) assay can be used. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein provided in antagonist form, such as soluble form, e.g. variant PD-L1-Fc or secretable immunomodulatory protein, block activity of the PD-1 inhibitory receptor and thereby increase MLR activity in the assay, such as observed by increased production of IFN-gamma in the assay. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein provided in agonist form, such as a localizing vIgD stack or conjugate containing a tumor-localizing moiety or an engineered cell expressing a transmembrane immunomodulatory protein as provided, may stimulate activity of the PD-1 inhibitory receptor and thereby decrease MLR activity, such as evidenced by decreased IFN-gamma production. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein provided in agonist form, such as a localizing vIgD stack or conjugate containing a tumor-localizing moiety or an engineered cell expressing a transmembrane immunomodulatory protein as provided, may block activity of the PD-1 inhibitory receptor and thereby increase MLR activity, such as increase IFN-gamma production.

**[0379]** Alternatively, in assaying for the ability of a variant PD-L1 to modulate an increase or decrease IFN-gamma expression in a primary T-cell assay, a co-immobilization assay can be used. In a co-immobilization assay, a TCR signal, provided in some embodiments by anti-CD3 antibody, is used in conjunction with a co-immobilized variant PD-L1 to determine the ability to increase or decrease IFN-gamma expression relative to a PD-L1 unmodified or wild-type control. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein, e.g. a co-immobilized variant PD-L1 (e.g., PD-L1-Fc), decreases IFN-gamma production in a coimmobilization assay.

**[0380]** In some embodiments, in assaying for the ability of a variant PD-L1 to modulate an increase or decrease IFN-gamma expression a T cell reporter assay can be used. In some embodiments, the T cell is a Jurkat T cell line or is derived from Jurkat T cell lines. In reporter assays, the reporter cell line (e.g. Jurkat reporter cell) also is generated to overexpress an inhibitory receptor that is the cognate binding partner of the variant IgSF domain polypeptide. For example, in the case of a variant PD-L1, the reporter cell line (e.g. Jurkat reporter cell) is generated to overexpress PD-1. In some embodiments, the reporter T cells also contain a reporter construct containing an inducible promoter responsive to T cell activation operably linked to a reporter. In some embodiments, the reporter is a fluorescent or luminescent reporter. In some embodiments, the reporter is luciferase. In some embodiments, the promoter is responsive to CD3 signaling. In some embodiments, the promoter is an NFAT promoter. In some embodiments, the promoter is responsive to costimulatory signaling, e.g. CD28 costimulatory signaling. In some embodiments, the promoter is an IL-2 promoter.

**[0381]** In aspects of a reporter assay, a reporter cell line is stimulated, such as by co-incubation with antigen presenting cells (APCs) expressing the wild-type ligand of the inhibitory receptor, e.g. PD-L1. In some embodiments, the APCs are artificial APCs. Artificial APCs are well known to a skilled artisan. In some embodiments, artificial APCs are derived from one or more mammalian cell line, such as K562, CHO or 293 cells.

**[0382]** In some embodiments, the Jurkat reporter cells are co-incubated with artificial APCs overexpressing

the inhibitory ligand in the presence of the variant IgSF domain molecule or immunomodulatory protein, e.g., variant PD-L1 polypeptide or immunomodulatory protein. In some embodiments, reporter expression is monitored, such as by determining the luminescence or fluorescence of the cells. In some embodiments, normal interactions between its inhibitory receptor and ligand result in a repression of or decrease in the reporter signal, such as compared to control, e.g. reporter expression by co-incubation of control T cells and APCs in which the inhibitory receptor and ligand interaction is not present, e.g. APCs that do not overexpress PD-L1. In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein provided herein antagonizes the interaction, e.g. when provided in soluble form as a variant PD-L1-Fc or when expressed from the APC as a secretable immunomodulatory protein, thereby resulting in an increase in the reporter signal compared to the absence of the variant PD-L1 polypeptide or immunomodulatory protein. In some cases, certain formats of a variant PD-L1 polypeptide or immunomodulatory protein as provided herein may provide an agonist activity, thereby decreasing reporter expression compared to the absence of the variant PD-L1 polypeptide or immunomodulatory protein.,

**[0383]** Use of proper controls is known to those of skill in the art, however, in the aforementioned embodiments, a control typically involves use of the unmodified PD-L1, such as a wild-type of native PD-L1 isoform from the same mammalian species from which the variant PD-L1 was derived or developed. In some embodiments, the wild-type or native PD-L1 is of the same form or corresponding form as the variant. For example, if the variant PD-L1 is a soluble form containing a variant ECD fused to an Fc protein, then the control is a soluble form containing the wild-type or native ECD of PD-L1 fused to the Fc protein. Irrespective of whether the binding affinity and/or selectivity to either one or more of PD-1 and CD80 is increased or decreased, a variant PD-L1 in some embodiments will increase IFN-gamma expression and, in alternative embodiments, decrease IFN-gamma expression in a T-cell assay relative to a wild-type PD-L1 control.

**[0384]** In some embodiments, a variant PD-L1 polypeptide or immunomodulatory protein, increases IFN-gamma expression (i.e., protein expression) relative to a wild-type or unmodified PD-L1 control by at least: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or higher. In other embodiments, a variant PD-L1 or immunomodulatory protein decreases IFN-gamma expression (i.e. protein expression) relative to a wild-type or unmodified PD-L1 control by at least: 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or higher. In some embodiments, the wild-type PD-L1 control is murine PD-L1, such as would typically be used for a variant PD-L1 altered in sequence from that of a wild-type murine PD-L1 sequence. In some embodiments, the wild-type PD-L1 control is human PD-L1, such as would typically be used for a variant PD-L1 altered in sequence from that of a wild-type human PD-L1 sequence such as an PD-L1 sequence comprising the sequence of amino acids of SEQ ID NO: 30 or 1728, or SEQ ID NO: 55 or 309.

## V. PHARMACEUTICAL FORMULATIONS

**[0385]** Provided herein are compositions containing any of the variant PD-L1 polypeptides, immunomodulatory proteins, conjugates, engineered cells or infectious agents described herein. The pharmaceutical composition can further comprise a pharmaceutically acceptable excipient. For example, the pharmaceutical composition can contain one or more excipients for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. In some aspects, a skilled artisan understands that a pharmaceutical composition containing cells may differ from a pharmaceutical composition containing a protein.

**[0386]** In some embodiments, the pharmaceutical composition is a solid, such as a powder, capsule, or

tablet. For example, the components of the pharmaceutical composition can be lyophilized. In some embodiments, the solid pharmaceutical composition is reconstituted or dissolved in a liquid prior to administration.

**[0387]** In some embodiments, the pharmaceutical composition is a liquid, for example variant PD-L1 polypeptides dissolved in an aqueous solution (such as physiological saline or Ringer's solution). In some embodiments, the pH of the pharmaceutical composition is between about 4.0 and about 8.5 (such as between about 4.0 and about 5.0, between about 4.5 and about 5.5, between about 5.0 and about 6.0, between about 5.5 and about 6.5, between about 6.0 and about 7.0, between about 6.5 and about 7.5, between about 7.0 and about 8.0, or between about 7.5 and about 8.5).

**[0388]** In some embodiments, the pharmaceutical composition comprises a pharmaceutically-acceptable excipient, for example a filler, binder, coating, preservative, lubricant, flavoring agent, sweetening agent, coloring agent, a solvent, a buffering agent, a chelating agent, or stabilizer. Examples of pharmaceutically-acceptable fillers include cellulose, dibasic calcium phosphate, calcium carbonate, microcrystalline cellulose, sucrose, lactose, glucose, mannitol, sorbitol, maltol, pregelatinized starch, corn starch, or potato starch. Examples of pharmaceutically-acceptable binders include polyvinylpyrrolidone, starch, lactose, xylitol, sorbitol, maltitol, gelatin, sucrose, polyethylene glycol, methyl cellulose, or cellulose. Examples of pharmaceutically-acceptable coatings include hydroxypropyl methylcellulose (HPMC), shellac, corn protein zein, or gelatin. Examples of pharmaceutically-acceptable disintegrants include polyvinylpyrrolidone, carboxymethyl cellulose, or sodium starch glycolate. Examples of pharmaceutically-acceptable lubricants include polyethylene glycol, magnesium stearate, or stearic acid. Examples of pharmaceutically-acceptable preservatives include methyl parabens, ethyl parabens, propyl paraben, benzoic acid, or sorbic acid. Examples of pharmaceutically-acceptable sweetening agents include sucrose, saccharine, aspartame, or sorbitol. Examples of pharmaceutically-acceptable buffering agents include carbonates, citrates, gluconates, acetates, phosphates, or tartrates.

**[0389]** In some embodiments, the pharmaceutical composition further comprises an agent for the controlled or sustained release of the product, such as injectable microspheres, bio-erodible particles, polymeric compounds (polylactic acid, polyglycolic acid), beads, or liposomes.

**[0390]** In some embodiments, the pharmaceutical composition is sterile. Sterilization may be accomplished by filtration through sterile filtration membranes or radiation. Where the composition is lyophilized, sterilization using this method may be conducted either prior to or following lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

**[0391]** In some embodiments, provided are pharmaceutical compositions containing the transmembrane immunomodulatory proteins, including engineered cells expressing such transmembrane immunomodulatory proteins. In some embodiments, the pharmaceutical compositions and formulations include one or more optional pharmaceutically acceptable carrier or excipient. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the present invention are preferably formulated for intravenous administration.

**[0392]** Such a formulation may, for example, be in a form suitable for intravenous infusion. A

pharmaceutically acceptable carrier may be a pharmaceutically acceptable material, composition, or vehicle that is involved in carrying or transporting cells of interest from one tissue, organ, or portion of the body to another tissue, organ, or portion of the body. For example, the carrier may be a liquid or solid filler, diluent, excipient, solvent, or encapsulating material, or some combination thereof. Each component of the carrier must be "pharmaceutically acceptable" in that it must be compatible with the other ingredients of the formulation. It also must be suitable for contact with any tissue, organ, or portion of the body that it may encounter, meaning that it must not carry a risk of toxicity, irritation, allergic response, immunogenicity, or any other complication that excessively outweighs its therapeutic benefits.

## VI. ARTICLES OF MANUFACTURE AND KITS

**[0393]** Also provided herein are articles of manufacture comprising the pharmaceutical compositions described herein in suitable packaging. Suitable packaging for compositions (such as ophthalmic compositions) described herein are known in the art, and include, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

**[0394]** Further provided are kits comprising the pharmaceutical compositions (or articles of manufacture) described herein, which may further comprise instruction(s) on methods of using the composition, such as uses described herein. The kits described herein may also include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein.

## VII. THERAPEUTIC APPLICATIONS

**[0395]** Provided herein are pharmaceutical compositions for use in methods for modulating an immune response, the pharmaceutical compositions containing a variant PD-L1 polypeptide, immunomodulatory protein, conjugate engineered cell or infectious agent described herein, including in connection with treating a disease or condition in a subject, such as in a human patient. The pharmaceutical compositions described herein (including pharmaceutical composition comprising the variant PD-L1 polypeptides, the immunomodulatory proteins, the conjugates, the engineered cells and the infectious agents described herein) can be used in a variety of therapeutic applications, such as the treatment of a disease. For example, in some embodiments the pharmaceutical composition is used to treat inflammatory or autoimmune disorders, cancer, organ transplantation, viral infections, and/or bacterial infections in a mammal. The pharmaceutical composition can modulate (e.g. increase or decrease) an immune response to treat the disease.

**[0396]** In some embodiments, the provided methods are applicable to therapeutic administration of variant PD-L1 polypeptides, the immunomodulatory proteins, the conjugates, the engineered cells and infectious agents described herein. It is within the level of a skilled artisan, in view of the provided disclosure, to choose a format for the indication depending on the type of modulation of the immune response, e.g. increase or decrease that is desired.

**[0397]** In some embodiments, a pharmaceutical composition provided herein that stimulates the immune response is administered, which can be useful, for example, in the treatment of cancer, viral infections, or bacterial infections. In some embodiments, the pharmaceutical composition contains a variant PD-L1 polypeptide in a format that exhibits antagonist activity of its cognate binding partner PD-1 and/or that

inhibits costimulatory signaling via PD-1. Exemplary formats of PD-L1 polypeptide for use in connection with such therapeutic applications include, for example, a variant PD-L1 polypeptide that is soluble (e.g. variant PD-L1-Fc fusion protein), an immunomodulatory protein or "stack" of a variant PD-L1 polypeptide and another IgSF domain, including soluble forms thereof that are Fc fusions, an engineered cell expressing a secretable immunomodulatory protein, or an infectious agent comprising a nucleic acid molecule encoding a secretable immunomodulatory protein, such as for expression and secretion of the secretable immunomodulatory protein in an infected cell (e.g. tumor cell or APC, e.g. dendritic cell). Among such methods are methods carried out by delivery of a variant PD-L1 polypeptide in a soluble format. Exemplary soluble formats are described herein, including formats in which an extracellular portion of a variant PD-L1 polypeptide containing an affinity modified IgSF domain (e.g. IgV) is linked, directly or indirectly, to a multimerization domain, e.g. an Fc domain or region. In some embodiments, such a therapeutic agent is a variant PD-L1-Fc fusion protein.

**[0398]** The provided methods to modulate an immune response can be used to treat a disease or condition, such as a tumor or cancer. In some embodiments, the pharmaceutical composition can be used to inhibit growth of mammalian cancer cells (such as human cancer cells). A method of treating cancer can include administering an effective amount of any of the pharmaceutical compositions described herein to a subject with cancer. The effective amount of the pharmaceutical composition can be administered to inhibit, halt, or reverse progression of cancers, including cancers that are sensitive to modulation of immunological activity, such as by the provided variants or immunomodulatory proteins. Human cancer cells can be treated in vivo, or ex vivo. In ex vivo treatment of a human patient, tissue or fluids containing cancer cells are treated outside the body and then the tissue or fluids are reintroduced back into the patient. In some embodiments, the cancer is treated in a human patient in vivo by administration of the therapeutic composition into the patient. Thus, the present invention provides ex vivo and in vivo methods to inhibit, halt, or reverse progression of the tumor, or otherwise result in a statistically significant increase in progression-free survival (i.e., the length of time during and after treatment in which a patient is living with cancer that does not get worse), or overall survival (also called "survival rate;" i.e., the percentage of people in a study or treatment group who are alive for a certain period of time after they were diagnosed with or treated for cancer) relative to treatment with a control.

**[0399]** The cancers that can be treated by the pharmaceutical compositions and the treatment methods described herein include, but are not limited to, melanoma, bladder cancer, hematological malignancies (leukemia, lymphoma, myeloma), liver cancer, brain cancer, renal cancer, breast cancer, pancreatic cancer (adenocarcinoma), colorectal cancer, lung cancer (small cell lung cancer and non-small-cell lung cancer), spleen cancer, cancer of the thymus or blood cells (i.e., leukemia), prostate cancer, testicular cancer, ovarian cancer, uterine cancer, a musculoskeletal cancer, a head and neck cancer, a gastrointestinal cancer, a germ cell cancer, or an endocrine and neuroendocrine cancer . In some embodiments, the cancer is Ewing's sarcoma. In some embodiments, the cancer is selected from melanoma, lung cancer, bladder cancer, and a hematological malignancy. In some embodiments, the cancer is a lymphoma, lymphoid leukemia, myeloid leukemia, cervical cancer, neuroblastoma, or multiple myeloma.

**[0400]** In some embodiments, the pharmaceutical composition is administered as a monotherapy (i.e., as a single agent) or as a combination therapy (i.e., in combination with one or more additional anticancer agents, such as a chemotherapeutic drug, a cancer vaccine, or an immune checkpoint inhibitor. In some embodiments, the pharmaceutical composition can also be administered with radiation therapy. In some aspects of the present disclosure, the immune checkpoint inhibitor is nivolumab, tremelimumab, pembrolizumab, ipilimumab, or the like.

**[0401]** In some embodiments, the pharmaceutical composition suppresses an immune response, which can be useful in the treatment of inflammatory or autoimmune disorders, or organ transplantation. In some

embodiments, the pharmaceutical composition contains a variant PD-L1 polypeptide in a format that exhibits agonist activity of its cognate binding partner PD-1 and/or that stimulates inhibitory signaling via PD-1. In some aspects, the variant PD-L1 polypeptide stimulates an inhibitory signal through CD80, such as expressed on a lymphocyte or APC. Exemplary formats of a PD-L1 polypeptide for use in connection with such therapeutic applications include, for example, an immunomodulatory protein or "stack" of a variant PD-L1 polypeptide and an IgSF domain or variant thereof that localizes to a cell or tissue of an inflammatory environment, a conjugate containing a variant PD-L1 polypeptide linked to a moiety that localizes to a cell or tissue of an inflammatory environment, an engineered cell expressing a transmembrane immunomodulatory protein, or an infectious agent comprising a nucleic acid molecule encoding a transmembrane immunomodulatory protein, such as for expression of the transmembrane immunomodulatory protein in an infected cell.

**[0402]** The provided methods to modulate an immune response can be used to treat a disease or condition, such as an inflammatory or autoimmune disorder. In some embodiments, the inflammatory or autoimmune disorder is antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, a vasculitis, an autoimmune skin disease, transplantation, a Rheumatic disease, an inflammatory gastrointestinal disease, an inflammatory eye disease, an inflammatory neurological disease, an inflammatory pulmonary disease, an inflammatory endocrine disease, or an autoimmune hematological disease.

**[0403]** In some embodiments, the inflammatory and autoimmune disorders that can be treated by the pharmaceutical composition described herein is Addison's Disease, allergies, alopecia areata, Alzheimer's, antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, ankylosing spondylitis, antiphospholipid syndrome (Hughes Syndrome), asthma, atherosclerosis, rheumatoid arthritis, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune inner ear disease, autoimmune lymphoproliferative syndrome, autoimmune myocarditis, autoimmune oophoritis, autoimmune orchitis, azoospermia, Behcet's Disease, Berger's Disease, bullous pemphigoid, cardiomyopathy, cardiovascular disease, celiac Sprue/coeliac disease, chronic fatigue immune dysfunction syndrome (CFIDS), chronic idiopathic polyneuritis, chronic inflammatory demyelinating, polyradicalneuropathy (CIDP), chronic relapsing polyneuropathy (Guillain-Barré syndrome), Churg-Strauss Syndrome (CSS), cicatricial pemphigoid, cold agglutinin disease (CAD), COPD (chronic obstructive pulmonary disease), CREST syndrome, Crohn's disease, dermatitis, herpetiformus, dermatomyositis, diabetes, discoid lupus, eczema, epidermolysis bullosa acquisita, essential mixed cryoglobulinemia, Evan's Syndrome, exopthalmos, fibromyalgia, Goodpasture's Syndrome, Graves' Disease, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA nephropathy, immunoproliferative disease or disorder, inflammatory bowel disease (IBD), interstitial lung disease, juvenile arthritis, juvenile idiopathic arthritis (JIA), Kawasaki's Disease, Lambert-Eaton Myasthenic Syndrome, lichen planus, lupus nephritis, lymphocytic hypophysitis, Ménière's Disease, Miller Fish Syndrome/acute disseminated encephalomyeloradiculopathy, mixed connective tissue disease, multiple sclerosis (MS), muscular rheumatism, myalgic encephalomyelitis (ME), myasthenia gravis, ocular inflammation, pemphigus foliaceus, pemphigus vulgaris, pernicious anaemia, polyarteritis nodosa, polychondritis, polyglandular syndromes (Whitaker's syndrome), polymyalgia rheumatica, polymyositis, primary agammaglobulinemia, primary biliary cirrhosis/autoimmune cholangiopathy, psoriasis, psoriatic arthritis, Raynaud's Phenomenon, Reiter's Syndrome/reactive arthritis, restenosis, rheumatic fever, rheumatic disease, sarcoidosis, Schmidt's syndrome, scleroderma, Sjörgen's Syndrome, stiff-man syndrome, systemic lupus erythematosus (SLE), systemic scleroderma, Takayasu arteritis, temporal arteritis/giant cell arteritis, thyroiditis, Type 1 diabetes, ulcerative colitis, uveitis, vasculitis, vitiligo, interstitial bowel disease or Wegener's Granulomatosis. In some embodiments, the inflammatory or autoimmune disorder is selected from interstitial bowel disease, transplant, Crohn's disease, ulcerative colitis, multiple sclerosis, asthma, rheumatoid arthritis, and psoriasis.

**[0404]** In some embodiments, the pharmaceutical composition is administered to modulate an autoimmune

condition. For example, suppressing an immune response can be beneficial in methods for inhibiting rejection of a tissue, cell, or organ transplant from a donor by a recipient. Accordingly, in some embodiments, the pharmaceutical compositions described herein are used to limit or prevent graft-related or transplant related diseases or disorders, such as graft versus host disease (GVHD). In some embodiments, the pharmaceutical compositions are used to suppress autoimmune rejection of transplanted or grafted bone marrow, organs, skin, muscle, neurons, islets, or parenchymal cells.

**[0405]** Pharmaceutical compositions comprising engineered cells and the methods described herein can be used in adoptive cell transfer applications. In some embodiments, cell compositions comprising engineered cells can be used in associated methods to, for example, modulate immunological activity in an immunotherapy approach to the treatment of, for example, a mammalian cancer or, in other embodiments the treatment of autoimmune disorders. The methods employed generally comprise a method of contacting a TIP of the present invention with a mammalian cell under conditions that are permissive to specific binding of the affinity modified IgSF domain and modulation of the immunological activity of the mammalian cell. In some embodiments, immune cells (such as tumor infiltrating lymphocytes (TILs), T-cells (including CD8+ or CD4+ T-cells), or APCs) are engineered to express as a membrane protein and/or as a soluble variant PD-L1 polypeptide, immunomodulatory protein, or conjugate as described herein. The engineered cells can then be contact a mammalian cell, such as an APC, a second lymphocyte or tumor cell in which modulation of immunological activity is desired under conditions that are permissive of specific binding of the affinity modified IgSF domain to a counter-structure on the mammalian cell such that immunological activity can be modulated in the mammalian cell. Cells can be contacted in vivo or ex vivo.

**[0406]** In some embodiments, the engineered cells are autologous cells. In other embodiments, the cells are allogeneic. In some embodiments, the cells are autologous engineered cells reinfused into the mammal from which it was isolated. In some embodiments, the cells are allogenic engineered cells infused into the mammal. In some embodiments, the cells are harvested from a patient's blood or tumor, engineered to express a polypeptide (such as the variant PD-L1polypeptide, immunomodulatory protein, or conjugate as described herein), expanded in an in vitro culture system (for example, by stimulating the cells), and reinfused into the patient to mediate tumor destruction. In some embodiments, the methods is conducted by adoptive cell transfer wherein cells expressing the TIP (e.g., a T-cell) are infused back into the patient. In some embodiments, the therapeutic compositions and methods of the invention are used in the treatment of a mammalian patient of cancers such as lymphoma, lymphoid leukemia, myeloid leukemia, cervical cancer, neuroblastoma, or multiple myeloma.

**[0407]** In some embodiments, the pharmaceutical composition is administered to a subject. In some embodiments, the subject is a human patient. Generally, dosages and routes of administration of the pharmaceutical composition are determined according to the size and condition of the subject, according to standard pharmaceutical practice. For example, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models such as mice, rats, rabbits, dogs, pigs, or monkeys. An animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. The exact dosage will be determined in light of factors related to the subject requiring treatment. Dosage and administration are adjusted to provide sufficient levels of the active compound or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, time and frequency of administration, drug combination(s), reaction sensitivities, and response to therapy.

**[0408]** Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or biweekly depending on the half-life and clearance rate of the particular formulation. The frequency of dosing will depend upon the pharmacokinetic parameters of the molecule in the formulation used. Typically,

a composition is administered until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as multiple doses (at the same or different concentrations/dosages) over time, or as a continuous infusion. Further refinement of the appropriate dosage is routinely made. Appropriate dosages may be ascertained through use of appropriate dose-response data. A number of biomarkers or physiological markers for therapeutic effect can be monitored including T cell activation or proliferation, cytokine synthesis or production (e.g., production of TNF-α, IFN-γ, IL-2), induction of various activation markers (e.g., CD25, IL-2 receptor), inflammation, joint swelling or tenderness, serum level of C-reactive protein, anti-collagen antibody production, and/or T cell-dependent antibody response(s).

**[0409]** In some embodiments, the pharmaceutical composition is administered to a subject through any route, including orally, transdermally, by inhalation, intravenously, intra-arterially, intramuscularly, direct application to a wound site, application to a surgical site, intraperitoneally, by suppository, subcutaneously, intradermally, transcutaneously, by nebulization, intrapleurally, intraventricularly, intra-articularly, intraocularly, or intraspinally.

**[0410]** In some embodiments, the dosage of the pharmaceutical composition is a single dose or a repeated dose. In some embodiments, the doses are given to a subject once per day, twice per day, three times per day, or four or more times per day. In some embodiments, about 1 or more (such as about 2 or more, about 3 or more, about 4 or more, about 5 or more, about 6 or more, or about 7 or more) doses are given in a week. In some embodiments, multiple doses are given over the course of days, weeks, months, or years. In some embodiments, a course of treatment is about 1 or more doses (such as about 2 or more does, about 3 or more doses, about 4 or more doses, about 5 or more doses, about 7 or more doses, about 10 or more doses, about 15 or more doses, about 25 or more doses, about 40 or more doses, about 50 or more doses, or about 100 or more doses).

**[0411]** In some embodiments, an administered dose of the pharmaceutical composition is about 1 µg of protein per kg subject body mass or more (such as about 2 µg of protein per kg subject body mass or more, about 5 µg of protein per kg subject body mass or more, about 10 µg of protein per kg subject body mass or more, about 25 µg of protein per kg subject body mass or more, about 50 µg of protein per kg subject body mass or more, about 100 µg of protein per kg subject body mass or more, about 250 µg of protein per kg subject body mass or more, about 500 µg of protein per kg subject body mass or more, about 1 mg of protein per kg subject body mass or more, about 2 mg of protein per kg subject body mass or more, or about 5 mg of protein per kg subject body mass or more).

**[0412]** In some embodiments, a therapeutic amount of a cell composition is administered. Typically, precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising engineered cells, e.g. T cells, as described herein may be administered at a dosage of $10^4$ to $10^9$ cells/kg body weight, such as $10^5$ to $10^6$ cells/kg body weight, including all integer values within those ranges. Engineered cell compositions, such as T cell compositions, may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, e.g., Rosenberg et al, New Eng. J. of Med. 319: 1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

**[0413]** In some embodiments, the pharmaceutical composition contains infectious agents containing

nucleic acid sequences encoding the immunomodulatory variant polypeptides. In some embodiments, the pharmaceutical composition contains a dose of infectious agents suitable for administration to a subject that is suitable for treatment. In some embodiments, the pharmaceutical composition contains an infectious agent that is a virus, at a single or multiple dosage amount, of between about between or between about $1\times10^5$ and about $1\times10^{12}$ plaque-forming units (pfu), $1\times10^6$ and $1\times10^{10}$ pfu, or $1\times10^7$ and $1\times10^{10}$ pfu, each inclusive, such as at least or at least about or at about $1\times10^6$, $1\times 10^7$, $1\times10^8$, $1\times10^9$, $2\times10^9$, $3\times10^9$, $4\times10^9$, $5\times10^9$pfu or about $1\times10^{10}$ pfu. In some embodiments, the pharmaceutical composition can contain a virus concentration of from or from about $10^5$ to about $10^{10}$ pfu/mL, for example, $5\times10^6$ to $5\times10^9$ or $1\times10^7$ to $1\times10^9$ pfu/mL, such as at least or at least about or at about $10^6$ pfu/mL, $10^7$ pfu/mL, $10^8$ pfu/mL or $10^9$ pfu/mL. In some embodiments, the pharmaceutical composition contains an infectious agent that is a bacterium, at a single or multiple dosage amount, of between about between or between about $1\times10^3$ and about $1\times10^9$ colony-forming units (cfu), $1\times10^4$ and $1\times10^9$ cfu, or $1\times10^5$ and $1\times10^7$ cfu, each inclusive, such as at least or at least about or at about $1\times10^4$, $1\times10^5$, $1\times10^6$, $1\times10^7$, $1\times10^8$ or $1\times10^9$ cfu. In some embodiments, the pharmaceutical composition can contain a bacterial concentration of from or from about $10^3$ to about $10^8$ cfu/mL, for example, $5\times10^5$ to $5\times10^7$ or $1\times10^6$ to $1\times10^7$ cfu/mL, such as at least or at least about or at about $10^5$ cfu/mL, $10^6$ cfu/mL, $10^7$ cfu/mL or $10^8$ cfu/mL.

**[0414]** A variety of means are known for determining if administration of a therapeutic composition of the invention sufficiently modulates immunological activity by eliminating, sequestering, or inactivating immune cells mediating or capable of mediating an undesired immune response; inducing, generating, or turning on immune cells that mediate or are capable of mediating a protective immune response; changing the physical or functional properties of immune cells; or a combination of these effects. Examples of measurements of the modulation of immunological activity include, but are not limited to, examination of the presence or absence of immune cell populations (using flow cytometry, immunohistochemistry, histology, electron microscopy, polymerase chain reaction (PCR)); measurement of the functional capacity of immune cells including ability or resistance to proliferate or divide in response to a signal (such as using T-cell proliferation assays and pepscan analysis based on 3H-thymidine incorporation following stimulation with anti-CD3 antibody, anti-T-cell receptor antibody, anti-CD28 antibody, calcium ionophores, PMA (phorbol 12-myristate 13-acetate) antigen presenting cells loaded with a peptide or protein antigen; B cell proliferation assays); measurement of the ability to kill or lyse other cells (such as cytotoxic T cell assays); measurements of the cytokines, chemokines, cell surface molecules, antibodies and other products of the cells (e.g., by flow cytometry, enzyme-linked immunosorbent assays, Western blot analysis, protein microarray analysis, immunoprecipitation analysis); measurement of biochemical markers of activation of immune cells or signaling pathways within immune cells (e.g., Western blot and immunoprecipitation analysis of tyrosine, serine or threonine phosphorylation, polypeptide cleavage, and formation or dissociation of protein complexes; protein array analysis; DNA transcriptional, profiling using DNA arrays or subtractive hybridization); measurements of cell death by apoptosis, necrosis, or other mechanisms (e.g., annexin V staining, TUNEL assays, gel electrophoresis to measure DNA laddering, histology; fluorogenic caspase assays, Western blot analysis of caspase substrates); measurement of the genes, proteins, and other molecules produced by immune cells (e.g., Northern blot analysis, polymerase chain reaction, DNA microarrays, protein microarrays, 2-dimensional gel electrophoresis, Western blot analysis, enzyme linked immunosorbent assays, flow cytometry); and measurement of clinical symptoms or outcomes such as improvement of autoimmune, neurodegenerative, and other diseases involving self-proteins or self-polypeptides (clinical scores, requirements for use of additional therapies, functional status, imaging studies) for example, by measuring relapse rate or disease severity (using clinical scores known to the ordinarily skilled artisan) in the case of multiple sclerosis, measuring blood glucose in the case of type I diabetes, or joint inflammation in the case of rheumatoid arthritis.

## VIII. EXAMPLES

**[0415]** The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Any examples which do not fall under the literal scope of the claims are included merely by way of background or comparative purposes.

### EXAMPLE 1

### Generation of Mutant DNA Constructs of IgSF Domains

**[0416]** Example 1 describes the generation of mutant DNA constructs of human PD-L1 IgSF domains for translation and expression on the surface of yeast as yeast display libraries.

### A. Degenerate Libraries

**[0417]** Constructs were generated based on a wildtype human PD-L1 sequence set forth in SEQ ID NO:309 containing the immunoglobulin-like V-type (IgV) IgV domain as follows:
FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEEDL

KVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGADYKRITVKV

NA

**[0418]** For libraries that target specific residues for complete or partial randomization with degenerate codons, degenerate codons, such as specific mixed base sets to code for various amino acid substitutions, were generated using an algorithm at the URL:
rosettadesign.med.unc.edu/SwiftLib/.

**[0419]** In general, positions to mutate were chosen from direct crystal structure information for PDL1::PD1 complex (eg PDB: 4ZQK). Alternatively, a homology models may be generated if structures of homologous protein complexes are available. Using the structural information, a structure viewer (available at the URL: spdbv.vital-it.ch) was used to identify contact or non-contact interface residues for mutagenesis with degenerate codons.

**[0420]** The next step in library design was the alignment of human, mouse, rat, and monkey PD-L1 sequences to identify which of the residues chosen for mutagenesis are conserved residues. Based on this analysis, conserved target residues were mutated with degenerate codons that only specified conservative amino acid changes plus the wild-type residue. Residues that were not conserved, were mutated more aggressively, but also including the wild-type residue. Degenerate codons that also encoded the wild-type residue were deployed to avoid excessive mutagenesis of target protein. For the same reason, only up to 20 positions were targeted for mutagenesis for each library. Mutational analysis was focused on contact and non-contact interfacial residues that were within 6 Å of the binding surface with their side chains directed toward the ligand/counter structure. To generate DNA encoding the targeted library, overlapping oligos of up to 80 nucleotides in length and containing degenerate codons at the residue positions targeted for mutagenesis, were ordered from Integrated DNA Technologies (Coralville, USA). The oligonucleotides

were dissolved in sterile water, mixed in equimolar ratios, heated to 95°C for five minutes and slowly cooled to room temperature for annealing. IgV domain-specific oligonucleotide primers that anneal to the start and end of the IgV domain gene sequence were then used to generate a first PCR product. IgV domain-specific oligonucleotides which overlap by 40bp with pBYDS03 cloning vector (Life Technologies USA), beyond and including the BamHI and KpnI cloning sites, were then used to amplify 100ng of PCR product from the prior step to generate a total of at least 12 µg of DNA for every elextroproation. Both PCR's were by polymerase chain reaction (PCR) using OneTaq 2x PCR master mix (New England Biolabs, USA). The second PCR products were purified using a PCR purification kit (Qiagen, Germany) and resuspended in sterile deionized water. Alternatively, Ultramers (Integrated DNA Technologies) of up to 200 bp in length were used in conjunction with megaprimer PCR (URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC146891/pdf/253371.pdf) to generate larger stretches of degenerate codons that could not be as easily generated using multiple small overlapping primers. Following the generation of full length product using megaprimer PCR, the mutant IgV domain library was PCR amplified again using DNA primers containing 40 bp overlap region with the modified pBYDS03 cloning variant for homologous recombination into yeast.

**[0421]** To prepare for library insertion, pBYDS03 vector was digested with BamHI and KpnI restriction enzymes (New England Biolabs, USA) and the large vector fragment was gel-purified and dissolved in sterile, deionized water. Electroporation-ready DNA for the next step was generated by mixing 12 µg of library DNA insert with 4 µg of linearized vector in a total volume of 50 µl deionized and sterile water. An alternative method to generate targeted libraries, is to carry out site-directed mutagenesis (Multisite kit, Agilent, USA) of the target IgV domain with oligonucleotides containing degenerate codons. This approach was used to generate sublibraries that only target a few specific stretches of DNA for mutagenesis. In these cases, sublibraries were mixed before proceeding to the selection steps. In general, library sizes are in the range of 10E7 ($10^7$) to 10E8 ($10^8$) clones, except that sublibraries are only in the range of 10E4 ($10^4$) to 10E5 ($10^5$).

**B. Random Libraries**

**[0422]** Random libraries were also constructed to identify variants of the IgV domain of PD-L1 set forth in SEQ ID NO:309 (containing the PD-L1 IgV domain corresponding to residues 19-132 as set forth in UniProt Accession No. Q9NZQ7 flanked by adjacent N- and C-terminal residues of the wildtype sequence). DNA encoding the wild-type IgV domain was cloned between the BamHI and KpnI sites of the yeast display vector pBYDS03. The DNA was then mutagenized with the Genemorph II Kit (Agilent, USA) so as to generate an average of three to five amino acid changes per library variant. Mutagenized DNA was then amplified by the two-step PCR and further processed as described above for targeted libraries.

**EXAMPLE 2**

**Introduction of DNA Libraries into Yeast**

**[0423]** Example 2 describes the introduction of PD-L1 DNA libraries into yeast.

**[0424]** To introduce degenerate and random library DNA into yeast, electroporation-competent cells of yeast strain BJ5464 (ATCC.org; ATCC number 208288) were prepared and electroporated on a Gene

Pulser II (Biorad, USA) with the electroporation-ready DNA from the steps above essentially as described (Colby, D.W. et al. 2004 Methods Enzymology 388, 348-358). The only exception is that transformed cells were grown in non-inducing minimal selective SCD-Leu medium to accommodate the LEU2 selective marker carried by modified plasmid pBYDS03. One liter of SCD-Leu media consists of 14.7 grams sodium citrate, 4.29 grams citric acid monohydrate, 20 grams dextrose, 6.7 grams yeast nitrogen base, and 1.6 grams yeast synthetic drop-out media supplement without leucine. The medium was filter sterilized before use using a 0.22 µm vacuum filter device.

**[0425]** Library size was determined by plating serial dilutions of freshly recovered cells on SCD-Leu agar plates and then extrapolating library size from the number of single colonies from plating that generated at least 50 colonies per plate. The remainder of the electroporated culture was grown to saturation and cells from this culture were subcultured 1/100 into the same medium once more and grown to saturation to minimize the fraction of untransformed cells and to allow for segregation of plasmid from cells that may contain two or more library variants. To maintain library diversity, this subculturing step was carried out using an inoculum that contained at least 10x more cells than the calculated library size. Cells from the second saturated culture were resuspended in fresh medium containing sterile 25% (weight/volume) glycerol to a density of 10E10/mL and frozen and stored at -80°C (frozen library stock).

**EXAMPLE 3**

**Yeast Selection**

**[0426]** Example 3 describes the selection of yeast cells expressing affinity-modified variants of PD-L1.

**[0427]** A number of cells equal to at least 10 times the estimated library size were thawed from individual library stocks, suspended to 0.1 × 10E6 cells/mL in non-inducing SCD-Leu medium, and grown overnight. The next day, a number of cells equal to 10 times the library size were centrifuged at 2000 RPM for two minutes and resuspended to 0.5 × 10E6 cells/mL in inducing SCDG-Leu media. One liter of SCDG-Leu induction media consists of 5.4 grams $Na_2HPO_4$, 8.56 grams $NaH_2PO_4 \cdot H_20$, 20 grams galactose, 2.0 grams dextrose, 6.7 grams yeast nitrogen base, and 1.6 grams yeast synthetic drop out media supplement without leucine dissolved in water and sterilized through a 0.22 µm membrane filter device. The culture was grown in induction medium for 1 day at room temperature to induce expression of library proteins on the yeast cell surface.

**[0428]** Cells were sorted two to three times using Protein A magnetic beads (New England Biolabs, USA) loaded with cognate ligand to reduce non-binders and enrich for all variant PD-L1 variants with the ability to bind their exogenous recombinant counter-structure proteins. This was then followed by one to two rounds of fluorescence activated cell sorting (FACS) using exogenous counter-structure protein staining to enrich the fraction of yeast cells that displays improved binders. Magnetic bead enrichment and selections by flow cytometry were carried out essentially as described in Miller K.D. et al., Current Protocols in Cytometry 4.7.1-4.7.30, July 2008.

**[0429]** With PD-L1 libraries, target ligand proteins were human rPD-1.Fc (i.e., recombinant PD-1-Fc fusion protein from R&D Systems, USA). Magnetic Protein A beads were obtained from New England Biolabs, USA. For two-color, flow cytometric sorting, a Bio-Rad S3e sorter was used. PD-L1 display levels were monitored with an anti-hemagglutinin antibody labeled with Alexafluor 488 (Life Technologies, USA). Ligand binding of Fc fusion protein to rPD-1.Fc, was detected with PE conjugated human Ig specific goat Fab

(Jackson ImmunoResearch, USA). Doublet yeast were gated out using forward scatter (FSC) / side scatter (SSC) parameters, and sort gates were based upon higher ligand binding detected in FL2 that possessed more limited tag expression binding in FL1. Alternatively, selections were performed with human rCD80.Fc (i.e., human recombinant CD80 Fc fusion protein from R&D Systems, USA). Selections were carried out largely as described for PD-1 above.

**[0430]** Yeast outputs from the flow cytometric sorts were assayed for higher specific binding affinity. Sort output yeast were expanded and re-induced to express the particular IgSF affinity modified domain variants they encode. This population then can be compared to the parental, wild-type yeast strain, or any other selected outputs, such as the bead output yeast population, by flow cytometry.

**[0431]** For PD-L1, the second round FACS outputs (F2) were compared to parental for binding rPD-1.Fc or rCD80.Fc by double staining each population with anti-HA (hemagglutinin) tag expression and the anti-human Fc secondary to detect ligand binding.

**[0432]** Selected variant PD-L1 IgV domains were further formatted as fusion proteins and tested for binding and functional activity as described below.

**EXAMPLE 4**

**Reformatting Selection Outputs as Fc-Fusions and in Various Immunomodulatory Protein Types**

**[0433]** Example 4 describes reformatting of selection outputs identified in Example 3 as immunomodulatory proteins containing an affinity modified (variant) immunoglobulin-like V-type (IgV) domain of PD-L1 fused to an Fc molecule (variant IgV domain -Fc fusion molecules).

**[0434]** Output cell pools from final flow cytometric PD-L1 sorts were grown to terminal density in SCD-Leu medium. Plasmid DNA from each output was isolated using a yeast plasmid DNA isolation kit (Zymoresearch, USA). For Fc fusions, PCR primers with added restriction sites suitable for cloning into the Fc fusion vector of choice were used to batch-amplify from the plasmid DNA preps the coding DNA for the mutant target IgV domains. After restriction digestion, the PCR products were ligated into Fc fusion vector followed by heat shock transformation into *E. coli* strain XL1 Blue (Agilent, USA) or NEB5alpha (New England Biolabs, USA) as directed by supplier. Alternatively, the outputs were PCR amplified with primers containing 40 bp overlap regions on either end with Fc fusion vector to carry out in vitro recombination using Gibson Assembly Mastermix (New England Biolabs), which was subsequently used in heat shock transformation into E. Coli strain NEB5alpha. Exemplary of an Fc fusion vector is pFUSE-hIgG1-Fc2 (Invivogen, USA).

**[0435]** Dilutions of transformation reactions were plated on LB-agar containing 100 µg/mL carbenicillin (Teknova, USA) to isolate single colonies for selection. Up to 96 colonies from each transformation were then grown in 96 well plates to saturation overnight at 37°C in LB-broth containing 100 µg/mL carbenicillin (Teknova cat # L8112) and a small aliquot from each well was submitted for DNA sequencing of the IgV domain insert in order to identify mutation(s) in all clones. Sample preparation for DNA sequencing was carried out using protocols provided by the service provider (Genewiz; South Plainfield, NJ). After removal of sample for DNA sequencing, glycerol was then added to the remaining cultures for a final glycerol content of 25% and plates were stored at -20°C for future use as master plates (see below). Alternatively, samples for DNA sequencing were generated by replica plating from grown liquid cultures to solid agar

plates using a disposable 96 well replicator (VWR, USA). These plates were incubated overnight to generate growth patches and the plates were submitted to Genewiz as specified by Genewiz.

**[0436]** After identification of clones of interest from analysis of Genewiz-generated DNA sequencing data, clones of interest were recovered from master plates and individually grown to density in liquid LB-broth containing 100 µg/mL carbenicillin (Teknova, USA) and cultures were then used for preparation of plasmid DNA of each clone using a standard kit such as the PureYield Plasmid Miniprep System (Promega, USA) or the MidiPlus kit (Qiagen). Identification of clones of interest from Genewiz sequencing data generally involved the following steps. First, DNA sequence data files were downloaded from the Genewiz website. All sequences were then manually curated so that they start at the beginning of the IgV domain coding region. The curated sequences were then batch-translated using a suitable program available at the URL: www.ebi.ac.uk/Tools/st/emboss_transeq/. The translated sequences were then aligned using a suitable program available at the URL:
multalin.toulouse.inra.fr/multalin/multalin.html. Alternatively, Genewiz sequences were processed to generate alignments using Ugene software (http://ugene.net).

**[0437]** Clones of interest were then identified from alignments using the following criteria: 1.) identical clone occurs at least two times in the alignment and 2.) a mutation occurs at least two times in the alignment and preferably in distinct clones. Clones that meet at least one of these criteria were assumed to be clones that have been enriched by the sorting process due to improved binding.

**[0438]** To generate recombinant immunomodulatory proteins that are Fc fusion proteins containing an affinity-modified variant of the PD-L1 IgV domain (e.g. variant PD-L1 IgV-Fc), the encoding DNA was generated to encode a protein as follows: variant (mutant) IgV domain followed by a linker of three alanines (AAA) followed by a human IgG1 Fc set forth in SEQ ID NO: 1157 containing the mutations R292C, N297G and V302C by EU numbering (corresponding to R77C, N82G and V87C with reference to wild-type human IgG1 Fc set forth in SEQ ID NO: 187). Since the construct does not include any antibody light chains that can form a covalent bond with a cysteine, the human IgG1 Fc also contains replacement of the cysteine residues to a serine residue at position 220 (C220S) by EU numbering (corresponding to position 5 (C5S) with reference to the wild-type or unmodified Fc set forth in SEQ ID NO: 187.

**EXAMPLE 5**

**Expression and Purification of Fc-Fusions**

**[0439]** Example 5 describes the high throughput expression and purification of Fc-fusion proteins containing variant IgV PD-L1 as described in the above Examples.

**[0440]** Recombinant variant Fc fusion proteins were produced from suspension-adapted human embryonic kidney (HEK) 293 cells using the with Expi293 expression system (Invitrogen, USA). 4µg of each plasmid DNA from the previous step was added to 200µL Opti-MEM (Invitrogen, USA) at the same time as 10.8µL ExpiFectamine was separately added to another 200µL Opti-MEM. After 5 minutes, the 200µL of plasmid DNA was mixed with the 200µL of ExpiFectamine and was further incubated for an additional 20 minutes before adding this mixture to cells. Ten million Expi293 cells were dispensed into separate wells of a sterile 10ml, conical bottom, deep 24 well growth plate (Thomson Instrument Company, USA) in a volume 4mL Expi293 media (Invitrogen, USA). Plates were shaken for 5 days at 120 RPM in a mammalian cell culture incubator set to 95% humidity and 8% $CO_2$. Following a 5 day incubation, cells were pelleted and culture

supernatants were retained.

**[0441]** Proteins were purified from supernatants using a high throughput 96 well Filter Plate (Thomson Catalog number931919), each well loaded with 60µL of Mab SelectSure settled bead(GE Healthcare cat. no.17543801). Protein was eluted with four consecutive 200µL fractions, of 50mM Acetate pH3.3. Each fraction's pH was adjusted to above pH 5.0 with 4µL 2M Tris pH 8.0. Fractions were pooled and quantitated using 280nm absorbance measured by Nanodrop instrument (Thermo Fisher Scientific, USA), and protein purity was assessed by loading 5 µg of protein on NUPAGE pre-cast, polyacrylamide gels (Life Technologies, USA) under denaturing and non-reducing conditions and subsequent gel electrophoresis. Proteins were visualized in gel using standard Coomassie staining.

**EXAMPLE 6**

**Assessment of Binding of Affinity-Matured IgSF Domain-Containing Molecules**

**A. Binding to Cell-Expressed Counter Structure**

**[0442]** This Example describes Fc-fusion binding studies of purified proteins from the above Examples to assess specificity and affinity of PD-L1 domain variant immunomodulatory proteins for a cognate binding partner.

**[0443]** Binding studies were carried out using JurkabII,-2 reporter cells (purchased from Promega Corp. USA) that were then transduced to stably express human PD-1 (Jurkat/PD-1 cells). For staining by flow cytometry, 100,000 Jurkat/PD-1 cells or negative control (Jurkat only) were plated in 96 well round bottom plates. Cells were spun down and resuspended in staining buffer (PBS (phosphate buffered saline), 1% BSA (bovine serum albumin), and 0.1% sodium azide) for 20 minutes to block non-specific binding. Afterwards, cells were centrifuged again and resuspended in 50µL staining buffer containing 100 nM to 46pM of each candidate PD-L1 variant Fc fusion protein. As controls, a full extracellular domain of wild-type PD-L1 (composed of one IgV and one IgC domain) fused to Fc ("Full length ECD of PD-L1") and a IgV domain of wild-type PD-L1 ("wild type PD-L1 IgV") were tested. Primary staining was performed on ice for 45 minutes, before washing cells twice in 150µL staining buffer. PE-conjugated anti-human Fc (Jackson ImmunoResearch, USA) was diluted 1: 150 in 50 µL staining buffer and added to cells and incubated another 30 minutes on ice. Secondary antibody was washed out twice, cells were fixed in 4% formaldehyde/PBS, and samples were analyzed on Intellicyt flow cytometer (Intellicyt Corp,, USA).

**[0444]** Mean Fluorescence Intensity (MFI) was calculated and compared to wildtype PD-L1 IgV as control with FlowJo Version 10 (FlowJo Version 10, USA). Results for the binding studies for exemplary tested variant PD-L1 IgV-Fc fusion molecules are shown in Table 10. Table 10 also indicates amino acid substitutions (replacements or insertions designated by "ins") in the IgV of the variant PD-L1 selected in the screen described above. In the Table, the exemplary amino acid substitutions and insertions in the IgV domain are designated by amino acid position number corresponding to amino acid positions in the respective reference unmodified mature PD-L1 extracellular domain (ECD) sequence set forth in SEQ ID NO:30 or 1728. The amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution (or inserted designated by "ins") listed after the number. Column 2 sets forth the SEQ ID NO identifier for each variant IgV domain contained in the variant IgV-Fc fusion molecule.

**[0445]** Also shown is the binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of 50 nM of each variant Fc-fusion molecule to Jurkat/PD-1 cells and the ratio of the MFI compared to the binding of the corresponding unmodified (wildtype) IgV-Fc fusion molecule not containing the amino acid substitution(s) to PD-1. As shown in Table 10, the selections resulted in the identification of a number of PD-L1 IgSF (e.g. IgV) domain variants that were affinity-modified to exhibit increased binding for PD-1.

**TABLE 10: Selected PD-L1 variants and binding data.**

| PD-L1 Mutation(s) | SEQ ID NO (IgV) | Binding to Jurkat/PD-1 Cells | |
|---|---|---|---|
| | | MFI at 50nM | Fold increase over wildtype PD-L1 IgV-Fc |
| K28N, M41V, N45T, H51N, K57E | 244 | 12585 | 2.4 |
| I20L, I36T, N45D, I47T | 245 | 3119 | 0.6 |
| I20L, M41K, K44E | 246 | 9206 | 1.8 |
| P6S, N45T, N78I, I83T | 247 | 419 | 0.1 |
| N78I | 248 | 2249 | 0.4 |
| M41K, N78I | 249 | Little or no protein produced | |
| N17D, N45T, V50A, D72G | 255 | Little or no protein produced | |
| N45T, V50A | 257 | 23887 | 4.6 |
| I20L, N45T, N78I | 258 | 29104 | 5.6 |
| N45T, N78I | 250 | 24865 | 4.7 |
| I20L, N45T | 251 | 24279 | 4.6 |
| I20L, N45T, V50A | 259 | 34158 | 6.5 |
| N45T | 252 | 6687 | 1.3 |
| M41K | 253 | 5079 | 1.0 |
| M41V, N45T | 260 | Little or no protein produced | |
| M41K, N45T | 261 | Little or no protein produced | |
| A33D, S75P, D85E | 262 | 685 | 0.1 |
| M18I, M41K, D43G, H51R, N78I | 263 | 20731 | 4.0 |
| V11E, I20L, I36T, N45D, H60R, S75P | 264 | 3313 | 0.6 |
| A33D, V50A | | Little or no protein produced | |
| S16G, A33D, K71E, S75P | 266 | Little or no protein produced | |
| E27G, N45T, M97I | 267 | 881 | 0.2 |
| E27G, N45T, K57R | 268 | 5022 | 1.0 |
| A33D, E53V | 269 | 650 | 0.1 |
| D43G, N45D, V58A | 270 | 63960 | 12.2 |
| E40G, D43V, N45T, V50A | 271 | 809 | 0.2 |
| Y14S, K28E, N45T | 272 | 16232 | 3.1 |
| A33D, N78S | 273 | 1725 | 0.3 |
| A33D, N78I | 274 | 8482 | 1.6 |
| A33D, N45T | 275 | 17220 | 3.3 |
| E27G, N45T, V50A | 277 | 25267 | 4.8 |
| N45T, V50A, N78S | 278 | 28572 | 5.4 |

| TABLE 10: Selected PD-L1 variants and binding data. | | | |
|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (IgV) | Binding to Jurkat/PD-1 Cells | |
| | | MFI at 50nM | Fold increase over wildtype PD-L1 IgV-Fc |
| N45T, V50A | 257 | 18717 | 3.6 |
| I20L, N45T, V110M | 279 | 464 | 0.1 |
| I20L, I36T, N45T, V50A | 280 | 7658 | 1.5 |
| N45T, L74P, S75P | 281 | 5251 | 1.0 |
| N45T, S75P | 282 | 12200 | 2.3 |
| S75P, K106R | 283 | 388 | 0.1 |
| S75P | 284 | 1230 | 0.2 |
| A33D, S75P | 285 | 306 | 0.1 |
| A33D, S75P, D104G | 286 | 251 | 0.0 |
| A33D, S75P | 287 | 1786 | 0.3 |
| I20L, E27G, N45T, V50A | 288 | 29843 | 5.7 |
| I20L, E27G, D43G, N45D, V58A, N78I | 289 | 69486 | 13.3 |
| I20L, D43G, N45D, V58A, N78I | 290 | 72738 | 13.9 |
| I20L, A33D, D43G, N45D, V58A, N78I | 291 | 80205 | 15.3 |
| I20L, D43G, N45D, N78I | 292 | 67018 | 12.8 |
| E27G, N45T, V50A, N78I | 293 | 30677 | 5.9 |
| N45T, V50A, N78I | 294 | 32165 | 6.1 |
| V11A, I20L, E27G, D43G, N45D, H51Y, S99G | 295 | 73727 | 14.1 |
| I20L, E27G, D43G, N45T, V50A | 296 | 36739 | 7.0 |
| 120L, K28E, D43G, N45D, V58A, Q89R, G101G-ins (G101GG) | 1727 | 80549 | 15.4 |
| I20L, I36T, N45D | 298 | 16870 | 3.2 |
| I20L, K28E, D43G, N45D, E53G, V58A, N78I | 299 | 139 | 0.0 |
| A33D, D43G, N45D, V58A, S75P | 300 | 58484 | 11.2 |
| K23R, D43G, N45D | 301 | 67559 | 12.9 |
| I20L, D43G, N45D, V58A, N78I, D90G, G101D | 302 | 259 | 0.0 |
| D43G, N45D, L56Q, V58A, G101G-ins (G101GG) | 303 | 88277 | 16.8 |
| I20L, K23E, D43G, N45D, V58A, N78I | 304 | 89608 | 17.1 |
| I20L, K23E, D43G, N45D, V50A, N78I | 305 | 88829 | 16.9 |
| T19I, E27G, N45I, V50A, N78I, M97K | 306 | 25496 | 4.9 |
| I20L, M41K, D43G, N45D | 307 | 599 | 0.1 |
| K23R, N45T, N78I | 308 | 84980 | 16.2 |
| Full length ECD PD-L1 | - | 18465 | 3.5 |
| Wild type PD-L1 IgV | 309 | 5243 | 1.0 |

| TABLE 10: Selected PD-L1 variants and binding data. | | | |
|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (IgV) | Binding to Jurkat/PD-1 Cells | |
| | | MFI at 50nM | Fold increase over wildtype PD-L1 IgV-Fc |
| Anti-PD-1 monoclonal antibody (nivolumab) | - | 79787 | 15.2 |
| Human IgG | - | 198 | 0.0 |

**B. ForteBio Octet Binding Assay**

**[0446]** Protein-protein interactions between PD-L1 domain variant immunomodulatory proteins and the binding partners PD-1 and CD80 were assessed using Fortebio binding assays. PD-1 or CD80 were loaded individually onto anti-human capture sensors (ForteBio Octet AHC) and Fc fusions of full length wildtype (unmodified) ECD of PD-L1, wildtype (unmodified) PD-L1 IgV-Fc fusion molecule, or variant PD-L1 IgV-Fc fusion molecules were bound to the receptors at a single concentration of 100nM.

**[0447]** Loading response of anti-human capture sensors of each binding protein being tested with the variant IgV-Fc fusion molecule was determined and compared to the response of wildtype PD-L1 IgV-Fc. A ratio between the response of each variant PD-L1 IgV-Fc fusion molecule to PD-1 and CD80 was also determined as set forth in Table 11A-B. In each of the Tables below, Column 2 sets forth the SEQ ID NO identifier for each variant IgV domain contained in the tested variant IgV-Fc fusion molecule.

**[0448]** As shown in Table 11A-B, the selections resulted in the identification of a number of PD-L1 IgSF (e.g. IgV) domain variants that were affinity-modified to exhibit altered binding for PD-1 and/or CD80.

| TABLE 11A: ForteBio Binding Data. | | | | | | |
|---|---|---|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (IgV) | PD-1: Fc Response | Fold over WT PD-L1 IgV-Fc | CD80:Fc Response | Fold over WT PD-L1 IgV-Fc | PD-1: CD80 ratio |
| K28N, M41V, N45T, H51N, K57E | 244 | 1.16 | 0.8 | 0.49 | 0.6 | 2.4 |
| I20L, I36T, N45D, I47T | 245 | 0.73 | 0.5 | 0.52 | 0.7 | 1.4 |
| I20L, I36T, N45D | 254 | 1.25 | 0.9 | 0.61 | 0.8 | 2.0 |
| I20L, M41K, K44E | 246 | 2.26 | 1.6 | 1.67 | 2.1 | 1.4 |
| P6S, N45T, N78I, I83T | 247 | 0.27 | 0.2 | 0.23 | 0.3 | 1.2 |
| 120L, F49S | 256 | 0.79 | 0.6 | 0.58 | 0.7 | 1.4 |
| N78L | 248 | 2.23 | 1.6 | 1.55 | 2.0 | 1.4 |
| N45T, V50A | 257 | 1.90 | 1.4 | 0.79 | 1.0 | 2.4 |
| I20L, N45T, N78I | 258 | 2.34 | 1.7 | 1.85 | 2.3 | 1.3 |
| N45T, N78I | 250 | 2.38 | 1.7 | 1.73 | 2.2 | 1.4 |

| TABLE 11A: ForteBio Binding Data. | | | | | | |
|---|---|---|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (IgV) | PD-1: Fc Response | Fold over WT PD-L1 IgV-Fc | CD80:Fc Response | Fold over WT PD-L1 IgV-Fc | PD-1: CD80 ratio |
| I20L, N45T | 251 | 2.30 | 1.7 | 1.58 | 2.0 | 1.5 |
| I20L, N45T, V50A | 259 | 2.32 | 1.7 | 1.22 | 1.5 | 1.9 |
| N45T | 252 | 1.34 | 1.0 | 0.65 | 0.8 | 2.0 |
| M41k | 253 | 2.25 | 1.6 | 1.70 | 2.2 | 1.3 |
| A33D, S75P, D85E | 262 | 0.84 | 0.6 | 0.55 | 0.7 | 1.5 |
| M18I, M41K, D43G, H51R, N78I | 263 | 2.33 | 1.7 | 2.14 | 2.7 | 1.1 |
| V11E, I20L, I36T, N45D, H60R, S75P | 264 | 0.93 | 0.7 | 0.56 | 0.7 | 1.7 |
| E27G, N45T, M97I | 267 | 2.10 | 1.5 | 1.05 | 1.3 | 2.0 |
| E27G, N45T, K57R | 268 | 2.31 | 1.7 | 1.57 | 2.0 | 1.5 |
| A33D, E53V | 269 | 1.09 | 0.8 | 0.62 | 0.8 | 1.7 |
| D43G, N45D, V58A | 270 | 1.55 | 1.1 | 0.71 | 0.9 | 2.2 |
| Y14S, K28E, N45T | 272 | 2.28 | 1.7 | 1.18 | 1.5 | 1.9 |
| A33D, N78S | 273 | 1.61 | 1.2 | 0.79 | 1.0 | 2.0 |
| A33D, N78I | 274 | 2.35 | 1.7 | 1.57 | 2.0 | 1.5 |
| A33D, N45T | 275 | 1.90 | 1.4 | 0.80 | 1.0 | 2.4 |
| A33D, N45T, N78I | 276 | 2.34 | 1.7 | 1.59 | 2.0 | 1.5 |
| E27G, N45T, V50A | 277 | 2.26 | 1.6 | 1.22 | 1.5 | 1.9 |
| N45T, V50A, N78S | 278 | 2.23 | 1.6 | 0.88 | 1.1 | 2.5 |
| N45T, V50A | 257 | 1.79 | 1.3 | 0.63 | 0.8 | 2.8 |
| I20L, N45T, V110M | 279 | 0.38 | 0.3 | 0.27 | 0.3 | 1.4 |
| I20L, I36T, N45T, V50A | 280 | 2.27 | 1.7 | 0.96 | 1.2 | 2.4 |
| N45T, L74P, S75P | 281 | 0.90 | 0.7 | 0.44 | 0.6 | 2.0 |
| N45T, S75P | 282 | 2.31 | 1.7 | 1.24 | 1.6 | 1.9 |
| S75P, K106R | 283 | 0.84 | 0.6 | 0.45 | 0.6 | 1.9 |
| S75P | 284 | 2.09 | 1.5 | 1.09 | 1.4 | 1.9 |
| A33D, S75P | 285 | 0.72 | 0.5 | 0.43 | 0.5 | 1.7 |

**TABLE 11A: ForteBio Binding Data.**

| PD-L1 Mutation(s) | SEQ ID NO (IgV) | PD-1: Fc Response | Fold over WT PD-L1 IgV-Fc | CD80:Fc Response | Fold over WT PD-L1 IgV-Fc | PD-1: CD80 ratio |
|---|---|---|---|---|---|---|
| A33D, S75P, D104G | 286 | 0.71 | 0.5 | 0.66 | 0.8 | 1.1 |
| A33D, S75P | 287 | 2.07 | 1.5 | 0.93 | 1.2 | 2.2 |
| Full length ECD PD-L1 | - | 2.53 | 1.8 | 1.46 | 1.8 | 1.7 |
| Wild type PD-L1 IgV | 309 | 1.37 | 1.0 | 0.79 | 1.0 | 1.7 |

**TABLE 11B. ForteBio Binding Data.**

| PD-L1 Mutation(s) | SEQ ID NO (IgV) | PD-1: Fc Response | Fold over WT PD-L1 IgV | CD80:Fc Response | Fold over WT PD-L1 IgV | PD-1: CD80 ratio |
|---|---|---|---|---|---|---|
| I20L, E27G, N45T, V50A | 288 | 1.08 | 2.0 | 0.39 | 3.2 | 2.7 |
| I20L, E27G, D43G, N45D, V58A, N78I | 289 | 1.20 | 2.2 | 0.62 | 5.1 | 1.9 |
| I20L, D43G, N45D, V58A, N78I | 290 | 1.27 | 2.4 | 0.54 | 4.5 | 2.3 |
| I20L, A33D, D43G, N45D, V58A, N78I | 291 | 1.22 | 2.3 | 0.47 | 3.9 | 2.6 |
| I20L, D43G, N45D, N78I | 292 | 1.25 | 2.3 | 0.49 | 4.0 | 2.6 |
| E27G, N45T, V50A, N78I | 293 | 1.12 | 2.1 | 0.48 | 3.9 | 2.4 |
| N45T, V50A, N78I | 294 | 1.24 | 2.3 | 0.31 | 2.5 | 4.0 |
| V11A, I20L, E27G, D43G, N45D, H51Y, S99G | 295 | 1.20 | 2.2 | 0.28 | 2.3 | 4.3 |
| I20L, E27G, D43G, N45T, V50A | 296 | 1.10 | 2.1 | 0.55 | 4.5 | 2.0 |
| I20L, K28E, D43G, N45D, V58A, Q89R, G101G-ins (G101GG) | 1727 | 1.20 | 2.2 | 0.06 | 0.5 | 18.9 |
| I20L, I36T, N45D | 298 | 0.72 | 1.3 | 0.10 | 0.8 | 7.2 |
| A33D, D43G, N45D, V58A, S75P | 300 | 0.67 | 1.2 | 0.09 | 0.7 | 7.5 |
| K23R, D43G, N45D | 301 | 1.08 | 2.0 | 0.27 | 2.2 | 4.1 |
| I20L, D43G, N45D, V58A, N78I, D90G, G101D | 302 | -0.02 | 0.0 | -0.04 | -0.3 | 0.5 |
| D43G, N45D, L56Q, V58A, G101G-ins (G101GG) | 303 | 1.24 | 2.3 | 0.07 | 0.6 | 18.4 |

| TABLE 11B. ForteBio Binding Data. | | | | | | |
|---|---|---|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (IgV) | PD-1: Fc Response | Fold over WT PD-L1 IgV | CD80:Fc Response | Fold over WT PD-L1 IgV | PD-1: CD80 ratio |
| I20L, K23E, D43G, N45D, V58A, N78I | 304 | 1.20 | 2.2 | 0.37 | 3.0 | 3.3 |
| I20L, K23E, D43G, N45D, V50A, N78I | 305 | 1.14 | 2.1 | 0.21 | 1.7 | 5.4 |
| T19I, E27G, N45I, V50A, N78I, M97K | 306 | 1.12 | 2.1 | 0.72 | 5.9 | 1.6 |
| I20L, M41K, D43G, N45D | 307 | 0.01 | 0.0 | 0.02 | 0.2 | 0.6 |
| K23R, N45T, N78I | 308 | 1.19 | 2.2 | 0.53 | 4.3 | 2.3 |
| Full length ECD PD-L1 | - | 1.44 | - | 0.80 | - | - |
| Wild type PD-L1 IgV | 309 | 0.54 | 1.0 | 0.12 | 1.0 | 4.4 |

**EXAMPLE 7**

**Assessment of Bioactivity of Affinity-Matured IgSF Domain-Containing Molecules Using Jurkat/IL2/PD-1 Reporter Assay**

**[0449]** This Example describes a Jurkat/Il,2/PD-1 reporter assay to assess bioactivity of PD-L1 domain variant immunomodulatory proteins for blockade of PD-1.

**[0450]** Jurkat effector cells expressing an IL-2-luciferase reporter and PD-1 on its surface were suspended in Jurkat Assay buffer (RPMI1640 + 5%FBS) at $2\times10^6$ cells/mL and 2 µg/mL anti-CD28 was added. Jurkat cells were then plated at 50 µL/well for a total of 100,000 cells per well.

**[0451]** Variant PD-L1 IgV-Fc fusion molecules and control proteins (full length PD-L1-Fc, wild type PD-L1 IgV-Fc) or anti-PD-1 monoclonal antibody (nivolumab) were diluted to 200nM and 25 µL of each protein was added to the plated Jurkat cells. The Jurkat cells with PD-L1 variant IgV-Fc fusion molecules or control proteins were incubated for 15 minutes at room temperature. K562 derived artificial antigen presenting cells (aAPC) cells displaying cell surface anti-CD3 single chain Fv (OKT3) and PD-L1were brought to $0.67\times10^6$ cells/mL and 25 µL was added to each well bringing the final volume of each well to 100 µL. Each well had a final ratio of 6:1 Jurkat:K562 cells, 1ug/mL anti-CD28 and test protein concentration of 50nM. Jurkat cells and K562 cells were incubated for 5-6 hours at 37 degrees Celsius in a humidified 5% $CO_2$ incubation chamber. Plates were then removed from the incubator and acclimated to room temperature for 15 minutes. 100 µL of a cell lysis and luciferase substrate solution (BioGlo®, Promega Corp, USA) was added to each well and the plates were placed on an orbital shaker for 10 minutes at room temperature. Luminescence was measured with a 1 second per well integration time using a BioTek Cytation luminometer (BioTek Corp., USA).

**[0452]** An average relative luminescence value was determined for each variant PD-L1 IgV Fc and a fold increase in IL-2 reporter signal was calculated for each variant compared to wildtype PD-L1 IgV-Fc variant

protein.

**[0453]** As shown in Table 12, luciferase activity of Jurkat effector cells expressing PD-1 and IL-2-luciferase reporter co-cultured with anti-CD3/PD-L1 aAPC and variant PD-L1 IgV-Fc molecules were altered (increased and decreased). The differences in luminescence values demonstrates variant PD-L1 IgV-Fc molecule binding to PD-1 and ability to block the interaction of PD-1 and PD-L1. Higher values are indicative of increased blockade of PD-1-mediated inhibition. In the Table, Column 2 sets forth the SEQ ID NO identifier for each variant IgV domain contained in the tested variant IgV-Fc fusion molecule.

**TABLE 12. Jurkat/IL2/PD-1 Reporter Assay**

| PD-L1 Mutation(s) | SEQ ID NO (IgV) | Average Relative Luminescence Units | Fold increase in IL2 reporter signal |
|---|---|---|---|
| K28N, M41V, N45T, H51N, K57E | 244 | 2348.00 | 1.05 |
| I20L, I36T, N45D, I47T | 245 | 2249.50 | 1.01 |
| I20L, I36T, N45D | 254 | 2109.50 | 0.95 |
| I20L, M41K, K44E | 246 | 2220.50 | 1.00 |
| P6S, N45T, N78I, I83T | 247 | 2246.00 | 1.01 |
| I20L, F49S | 256 | 2226.50 | 1.00 |
| N78L | 248 | 2243.50 | 1.01 |
| N45T, V50A | 257 | 2076.50 | 0.93 |
| I20L, N45T, N78I | 258 | 2066.00 | 0.93 |
| N45T, N78I | 250 | 2179.50 | 0.98 |
| I20L, N45T | 251 | 2176.50 | 0.98 |
| I20L, N45T, V50A | 259 | 2226.50 | 1.00 |
| N45T | 252 | 2193.00 | 0.98 |
| M41K | 253 | 2154.00 | 0.97 |
| A33D, S75P, D85E | 262 | 2217.50 | 0.99 |
| M18I, M41K, D43G, H51R, N78I | 263 | 2124.50 | 0.95 |
| V11E, I20L, I36T, N45D, H60R, S75P | 264 | 2283.00 | 1.02 |
| E27G, N45T, M97I | 267 | 1971.00 | 0.88 |
| E27G, N45T, K57R | 268 | 2034.00 | 0.91 |
| A33D, E53V | 269 | 2142.00 | 0.96 |
| D43G, D45D, V58A | 270 | 2464.00 | 1.11 |
| E40G, D43V, N45T, V50A | 271 | 2216.00 | 0.99 |
| Y14S, K28E, N45T | 272 | 2151.50 | 0.97 |
| A33D, N78S | 273 | 2056.50 | 0.92 |
| A33D, N78I | 274 | 2057.00 | 0.92 |
| A33D, N45T | 275 | 1995.00 | 0.90 |
| A33D, N45T, N78I | 276 | 1973.00 | 0.89 |
| E27G, N45T, V50A | 277 | 2004.00 | 0.90 |
| N45T, V50A, N78S | 278 | 2224.50 | 1.00 |

| TABLE 12. Jurkat/IL2/PD-1 Reporter Assay | | | |
|---|---|---|---|
| **PD-L1 Mutation(s)** | **SEQ ID NO (IgV)** | **Average Relative Luminescence Units** | **Fold increase in IL2 reporter signal** |
| N45T, V50A | 257 | 2201.50 | 0.99 |
| I20L, N45T, V110M | 279 | 2210.00 | 0.99 |
| I20L, I36T, N45T, V50A | 280 | 2157.50 | 0.97 |
| N45T, L74P, S75P | 281 | 2031.00 | 0.91 |
| N45T, S75P | 282 | 1963.50 | 0.88 |
| A33D, V50A | 265 | 2251.50 | 1.01 |
| S75P, K106R | 283 | 2127.50 | 0.95 |
| S75P | 284 | 2233.50 | 1.00 |
| A33D, S75P | 285 | 2168.50 | 0.97 |
| A33D, S75P, D104G | 286 | 2168.50 | 0.97 |
| A33D, S75P | 287 | 2044.50 | 0.92 |
| I20L, E27G, N45T, V50A | 288 | 1804.50 | 0.81 |
| I20L, E27G, D43G, N45D, V58A, N78I | 289 | 4588.50 | 2.06 |
| I20L, D43G, N45D, V58A, N78I | 290 | 4191.50 | 1.88 |
| I20L, A33D, D43G, N45D, V58A, N78I | 291 | 4384.50 | 1.97 |
| I20L, D43G, N45D, N78I | 292 | 3471.50 | 1.56 |
| E27G, N45T, V50A, N78I | 293 | 1960.00 | 0.88 |
| N45T, V50A, N78I | 294 | 1899.00 | 0.85 |
| V11A, I20L, E27G, D43G, N45D, H51Y, S99G | 295 | 3381.50 | 1.52 |
| I20L, E27G, D43G, N45T, V50A | 296 | 2013.50 | 0.90 |
| I20L, K28E, D43G, N45D, V58A, Q89R, G101G-ins (G101GG) | 1727 | 4294.00 | 1.93 |
| I20L, I36T, N45D, | 298 | 2210.50 | 0.99 |
| A33D, D43G, N45D, V58A, S75P | 300 | 2312.50 | 1.04 |
| K23R, D43G, N45D | 301 | 2361.00 | 1.06 |
| I20L, D43G, N45D, V58A, N78I, D90G, G101D | 302 | 1998.50 | 0.90 |
| D43G, N45D, L56Q, V58A, G101Gins (G101GG) | 303 | 3926.00 | 1.76 |
| I20L, K23E, D43G, N45D, V58A, N78I | 304 | 3506.00 | 1.57 |
| I20L, K23E, D43G, N45D, V50A, N78I | 305 | 3586.00 | 1.61 |
| T19I, E27G, N45I, V50A, | 306 | 2047.00 | 0.92 |

| TABLE 12. Jurkat/IL2/PD-1 Reporter Assay | | | |
|---|---|---|---|
| **PD-L1 Mutation(s)** | **SEQ ID NO (IgV)** | **Average Relative Luminescence Units** | **Fold increase in IL2 reporter signal** |
| N78I, M97K | | | |
| I20L, M41K, D43G, N45D | 307 | 2109.50 | 0.95 |
| K23R, N45T, N78I | 308 | 2690.50 | 1.21 |
| Full length PD-L1 Fc | - | 1945.00 | 0.87 |
| Wild type PD-L1 IgV | 309 | 2229.00 | 1.00 |
| Anti-PD-1 monoclonal antibody (nivolumab) | - | 8892.00 | 3.99 |

**EXAMPLE 8**

**Additional Affinity Modified IgSF Domains**

**[0454]** This examples describe the design, creation, and screening of additional affinity modified CD80 (B7-1), PD-L2, CD155 and CD112, and CD86 (B7-2) immunomodulatory proteins, which are other components of the immune synapse (IS) that have a demonstrated dual role in both immune activation and inhibition. Affinity-modified NKp30 variants also were generated and screened. These examples demonstrate that affinity modification of IgSF domains yields proteins that can act to both increase and decrease immunological activity. Various combinations of those domains can be fused in pairs (i.e., stacked) with a variant affinity modified PD-L1 to form a Type II immunomodulatory protein to achieve immunomodulatory activity.

**[0455]** Mutant DNA constructs encoding a variant of the IgV domain of human CD80, or IgV domains of PD-L2, CD155 and CD112 for translation and expression as yeast display libraries were generated substantially as described in Example 1. Target libraries that target specific residues for complete or partial randomization with degenerate codons and/or random libraries were constructed to identify variants of the IgV of CD80 (SEQ ID NO:2030), variants of the IgV of PD-L2 (SEQ ID NO: 1263), variants of the IgV of CD155 (SEQ ID NO:353), and variants of the IgV of CD112 (SEQ ID NO:761) substantially as described in Example 1. Similar methods also were used to generate libraries of the IgC-like domain of NKp30 (SEQ ID NO:1190).

**[0456]** The degenerate or random library DNA was introduced into yeast substantially as described in Example 2 to generate yeast libraries. The libraries were used to select yeast expressing affinity modified variants of CD80, PD-L2, CD155, CD112, CD86 (B7-2), and NKp30 substantially as described in Example 3. Cells were processed to reduce non-binders and to enrich for CD80, PD-L2, CD155 or CD112, CD86 (B7-2), and NKp30 variants with the ability to bind their exogenous recombinant counter-structure proteins substantially as described in Example 3.

**[0457]** With CD80, CD86 and NKp30 libraries, target ligand proteins were sourced from R&D Systems (USA) as follows: human rCD28.Fc (i.e., recombinant CD28-Fc fusion protein), rPDL1.Fc, rCTLA4.Fc, and rB7H6.Fc. Two-color flow cytometry was performed substantially as described in Example 3. Yeast outputs from the flow cytometric sorts were assayed for higher specific binding affinity. Sort output yeast were

expanded and re-induced to express the particular IgSF affinity modified domain variants they encode. This population then can be compared to the parental, wild-type yeast strain, or any other selected outputs, such as the bead output yeast population, by flow cytometry.

**[0458]** In the case of NKp30 yeast variants selected for binding to B7-H6, the F2 sort outputs gave MFI values of 533 when stained with 16.6nM rB7H6.Fc, whereas the parental NKp30 strain MFI was measured at 90 when stained with the same concentration of rB7H6.Fc (6-fold improvement).

**[0459]** Among the NKp30 variants that were identified, was a variant that contained mutations L30V/A60V/S64P/S86G with reference to positions in the NKp30 extracellular domain corresponding to positions set forth in SEQ ID NO: 54.

**[0460]** For CD80 variants provided in Table 13A-B, CD80 libraries consisted of positive selection with the desired counter structure CTLA4 and negative selection with the counter structure CD28.

**[0461]** For CD155 variants provided in Table 14A, CD155 libraries were selected against each of TIGIT, CD96, and CD226, separately. For CD155 variants provided in Table 14B-F, selection involved two positive selections with the desired counter structures TIGIT and CD96 followed by one negative selection with the counter structure CD226 to select away from CD226 and improve binding specificity of the variant CD155. Selection was performed essentially as described in Example 3 above except the concentrations of the counter structures (TIGIT/CD96) and selection stringency of the positive sorts were varied to optimize lead identification. The concentration of CD226 for the negative selection was kept at 100 nM.

**[0462]** For CD112 variants provided in Table 15A, CD112 libraries were selected against each of TIGIT, CD112R, and CD226, separately. For additional CD112 variants provided in Table 15B-15C, selection involved two positive selections with the desired counter structures TIGIT and CD112R followed by one negative selection with the counter structure CD226 to select away from CD226 and improve binding specificity of the variant CD112. Selection was performed essentially as described in Example 3 above except the concentrations of the counter structures (TIGIT/CD112R) and selection stringency of the positive sorts were varied to optimize lead identification. The concentration of CD226 for the negative selection was kept at 100 nM.

**[0463]** For PD-L2 variants provided in Table 16A-B, yeast display targeted or random PD-L2 libraries were selected against PD-1. This was then followed by two to three rounds of flow cytometry sorting using exogenous counter-structure protein staining to enrich the fraction of yeast cells that displays improved binders. Magnetic bead enrichment and selections by flow cytometry are essentially as described in Miller K.D. et al., Current Protocols in Cytometry 4.7.1-4.7.30, July 2008.

**[0464]** Exemplary selection outputs were reformatted as immunomodulatory proteins containing an affinity modified (variant) IgV of CD80, variant IgV of PD-L2, variant IgV of CD155, variant IgV of CD112, each fused to an Fc molecule (variant ECD-Fc fusion molecules or variant IgV-Fc fusion molecules) substantially as described in Example 4 and the Fc-fusion protein was expressed and purified substantially as described in Example 5.

**[0465]** Binding of exemplary IgSF domain variants to cell-expressed counter structures was then assessed substantially as described in Example 6. Cells expressing cognate binding partners were produced and binding studies and flow cytometry were carried out substantially as described in Example 6. In addition, the bioactivity of the Fc-fusion variant protein was characterized by either mixed lymphocyte reaction (MLR) or anti-CD3 coimmobilization assay substantially as described in Example 6.

**[0466]** As above, for each Table, the exemplary amino acid substitutions are designated by amino acid position number corresponding to the respective reference unmodified ECD sequence (Table 2). The amino acid position is indicated in the middle, with the corresponding unmodified (e.g. wild-type) amino acid listed before the number and the identified variant amino acid substitution listed (or inserted designated by a) after the number.

**[0467]** Also shown is the binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of each variant Fc-fusion molecule to cells engineered to express the cognate counter structure ligand and the ratio of the MFI compared to the binding of the corresponding unmodified Fc fusion molecule not containing the amino acid substitution(s) to the same cell-expressed counter structure ligand. The functional activity of the variant Fc-fusion molecules to modulate the activity of T cells also is shown based on the calculated levels of IFN-gamma in culture supernatants (pg/mL) generated either i) with the indicated variant Fc fusion molecule coimmoblized with anti-CD3 or ii) with the indicated variant Fc fusion molecule in an MLR assay. The Tables also depict the ratio of IFN-gamma produced by each variant ECD-Fc or IgV-Fc compared to the corresponding unmodified ECD-Fc or IgV-Fc in the functional assays.

**[0468]** As shown in Tables 13A-16F, the selections resulted in the identification of a number of PD-L2, CD155, CD112, and CD80 IgSF domain variants that were affinity-modified to exhibit increased binding for at least one, and in some cases more than one, cognate counter structure ligand. In addition, the results showed that affinity modification of the variant molecules also exhibited improved activities to both increase and decrease immunological activity depending on the format of the molecule.

**TABLE 13A: Variant CD80 Binding to HEK293 Cells Transfected with CTLA4, CD28 or PD-L1**

| CD80 mutation(s) | SEQ ID NO (IgV) | CTLA4 | | CD28 | | PD-L1 | | |
|---|---|---|---|---|---|---|---|---|
| | | MFI at 66.6 nM | Fold change to WT | MFI at 66.6 nM | Fold change to WT | MFI at 22.2 nM | Fold change to WT | Ratio of CTLA4: CD28 |
| L70P | 1080 | Not tested | | | | | | |
| I30F/L70P | 1081 | Not tested | | | | | | |
| Q27H/T41S/A71D | 1082 | 368176 | 2.3 | 25051 | 1.01 | 24181 | N/A | 14.7 |
| I30T/L70R | 1083 | 2234 | 0.0 | 2596 | 0.10 | 5163 | N/A | 0.9 |
| T13R/C16R/L70Q/A71D | 1084 | 197357 | 1.2 | 16082 | 0.65 | 9516 | N/A | 12.3 |
| T57I | 1085 | 393810 | 2.4 | 23569 | 0.95 | 3375 | N/A | 16.7 |
| M43I/C82R | 1086 | 3638 | 0.0 | 3078 | 0.12 | 7405 | N/A | 1.2 |
| V22L/M38V/M47T/A71D/ L85M | 1087 | 175235 | 1.1 | 3027 | 0.12 | 6144 | N/A | 57.9 |
| I30V/T57I/L70P/A71D/ A91T | 1088 | 116085 | 0.7 | 10129 | 0.41 | 5886 | N/A | 11.5 |
| V22I/L70M/A71D | 1089 | 163825 | 1.0 | 22843 | 0.92 | 33404 | N/A | 7.2 |
| N55D/L70P/E77G | 1090 | Not tested | | | | | | |
| T57A/I69T | 1091 | Not tested | | | | | | |
| N55D/K86M | 1092 | 3539 | 0.0 | 3119 | 0.13 | 5091 | N/A | 1.1 |
| L72P/T79I | 1093 | 50176 | 0.3 | 3397 | 0.14 | 6023 | N/A | 14.8 |
| L70P/F92S | 1094 | 4035 | 0.0 | 2948 | 0.12 | 6173 | N/A | 1.4 |
| T79P | 1095 | 2005 | 0.0 | 2665 | 0.11 | 4412 | N/A | 0.8 |

**TABLE 13A: Variant CD80 Binding to HEK293 Cells Transfected with CTLA4, CD28 or PD-L1**

| CD80 mutation(s) | SEQ ID NO (IgV) | CTLA4 | | CD28 | | PD-L1 | | |
|---|---|---|---|---|---|---|---|---|
| | | MFI at 66.6 nM | Fold change to WT | MFI at 66.6 nM | Fold change to WT | MFI at 22.2 nM | Fold change to WT | Ratio of CTLA4: CD28 |
| E35D/M47I/L65P/D90N | 1096 | 4411 | 0.0 | 2526 | 0.10 | 4034 | N/A | 1.7 |
| L25 S/E35D/M47I/D90N | 1097 | 61265 | 0.4 | 4845 | 0.20 | 20902 | N/A | 12.6 |
| Q27X*/S44P/I67T/P74S/E81G/E95D | 1098 | 195637 | 1.2 | 17524 | 0.71 | 17509 | N/A | 11.2 |
| A71D | 1099 | 220090 | 1.4 | 16785 | 0.68 | 29642 | N/A | 13.1 |
| T13A/Q27X*/I61N/A71D | 1100 | 195061 | 1.2 | 17519 | 0.71 | 21717 | N/A | 11.1 |
| E81K/A91S | 1101 | 98467 | 0.6 | 3309 | 0.13 | 44557 | N/A | 29.8 |
| A12V/M47V/L70M | 1102 | 81616 | 0.5 | 7400 | 0.30 | 31077 | N/A | 11.0 |
| K34E/T41A/L72V | 1103 | 88982 | 0.6 | 3755 | 0.15 | 35293 | N/A | 23.7 |
| T41S/A71D/V84A | 1104 | 103010 | 0.6 | 5573 | 0.22 | 83541 | N/A | 18.5 |
| E35D/A71D | 1105 | 106069 | 0.7 | 18206 | 0.73 | 40151 | N/A | 5.8 |
| E35D/M47I | 1106 | 353590 | 2.2 | 14350 | 0.58 | 149916 | N/A | 24.6 |
| K36R/G78A | 1107 | 11937 | 0.1 | 2611 | 0.11 | 5715 | N/A | 4.6 |
| Q33E/T41A | 1108 | 8292 | 0.1 | 2442 | 0.10 | 3958 | N/A | 3.4 |
| M47V/N48H | 1109 | 207012 | 1.3 | 14623 | 0.59 | 145529 | N/A | 14.2 |
| M47L/V68A | 1110 | 74238 | 0.5 | 13259 | 0.53 | 11223 | N/A | 5.6 |
| S44P/A71D | 1111 | 8839 | 0.1 | 2744 | 0.11 | 6309 | N/A | 3.2 |
| Q27H/M43I/A71D/R73 S | 1112 | 136251 | 0.8 | 12391 | 0.50 | 8242 | N/A | 11.0 |
| E35D/T57I/L70Q/A71D | 1114 | 121901 | 0.8 | 21284 | 0.86 | 2419 | N/A | 5.7 |
| M47I/E88D | 1115 | 105192 | 0.7 | 7337 | 0.30 | 97695 | N/A | 14.3 |
| M42I/I61V/A71D | 1116 | 54478 | 0.3 | 6074 | 0.24 | 4226 | N/A | 9.0 |
| P51A/A71D | 1117 | 67256 | 0.4 | 4262 | 0.17 | 5532 | N/A | 15.8 |
| H18Y/M47I/T57I/A71G | 1118 | 136455 | 0.8 | 20081 | 0.81 | 13749 | N/A | 6.8 |
| V20I/M47V/T57I/V84I | 1119 | 183516 | 1.1 | 26922 | 1.08 | 3583 | N/A | 6.8 |
| WT | 2030 | 161423 | 1.0 | 24836 | 1.00 | Not tested | N/A | 6.5 |

*Stop codon at indicated position

**TABLE 13B: Variant CD80 Binding to HEK293 Cells Transfected with CTLA4, CD28 or PD-L1**

| CD80 mutation(s) | SEQ ID NO (IgV) | CTLA4 | | CD28 | | PD-L1 | | |
|---|---|---|---|---|---|---|---|---|
| | | MFI at 66.6 nM | Fold change to WT | MFI at 66.6 nM | Fold change to WT | MFI at 22.2 nM | Fold change to WT | Ratio of CTLA4: CD28 |
| V20I/M47V/A71D | 1120 | 149937 | 7.23 | 15090 | 9.33 | 9710 | 5.48 | 9.9 |
| A71D/L72V/E95K | 1121 | 140306 | 6.77 | 6314 | 3.90 | 8417 | 4.75 | 22.2 |

| TABLE 13B: Variant CD80 Binding to HEK293 Cells Transfected with CTLA4, CD28 or PD-L1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| V22L/E35G/A71D/L72P | 1122 | 152588 | 7.36 | 8150 | 5.04 | 1403 | 0.79 | 18.7 |
| E35D/A71D | 1123 | 150330 | 7.25 | 14982 | 9.26 | 13781 | 7.77 | 10.0 |

| CD80 mutation(s) | SEQ ID NO (IgV) | CTLA4 | | CD28 | | PD-L1 | | |
|---|---|---|---|---|---|---|---|---|
| | | MFI at 66.6 nM | Fold change to WT | MFI at 66.6 nM | Fold change to WT | MFI at 22.2 nM | Fold change to WT | Ratio of CTLA4: CD28 |
| E35D/I67L/A71D | 1124 | 146087 | 7.04 | 11175 | 6.91 | 9354 | 5.28 | 13.1 |
| T13R/M42V/M47I/A71D | 1126 | 108900 | 5.25 | 16713 | 10.33 | 1869 | 1.05 | 6.5 |
| E35D | 1127 | 116494 | 5.62 | 3453 | 2.13 | 25492 | 14.38 | 33.7 |
| E35D/M47I/L70M | 1128 | 116531 | 5.62 | 14395 | 8.90 | 49131 | 27.71 | 8.1 |
| E35D/A71/L72V | 1129 | 134252 | 6.47 | 11634 | 7.19 | 13125 | 7.40 | 11.5 |
| E35D/M43L/L70M | 1130 | 102499 | 4.94 | 3112 | 1.92 | 40632 | 22.92 | 32.9 |
| A26P/E35D/M43I/L85Q/ E88D | 1131 | 83139 | 4.01 | 5406 | 3.34 | 9506 | 5.36 | 15.4 |
| E35D/D46V/L85Q | 1132 | 85989 | 4.15 | 7510 | 4.64 | 38133 | 21.51 | 11.4 |
| Q27L/E35D/M47I/T57I/ L70Q/E88D | 1133 | 59793 | 2.88 | 14011 | 8.66 | 1050 | 0.59 | 4.3 |
| Q27H/E35G/A71D/L72P/ T79I | 1125 | 85117 | 4.10 | 10317 | 6.38 | 1452 | 0.82 | 8.3 |
| M47V/I69F/A71D/V83I | 1134 | 76944 | 3.71 | 15906 | 9.83 | 3399 | 1.92 | 4.8 |
| E35D/T57A/A71D/L85Q | 1135 | 85724 | 4.13 | 3383 | 2.09 | 1764 | 0.99 | 25.3 |
| H18Y/A26T/E35D/A71D/ L85Q | 1136 | 70878 | 3.42 | 6487 | 4.01 | 8026 | 4.53 | 10.9 |
| E35D/M47L | 1137 | 82410 | 3.97 | 11508 | 7.11 | 58645 | 33.08 | 7.2 |
| E23D/M42V/M43I/I58V/ L70R | 1138 | 37331 | 1.80 | 10910 | 6.74 | 2251 | 1.27 | 3.4 |
| V68M/L70M/A71D/E95K | 1139 | 56479 | 2.72 | 10541 | 6.51 | 38182 | 21.53 | 5.4 |
| N55I/T57I/I69F | 1140 | 2855 | 0.14 | 1901 | 1.17 | 14759 | 8.32 | 1.5 |
| E35D/M43I/A71D | 1141 | 63789 | 3.08 | 6369 | 3.94 | 27290 | 15.39 | 10.0 |
| T41S/T57I/L70R | 1142 | 59844 | 2.89 | 4902 | 3.03 | 19527 | 11.01 | 12.2 |
| H18Y/A71D/L72P/E88V | 1143 | 68391 | 3.30 | 8862 | 5.48 | 1085 | 0.61 | 7.7 |
| V20I/A71D | 1144 | 60323 | 2.91 | 10500 | 6.49 | 3551 | 2.00 | 5.7 |
| E23G/A26S/E3SD/T62N/ A71D/L72V/L85M | 1145 | 59025 | 2.85 | 5484 | 3.39 | 10662 | 6.01 | 10.8 |
| A12T/E24D/E35D/D46V/ I61V/L72P/E95V | 1146 | 63738 | 3.07 | 7411 | 4.58 | 1221 | 0.69 | 8.6 |
| V22L/E35D/M43L/A71G/ D76H | 1147 | 2970 | 0.14 | 1498 | 0.93 | 1851 | 1.04 | 2.0 |
| E35G/K54E/A71D/L72P | 1148 | 71899 | 3.47 | 3697 | 2.29 | 1575 | 0.89 | 19.4 |
| L70Q/A71D | 1149 | 45012 | 2.17 | 18615 | 11.50 | 1692 | 0.95 | 2.4 |
| A26E/E35D/M47L/L85Q | 1150 | 40325 | 1.94 | 2266 | 1.40 | 55548 | 31.33 | 17.8 |

| CD80 mutation(s) | SEQ ID NO (IgV) | CTLA4 | | CD28 | | PD-L1 | | |
|---|---|---|---|---|---|---|---|---|
| | | MFI at 66.6 nM | Fold change to WT | MFI at 66.6 nM | Fold change to WT | MFI at 22.2 nM | Fold change to WT | Ratio of CTLA4: CD28 |
| D46E/A71D | 1151 | 69674 | 3.36 | 16770 | 10.36 | 22777 | 12.85 | 4.2 |
| Y31H/E35D/T41S/V68L/ K93R/R94W | 1152 | 3379 | 0.16 | 2446 | 1.51 | 18863 | 10.64 | 1.4 |
| WT CD80 IgV Fc | 2030 (IgV) | 20739 | 1.00 | 1618 | 1.00 | 1773 | 1.00 | 12.8 |
| WT CD80 ECD Fc | - | 72506 | 3.50 | 3072 | 1.90 | 4418 | 2.49 | 23.6 |

**TABLE 14A: Variant CD155 selected against cognate binding partners. Molecule sequences, binding data, and costimulatory bioactivity data.**

| CD155 mutations | SEQ ID NO (IgV) | CD226 tfxn MFI (CD226 MFI parental ratio) | TIGIT tfxn MFI (TIGIT MFI parental ratio) | CD96 MFI (CD96 MFI parental ratio) | Mock Expi293 MFI (Mock MFI parental ratio) | Anti-CD3 IFN-gamma (pg/mL) (Anti-CD3 IFN-gamma parental ratio) |
|---|---|---|---|---|---|---|
| P18S, P64S, F91S | 354 | 497825 (133.7) | 247219 (91.1) | 140065 (45.4) | 3528 (1.2) | 270.1 (0.7) |
| P18S, F91S, L104P | 355 | 26210 (7.0) | 75176 (27.7) | 10867 (3.5) | 2130 (0.7) | 364.2 (0.9) |
| L44P | 356 | 581289 (156.1) | 261931 (96.5) | 152252 (49.4) | 3414 (1.2) | 277.6 (0.7) |
| A56V | 357 | 455297 (122.3) | 280265 (1032) | 161162 (52.2) | 2601 (0.9) | 548.2 (1.4) |
| P18L, L79V, F91S | 358 | 5135 (1.4) | 4073 (1.5) | 3279 (1.1) | 2719 (0.9) | 1241.5 (3.2) |
| P18S, F91S | 359 | 408623 (109.8) | 284190 (104.7) | 147463 (47.8) | 3348 (1.1) | 760.6 (2.0) |
| P18T, F91S | 360 | 401283 (107.8) | 223985 (82.5) | 157644 (51.1) | 3065 (1.1) | 814.7 (2.1) |
| P18T, S42P, F91S | 361 | 554105 (148.8) | 223887 (82.5) | 135395 (43.9) | 3796 (1.3) | 539.7 (1.4) |
| G7E, P18T, Y30C, F91S | 362 | 12903 (3.5) | 12984 (4.8) | 7906 (2.6) | 2671 (0.9) | 275.9 (0.7) |
| P18T, F91S, G111D | 363 | 438327 (117.7) | 287315 (105.8) | 167583 (54.3) | 4012 (1.4) | 307.2 (0.8) |
| P18S, F91P | 364 | 4154 (1.1) | 3220 (1.2) | 2678 (0.9) | 2816 (1.0) | 365.7 (0.9) |
| P18T, F91S, F108L | 365 | 394546 (106.0) | 298680 (110.0) | 193122 (62.6) | 2926 (1.0) | 775.4 (2.0) |

**TABLE 14A: Variant CD155 selected against cognate binding partners. Molecule sequences, binding data, and costimulatory bioactivity data.**

| CD155 mutations | SEQ ID NO (IgV) | CD226 tfxn MFI (CD226 MFI parental ratio) | TIGIT tfxn MFI (TIGIT MFI parental ratio) | CD96 MFI (CD96 MFI parental ratio) | Mock Expi293 MFI (Mock MFI parental ratio) | Anti-CD3 IFN-gamma (pg/mL) (Anti-CD3 IFN-gamma parental ratio) |
|---|---|---|---|---|---|---|
| P18T, T45A, F91S | 366 | 435847 (117.1) | 222044 (81.8) | 191026 (61.9) | 2948 (1.0) | 1546.8 (4.0) |
| P18T, F91S, R94H | 367 | 3589 (1.0) | 2942 (1.1) | 2509 (0.8) | 2390 (0.8) | 1273.2 (3.3) |
| P18S, Y30C, F91S | 368 | 382352 (102.7) | 276358 (101.8) | 56934 (18.5) | 3540 (1.2) | 426.5 (1.1) |
| A81V, L83P | 369 | 4169 (1.1) | 2912 (1.1) | 2616 (0.8) | 2993 (1.0) | 339.7 (0.9) |
| L88P | 370 | 65120 (17.5) | 74845 (27.6) | 35280 (11.4) | 2140 (0.7) | 969.2 (2.5) |
| Wild type | 353 | 3723 (1.0) | 2715 (1.0) | 3085 (1.0) | 2913 (1.0) | 389.6 (1.0) |
| R94H | 371 | 18905 (5.1) | 104013 (38.3) | 11727 (3.8) | 1663 (0.6) | 372.6 (1.0) |
| A13E, P18S, A56V, F91S | 372 | 357808 (96.1) | 179060 (66.0) | 118570 (38.4) | 2844 (1.0) | 349.2 (0.9) |
| P18T, F91S, V115A | 373 | 38487 (10.3) | 46313 (17.1) | 22718 (7.4) | 2070 (0.7) | 1574.5 (4.0) |
| P18T, Q60K | 374 | 238266 (64.0) | 173730 (64.0) | 154448 (50.1) | 4778 (1.6) | 427.2 (1.1) |

**TABLE 14B: Additional CD155 Variants and Binding Data.**

| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD |
| S52M | 569 | 1865.3 | 0.00 | 1901.0 | 0.01 | 1553.4 | 0.87 | 1609.8 | 0.02 |
| T45Q, S52L, L104E, G111R | 570 | 2287.0 | 0.01 | 2390.4 | 0.01 | 1735.1 | 0.97 | 1575.1 | 0.02 |
| S42G | 571 | 4837.5 | 0.01 | 2448.1 | 0.01 | 1815.4 | 1.02 | 1699.6 | 0.02 |
| Q62F | 572 | 2209.5 | 0.01 | 2572.1 | 0.01 | 2706.5 | 1.52 | 2760.7 | 0.03 |
| S52Q | 573 | 2288.1 | 0.01 | 2022.3 | 0.01 | 1790.1 | 1.00 | 1822.3 | 0.02 |
| S42A, L104Q, G111R | 574 | 1923.7 | 0.00 | 1901.7 | 0.01 | 1815.1 | 1.02 | 1703.8 | 0.02 |

| TABLE 14B: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD |
| S42A, S52Q, L104Q, G111R | 575 | 1807.5 | 0.00 | 2157.2 | 0.01 | 1894.4 | 1.06 | 1644.0 | 0.02 |
| S52W, L104E | 576 | 1938.2 | 0.00 | 1905.6 | 0.01 | 2070.6 | 1.16 | 1629.5 | 0.02 |
| S42C | 577 | 1914.0 | 0.00 | 2096.1 | 0.01 | 1685.0 | 0.95 | 1592.4 | 0.02 |
| S52W | 578 | 1991.6 | 0.00 | 2037.3 | 0.01 | 1612.8 | 0.90 | 1712.9 | 0.02 |
| S52M, L104Q | 579 | 2666.6 | 0.01 | 2252.2 | 0.01 | 1706.0 | 0.96 | 1633.1 | 0.02 |
| S42L, S52L, Q62F, L104Q | 580 | 2021.4 | 0.00 | 2643.8 | 0.02 | 1730.1 | 0.97 | 2318.7 | 0.02 |
| S42W | 581 | 2434.5 | 0.01 | 2133.4 | 0.01 | 2325.7 | 1.30 | 2555.4 | 0.03 |
| S42Q | 582 | 2073.5 | 0.00 | 2225.9 | 0.01 | 1905.1 | 1.07 | 2143.1 | 0.02 |
| S52L | 583 | 2224.8 | 0.01 | 2676.3 | 0.02 | 2038.6 | 1.14 | 2043.2 | 0.02 |
| S52R | 584 | 4395.4 | 0.01 | 3964.4 | 0.02 | 2741.7 | 1.54 | 4846.9 | 0.05 |
| L104E | 585 | 3135.4 | 0.01 | 2264.2 | 0.01 | 1803.5 | 1.01 | 1556.7 | 0.02 |
| G111R | 586 | 2082.7 | 0.00 | 2791.3 | 0.02 | 2470.9 | 1.39 | 3317.1 | 0.03 |
| S52E | 587 | 2655.4 | 0.01 | 2599.8 | 0.02 | 1904.9 | 1.07 | 1799.0 | 0.02 |
| Q62Y | 588 | 2528.6 | 0.01 | 2621.4 | 0.02 | 1918.4 | 1.08 | 1827.5 | 0.02 |
| T45Q, S52M, L104E | 589 | 79498.2 | 0.19 | 143238.5 | 0.83 | 2600.6 | 1.46 | 6310.4 | 0.06 |
| S42N, L104Q, G111R | 590 | 2432.1 | 0.01 | 2311.3 | 0.01 | 1847.4 | 1.04 | 1958.3 | 0.02 |
| S52M, V57L | 591 | 1760.7 | 0.00 | 2431.6 | 0.01 | 2006.9 | 1.13 | 1858.7 | 0.02 |
| S42N, S52Q, Q62F | 592 | 2402.7 | 0.01 | 2152.0 | 0.01 | 1855.0 | 1.04 | 1737.6 | 0.02 |
| S42A, S52L, L104E, G111R | 593 | 2262.7 | 0.01 | 1889.4 | 0.01 | 1783.2 | 1.00 | 1606.2 | 0.02 |
| S42W, S52Q, V57L, Q62Y | 594 | 1961.4 | 0.00 | 2138.3 | 0.01 | 1844.9 | 1.03 | 1699.6 | 0.02 |
| L104Q | 595 | 10314.4 | 0.02 | 3791.4 | 0.02 | 2119.9 | 1.19 | 1542.6 | 0.02 |

| TABLE 14B: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD |
| S42L, S52Q, L104E | 596 | 1946.9 | 0.00 | 6474.3 | 0.04 | 1749.0 | 0.98 | 1702.2 | 0.02 |
| S42C, S52L | 597 | 1762.5 | 0.00 | 2147.3 | 0.01 | 1663.4 | 0.93 | 1484.7 | 0.01 |
| S42W, S52R, Q62Y, L104Q | 598 | 1918.8 | 0.00 | 2300.1 | 0.01 | 1824.6 | 1.02 | 1756.0 | 0.02 |
| T45Q, S52R, L104E | 599 | 121636.9 | 0.29 | 142381.2 | 0.82 | 2617.9 | 1.47 | 3748.2 | 0.04 |
| S52R, Q62F, L104Q, G111R | 600 | 2969.2 | 0.01 | 3171.6 | 0.02 | 1725.4 | 0.97 | 2362.3 | 0.02 |
| T45Q, S52L, V57L, L104E | 601 | 2857.7 | 0.01 | 5943.5 | 0.03 | 1496.8 | 0.84 | 1533.3 | 0.02 |
| S52M, Q62Y | 602 | 1926.6 | 0.00 | 2000.3 | 0.01 | 1771.6 | 0.99 | 1651.1 | 0.02 |
| Q62F, L104E, G111R | 603 | 1966.4 | 0.00 | 2043.5 | 0.01 | 1701.9 | 0.95 | 1524.8 | 0.02 |
| T45Q, S52Q | 604 | 4812.8 | 0.01 | 5787.5 | 0.03 | 1765.6 | 0.99 | 2451.3 | 0.02 |
| S52L, L104E | 605 | 4317.8 | 0.01 | 2213.9 | 0.01 | 1756.9 | 0.99 | 1829.3 | 0.02 |
| S42V, S52E | 606 | 2055.0 | 0.00 | 2272.6 | 0.01 | 1808.0 | 1.01 | 2530.2 | 0.03 |
| T45Q, S52R, G111R | 607 | 4092.3 | 0.01 | 2075.2 | 0.01 | 1793.6 | 1.01 | 2336.6 | 0.02 |
| S42G, S52Q, L104E, G111R | 608 | 2010.1 | 0.00 | 2019.2 | 0.01 | 1706.4 | 0.96 | 1707.6 | 0.02 |
| S42N, S52E, V57L, L104E | 609 | 1784.2 | 0.00 | 1743.6 | 0.01 | 1690.1 | 0.95 | 1538.7 | 0.02 |
| Wildtype | 353 | 1964.7 | 0.00 | 2317.1 | 0.01 | 2169.6 | 1.22 | 1893.4 | 0.02 |
| S42C, S52M, Q62F | 610 | 1861.0 | 0.00 | 2084.2 | 0.01 | 1592.3 | 0.89 | 1481.3 | 0.01 |

**TABLE 14B: Additional CD155 Variants and Binding Data.**

| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD | MFI at 100nM | Fold ↑ to WT ECD |
| S42L | 611 | 1930.4 | 0.00 | 2187.2 | 0.01 | 1743.2 | 0.98 | 1618.4 | 0.02 |
| Wildtype | 353 | 2182.6 | 0.01 | 2374.5 | 0.01 | 1743.1 | 0.98 | 1680.4 | 0.02 |
| S42A | 612 | 1929.2 | 0.00 | 2188.6 | 0.01 | 1733.7 | 0.97 | 1623.6 | 0.02 |
| S42G, S52L, Q62F, L104Q | 613 | 1924.3 | 0.00 | 2157.6 | 0.01 | 1661.3 | 0.93 | 1642.1 | 0.02 |
| S42N | 614 | 1817.4 | 0.00 | 1910.9 | 0.01 | 1699.7 | 0.95 | 1691.5 | 0.02 |
| CD155 IgV Fc | 353 (IgV) | 4690 | 0.01 | 4690 | 0.03 | 2941 | 1.65 | 3272 | 0.03 |
| Wildtype CD155 ECD-Fc | 47 (ECD) | 423797 | 1.00 | 172839 | 1.00 | 1783 | 1.00 | 99037 | 1.00 |
| Anti-human Fc PE | | 1506.3 | 0.00 | 3774 | 0.02 | 1587 | 0.89 | 1618 | 0.02 |

**TABLE 14C: Additional CD155 Variants and Binding Data.**

| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
|---|---|---|---|---|---|---|---|
| | | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD |
| P18T, S65A, S67V, F91S | 615 | 297843 | 1.99 | 351195 | 3.22 | 128180 | 1.68 |
| P18T, T45Q, T61R, S65N, S67L | 617 | 224682 | 1.50 | 270175 | 2.48 | 22820 | 0.30 |
| P18F, T39A, T45Q, T61R, S65N, S67L, E73G, R78G | 616 | Little to no protein produced | | | | | |
| P18F, S65A, S67V, F91S | 618 | 534106 | 3.57 | 350410 | 3.21 | 144069 | 1.89 |
| P18F, T45Q, T61R, S65N, S67L, F91S, L104P | 619 | Little to no protein produced | | | | | |
| P18S, L79P, L104M | 620 | 342549 | 2.29 | 320823 | 2.94 | 107532 | 1.41 |
| P18S, L104M | 621 | 449066 | 3.00 | 295126 | 2.70 | 121266 | 1.59 |
| L79P, L104M | 622 | 3210 | 0.02 | 8323 | 0.08 | 2894 | 0.04 |
| P18T, T45Q, L79P | 623 | 542878 | 3.63 | 371498 | 3.40 | 193719 | 2.55 |
| P18T, T45Q, T61R, S65H, S67H | 624 | 312337 | 2.09 | 225439 | 2.07 | 152903 | 2.01 |
| P18T, A81E | 625 | Little to no protein produced | | | | | |
| P18S, D23Y, E37P, S52G, Q62M, G80S, | 626 | Little to no protein produced | | | | | |

| TABLE 14C: Additional CD155 Variants and Binding Data. | | | | | | | |
|---|---|---|---|---|---|---|---|
| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
| | | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD |
| A81P, G99Y, S112N | | | | | | | |
| A13R, D23Y, E37P, S42P, Q62Y, A81E | 627 | 4161 | 0.03 | 11673 | 0.11 | 5762 | 0.08 |
| A13R, D23Y, E37P, G99Y, S112N | 627 | Little to no protein produced | | | | | |
| A13R, D23Y, E37P, Q62M, A77V, G80S, A81P, G99Y | 629 | Little to no protein produced | | | | | |
| P18L, E37S, Q62M, G80S, A81P, G99Y, S112N | 630 | 5900 | 0.04 | 14642 | 0.13 | 3345 | 0.04 |
| P18S, L104T | 631 | 321741 | 2.15 | 367470 | 3.37 | 108569 | 1.43 |
| P18S, Q62H, L79Q, F91S | 632 | 283357 | 1.89 | 324877 | 2.98 | 125541 | 1.65 |
| P18S, F91S | 359 | 222780 | 1.49 | 300049 | 2.75 | 48542 | 0.64 |
| T45Q, S52K, Q62F, L104Q, G111R | 633 | Little to no protein produced | | | | | |
| T45Q, S52Q, Q62Y, L104Q, G111R | 634 | Little to no protein produced | | | | | |
| T45Q, S52Q, Q62Y, L104E, G111R | 635 | Little to no protein produced | | | | | |
| V57A, T61M, S65W, S67A, E96D, L104T | 636 | Little to no protein produced | | | | | |
| P18L, V57T, T61S, S65Y, S67A, L 104T | 637 | 278178 | 1.86 | 276870 | 2.54 | 121499 | 1.60 |
| P18T, T45Q | 638 | 326769 | 2.18 | 357515 | 3.28 | 92389 | 1.21 |
| P18L, V57A, T61M, S65W, S67A, L104T | 639 | Little to no protein produced | | | | | |
| T61M, S65W, S67A, L104T | 640 | 360915 | 2.41 | 417897 | 3.83 | 148954 | 1.96 |
| P18S, V41A, S42G, T45G, L104N | 641 | 3821 | 0.03 | 11449 | 0.10 | 3087 | 0.04 |
| P18H, S42G, T45I, S52T, G53R, S54H, V57L, H59E, T61S, S65D, E68G, L104N | 642 | 5066 | 0.03 | 177351 | 1.63 | 3700 | 0.05 |
| P18S, S42G, T45V, F58L, S67W, L104N | 643 | 14137 | 0.09 | 15175 | 0.14 | 15324 | 0.20 |
| P18S, T45I, L104N | 644 | 141745 | 0.95 | 298011 | 2.73 | 97246 | 1.28 |
| P18S, S42G, T45G, L104N, V106A | 645 | 29387 | 0.20 | 117965 | 1.08 | 15884 | 0.21 |

| TABLE 14C: Additional CD155 Variants and Binding Data. | | | | | | | |
|---|---|---|---|---|---|---|---|
| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
| | | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD |
| P18H, H40R, S42G, T45I, S52T, G53R, S54H, V57L, H59E, T61S, S65D, E68G, L104Y, V106L, F108H | 646 | 12335 | 0.08 | 14657 | 0.13 | 15779 | 0.21 |
| E37V, S42G, T45G, L104N | 647 | Little to no protein produced | | | | | |
| P18S, T45Q, L79P, L104T | 648 | 206674 | 1.38 | 285512 | 2.62 | 87790 | 1.15 |
| P18L, Q62R | 649 | 66939 | 0.45 | 25063 | 0.23 | 10928 | 0.14 |
| A13R, D23Y, E37P, S42L, S52G, Q62Y, A81E | 650 | Little to no protein produced | | | | | |
| P18L, H49R, L104T, D116N | 651 | 167980 | 1.12 | 214677 | 1.97 | 62451 | 0.82 |
| A13R, D23Y, E37P, Q62M, G80S, A81P, L104T | 652 | Little to no protein produced | | | | | |
| S65T, L104T | 653 | 205942 | 1.38 | 187147 | 1.71 | 65207 | 0.86 |
| A13R, D23Y, E37P, S52G, V57A, Q62M, K70E, L104T | 654 | Little to no protein produced | | | | | |
| P18L, A47V, Q62Y, E73D, L104T | 655 | 146142 | 0.98 | 248926 | 2.28 | 73956 | 0.97 |
| H40T, V41M, A47V, S52Q, Q62L, S65T, E73R, D97G, E98S, L104T, D116N | 656 | Little to no protein produced | | | | | |
| P18L, S42P, T45Q, T61G, S65H, S67E, L104T, D116N | 657 | 153536 | 1.03 | 402503 | 3.69 | 53044 | 0.70 |
| P18S, H40T, V41M, A47V, S52Q, Q62L, S65T, E73R, L104M, V106A | 658 | Little to no protein produced | | | | | |
| H40T, V41M, A47V, S52Q, Q62L, S65T, E68G, E73R, D97G, E98S, L104T | 659 | Little to no protein produced | | | | | |
| T45Q, S52E, L104E | 660 | Little to no protein produced | | | | | |
| T45Q, S52E, Q62F, L104E | 661 | 132850 | 0.89 | 276434 | 2.53 | 14558 | 0.19 |

**TABLE 14C: Additional CD155 Variants and Binding Data.**

| CD155 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
|---|---|---|---|---|---|---|---|
| | | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD | MFI at 100nM | Fold Increase to WT ECD |
| Wildtype CD155 ECD-Fc | 47 (ECD) | 149692 | 1.00 | 109137 | 1.00 | 76083 | 1.00 |
| Anti-human Fc PE | - | 2287 | 0.02 | 4799 | 0.04 | 2061 | 0.03 |

**TABLE 14D: Additional CD155 Variants and Binding Data.**

| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
|---|---|---|---|---|---|---|---|
| | | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV |
| P18F, T26M, L44V, Q62K, L79P, F91S, L104M, G111D | 662 | 117327 | 1.2 | 1613 | 0.1 | 1629 | 0.1 |
| P18S, T45S, T61K, S65W, S67A, F91S, G111R | 663 | 124936 | 1.3 | 2114 | 0.1 | 2223 | 0.1 |
| P18S, L79P, L104M, T107M | 664 | 110512 | 1.1 | 18337 | 0.9 | 22793 | 1.3 |
| P18S, S65W, S67A, M90V, V95A, L104Q, G111R | 665 | 101726 | 1.0 | 1605 | 0.1 | 2571 | 0.1 |
| Wildtype CD155-ECD | 47 (ECD) | 98935 | 1.0 | 20029 | 1.0 | 17410 | 1.0 |

**TABLE 14E: Additional CD155 Variants and Binding Data.**

| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
|---|---|---|---|---|---|---|---|
| | | MFI at 11.1nM | Fold Change from CD155-ECD | MFI at 11.1nM | Fold Change from CD155-ECD | MFI at 11.1nM | Fold Change from CD155-ECD |
| P18S, A47G, L79P, F91S, L104M, T107A, R113W | 1528 | 56,409 | 1.19 | 1,191 | 0.08 | 25,362 | 1.49 |
| P18T, D23G, S24A, N35D, H49L, L79P, F91S, L104M, G111R | 1529 | 128,536 | 2.72 | 987 | 0.06 | 3,497 | 0.20 |
| V9L, P18S, Q60R, V75L, L79P, R89K, F91S, L104E, G111R | 1530 | 125,329 | 2.65 | 986 | 0.06 | 959 | 0.06 |
| P18S, H49R, E73D, L79P, N85D, F91S, V95A, L104M, | 1531 | Little to no protein produced | | | | | |

| TABLE 14E: Additional CD155 Variants and Binding Data. | | | | | | | |
|---|---|---|---|---|---|---|---|
| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
| | | MFI at 11.1nM | Fold Change from CD155-ECD | MFI at 11.1nM | Fold Change from CD155-ECD | MFI at 11.1nM | Fold Change from CD155-ECD |
| G111R | | | | | | | |
| V11A, P18S, L79P, F91S, L104M, G111R | 1532 | 48,246 | 1.02 | 974 | 0.06 | 923 | 0.05 |
| V11A, P18S, S54R, Q60P, Q62K, L79P, N85D, F91S, T107M | 1533 | 190,392 | 4.02 | 1,019 | 0.07 | 1,129 | 0.07 |
| P18T, S52P, S65A, S67V, L79P, F91S, L104M, G111R | 1534 | 121,611 | 2.57 | 986 | 0.06 | 16,507 | 0.97 |
| P18T, M36T, L79P, F91S, G111R | 1535 | 150,015 | 3.17 | 1,029 | 0.07 | 2,514 | 0.15 |
| D8G, P18S, M36I, V38A, H49Q, A76E, F91S, L104M, T107A, R113W | 1536 | 79,333 | 1.68 | 1,026 | 0.07 | 2,313 | 0.14 |
| P18S, S52P, S65A, S67V, L79P, F91S, L104M, T107S, R113W | 1537 | 23,766 | 0.50 | 1,004 | 0.07 | 1,080 | 0.06 |
| T15I, P18T, L79P, F91S, L104M, G111R | 1538 | 55,498 | 1.17 | 1,516 | 0.10 | 1,030 | 0.06 |
| P18F, T26M, L44V, Q62K, L79P, E82D, F91S, L104M, G111D | 1539 | 213,640 | 4.51 | 991 | 0.06 | 1,276 | 0.07 |
| P18T, E37G, G53R, Q62K, L79P, F91S, E98D, L104M, T107M | 1540 | 251,288 | 5.31 | 2,001 | 0.13 | 45,878 | 2.69 |
| P18L, K70E, L79P, F91S, V95A, G111R | 1541 | 62,608 | 1.32 | 1,117 | 0.07 | 973 | 0.06 |
| V9I, Q12K, P18F, S65A, S67V, L79P, L104T, G111R, S112I | 1542 | 81,932 | 1.73 | 803 | 0.05 | 68,295 | 4.00 |
| P18F, S65A, S67V, F91S, L104M, G111R | 1543 | 30,661 | 0.65 | 901 | 0.06 | 3,193 | 0.19 |
| V9I, V10I, P18S, F20S, T45A, L79P, F91S, L104M, F108Y, G111R, | 1544 | 151,489 | 3.20 | 973 | 0.06 | 974 | 0.06 |

**TABLE 14E: Additional CD155 Variants and Binding Data.**

| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD96 | |
|---|---|---|---|---|---|---|---|
| | | MFI at 11.1nM | Fold Change from CD155-ECD | MFI at 11.1nM | Fold Change from CD155-ECD | MFI at 11.1nM | Fold Change from CD155-ECD |
| S112V | | | | | | | |
| V9L, P18L, L79P, M90I, F91S, T102S, L104M, G111R | 1545 | 155,279 | 3.28 | 910 | 0.06 | 10,568 | 0.62 |
| P18C, T26M, L44V, M55I, Q62K, L79P, F91S, L104M, T107M | 1546 | 137,521 | 2.91 | 973 | 0.06 | 111,085 | 6.51 |
| V9I, P18T, D23G, L79P, F91S, G111R | 1547 | 151,426 | 3.20 | 897 | 0.06 | 2,725 | 0.16 |
| P18F, L79P, M90L, F91S, V95A, L104M, G111R | 1548 | 125,639 | 2.66 | 917 | 0.06 | 3,939 | 0.23 |
| P18F, L79P, M90L, F91S, V95A, L104M, G111R | 1548 | 115,156 | 2.43 | 1,073 | 0.07 | 2,464 | 0.14 |
| P18T, M36T, S65A, S67E, L79Q, A81T, F91S, G111R | 1549 | 10,616 | 0.22 | 1,130 | 0.07 | 963 | 0.06 |
| V9L, P18T, Q62R, L79P, F91S, L104M, G111R | 1550 | 195,111 | 4.12 | 835 | 0.05 | 1,497 | 0.09 |
| CD155-ECD-Fc | 47 (ECD) | 47,319 | 1.00 | 15,421 | 1.00 | 17,067 | 1.00 |
| Fc Control | 1155 | 2,298 | 0.05 | 1,133 | 0.07 | 996 | 0.06 |

**TABLE 14F: Additional CD155 Variants and Binding Data.**

| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI at 25nM | Fold Change from CD155 - ECD | MFI at 25nM | Fold Change from CD155 - ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| P18T, G19D, M36T, S54N, L79P, L83Q, F91S, T107M, F108Y | 1669 | 905 | 0.02 | 748 | 0.02 | 1276 | 1.56 | 726 | 0.01 |
| V9L, P18L, M55V, | 1670 | | | | | | | | |

| TABLE 14F: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CD155 Mutations** | **SEQ ID NO (IgV)** | **TIGIT** | | **CD226** | | **CD112R** | | **CD96** | |
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| S69L, L79P, A81E, F91S, T107M | | 58656 | 1.34 | 11166 | 0.29 | 920 | 1.13 | 67364 | 1.39 |
| P18F, H40Q, T61K, Q62K, L79P, F91S, L104M, T107V | 1671 | 108441 | 2.48 | 853 | 0.02 | 918 | 1.13 | 8035 | 0.17 |
| P18S, Q32R, Q62K, R78G, L79P, F91S, T107A, R113W | 1672 | 5772 | 0.13 | 701 | 0.02 | 843 | 1.03 | 831 | 0.02 |
| Q12H, P18T, L21S, G22S, V57A, Q62R, L79P, F91S, T107M | 1673 | 1084 | 0.02 | 687 | 0.02 | 876 | 1.07 | 818 | 0.02 |
| V9I, P18S, S24P, H49Q, F58Y, Q60R, Q62K, L79P, F91S, T107M | 1674 | 69926 | 1.60 | 1089 | 0.03 | 1026 | 1.26 | 43856 | 0.90 |
| P18T, W46C, H49R, S65A, S67V, A76T, L79P, S87T, L104M | 1675 | 918 | 0.02 | 640 | 0.02 | 803 | 0.98 | 717 | 0.01 |
| P18S, S42T, E51G, L79P, F91S, G92W, T107M | 1676 | 12630 | 0.29 | 707 | 0.02 | 857 | 1.05 | 1050 | 0.02 |

**TABLE 14F: Additional CD155 Variants and Binding Data.**

| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| P18S, S42T, E51G, L79P, F91S, G92W, T107M | 1676 | 7476 | 0.17 | 851 | 0.02 | 935 | 1.15 | 924 | 0.02 |
| V10F, T15S, P18L, R48Q, L79P, F91S, T107M, V115M | 1677 | 1168 | 0.03 | 792 | 0.02 | 901 | 1.10 | 998 | 0.02 |
| P18S, L21M, Y30F, N35D, R84W, F91S, T107M, D116G | 1678 | 1377 | 0.03 | 743 | 0.02 | 946 | 1.16 | 1033 | 0.02 |
| P18F, E51V, S54G, Q60R, L79Q, E82G, S87T, M90I, F91S, G92R, T107M | 1679 | 46090 | 1.05 | 15701 | 0.41 | 1012 | 1.24 | 61814 | 1.27 |
| Q16H, P18F, F91S, T107M | 1680 | Little to no protein produced | | | | | | | |
| P18T, D23G, Q60R, S67L, L79P, F91S, T107M, V115A | 1681 | 64091 | 1.47 | 30931 | 0.81 | 874 | 1.07 | 108875 | 2.24 |
| D8G, V9I, V11A, P18T, T26M, S52P, L79P, F91S, G92A, | 1682 | 52508 | 1.20 | 9483 | 0.25 | 817 | 1.00 | 97770 | 2.01 |

| TABLE 14F: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CD155 Mutations** | **SEQ ID NO (IgV)** | **TIGIT** | | **CD226** | | **CD112R** | | **CD96** | |
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| T107L, V115A | | | | | | | | | |
| V9I, P18F, A47E, G50S, E68G, L79P, F91S, T107M | 1683 | 55167 | 1.26 | 54341 | 1.43 | 752 | 0.92 | 102115 | 2.10 |
| P18S, M55I, Q62K, S69P, L79P, F91S, T107M | 1684 | Little to no protein produced | | | | | | | |
| P18T, T39S, S52P, S54R, L79P, F91S, T107M | 1685 | 45927 | 1.05 | 744 | 0.02 | 1038 | 1.27 | 1225 | 0.03 |
| P18S, D23N, L79P, F91S, T107M, S114N | 1686 | Little to no protein produced | | | | | | | |
| P18S, P34S, E51V, L79P, F91S, G111R | 1687 | 7917 | 0.18 | 769 | 0.02 | 853 | 1.04 | 892 | 0.02 |
| P18S, H59N, V75A, L79P, A81T, F91S, L104M, T107M | 1688 | 800 | 0.02 | 676 | 0.02 | 915 | 1.12 | 759 | 0.02 |
| P18S, W46R, E68D, L79P, F91S, T107M, R113G | 1689 | 1359 | 0.03 | 717 | 0.02 | 798 | 0.98 | 737 | 0.02 |
| V9L, P18F, T45A, S65A, | 1690 | | | | | | | | |

| TABLE 14F: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD155 Mutations | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| S67V, R78K, L79V, F91S, T107M, S114T | | 130274 | 2.98 | 153569 | 4.04 | 812 | 1.00 | 85605 | 1.76 |
| P18T, M55L, T61R, L79P, F91S, V106I, T107M | 1691 | 133399 | 3.05 | 1906 | 0.05 | 827 | 1.01 | 57927 | 1.19 |
| T15I, P18S, V33M, N35F, T39S, M55L, R78S, L79P, F91S, T107M | 1692 | 7550 | 0.17 | 1015 | 0.03 | 789 | 0.97 | 2709 | 0.06 |
| P18S, Q62K, K70E, L79P, F91S, G92E, R113W | 1693 | 11173 | 0.26 | 691 | 0.02 | 735 | 0.90 | 1951 | 0.04 |
| P18F, F20I, T26M, A47V, E51K, L79P, F91S | 1694 | 136088 | 3.11 | 54026 | 1.42 | 1401 | 1.72 | 96629 | 1.99 |
| P18T, D23A, Q60H, L79P, M90V, F91S, T107M | 1695 | 43795 | 1.00 | 98241 | 2.58 | 888 | 1.09 | 70891 | 1.46 |
| P18S, D23G, C29R, N35D, E37G, M55I, Q62K, S65A, S67G, R78G, | 1696 | 1599 | 0.04 | 1030 | 0.03 | 1115 | 1.37 | 1944 | 0.04 |

| TABLE 14F: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CD155 Mutations** | **SEQ ID NO (IgV)** | **TIGIT** | | **CD226** | | **CD112R** | | **CD96** | |
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| L79P, F91S, L104M, T107M, Q110R | | | | | | | | | |
| A13E, P18S, M36R, Q62K, S67T, L79P, N85D, F91S, T107M | 1697 | Little to no protein produced | | | | | | | |
| V9I, P18T, H49R, L79P, N85D, F91S, L104T, T107M | 1698 | 46375 | 1.06 | 76851 | 2.02 | 794 | 0.97 | 80210 | 1.65 |
| V9A, P18F, T61S, Q62L, L79P, F91S, G111R | 1699 | 26109 | 0.60 | 891 | 0.02 | 825 | 1.01 | 2633 | 0.05 |
| D8E, P18T, T61A, L79P, F91S, T107M | 1700 | Little to no protein produced | | | | | | | |
| P18S, V41A, H49R, S54C, L79S, N85Y, L88P, F91S, L104M, T107M | 1701 | 1098 | 0.03 | 830 | 0.02 | 876 | 1.07 | 1678 | 0.03 |
| V11E, P18H, F20Y, V25E, N35S, H49R, L79P, F91S, T107M, G111R | 1702 | 979 | 0.02 | 846 | 0.02 | 844 | 1.03 | 928 | 0.02 |

| TABLE 14F: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CD155 Mutations** | **SEQ ID NO (IgV)** | **TIGIT** | | **CD226** | | **CD112R** | | **CD96** | |
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| V11A, P18F, D23A, L79P, G80D, V95A, T107M | 1703 | 45249 | 1.04 | 913 | 0.02 | 830 | 1.02 | 33883 | 0.70 |
| P18S, K70R, L79P, F91S, G111R | 1704 | 16180 | 0.37 | 793 | 0.02 | 854 | 1.05 | 1182 | 0.02 |
| P18T, D23A, Q60H, L79P, M90V, F91S, T107M | 1695 | 175673 | 4.02 | 161958 | 4.26 | 879 | 1.08 | 50981 | 1.05 |
| V9L, V11M, P18S, N35S, S54G, Q62K, L79P, L104M, T107M, V115M | 1705 | 2999 | 0.07 | 2315 | 0.06 | 893 | 1.09 | 925 | 0.02 |
| V9L, P18Y, V25A, V38G, M55V, A77T, L79P, M90I, F91S, L104M | 1706 | 138011 | 3.16 | 26015 | 0.68 | 919 | 1.13 | 17970 | 0.37 |
| V10G, P18T, L72Q, L79P, F91S, T107M | 1707 | 4253 | 0.10 | 1584 | 0.04 | 863 | 1.06 | 3643 | 0.07 |
| P18S, H59R, A76G, R78S, L79P | 1708 | 130622 | 2.99 | 79435 | 2.09 | 1009 | 1.24 | 44493 | 0.91 |
| V9A, P18S, M36T, S65G, L79P, F91S, | 1709 | 92503 | 2.12 | 989 | 0.03 | 886 | 1.09 | 7850 | 0.16 |

| TABLE 14F: Additional CD155 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CD155 Mutations** | **SEQ ID NO (IgV)** | **TIGIT** | | **CD226** | | **CD112R** | | **CD96** | |
| | | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD | MFI at 25nM | Fold Change from CD155 -ECD |
| L104T, G111R, S112I | | | | | | | | | |
| P18T, S52A, V57A, Q60R, Q62K, S65C, L79P, F91T, N100Y, T107M | 1710 | 187338 | 4.29 | 10579 | 0.28 | 908 | 1.11 | 3791 | 0.08 |
| V11A, P18F, N35D, A47E, Q62K, L79P, F91S, G99D, T107M, S114N | 1711 | Little to no protein produced | | | | | | | |
| V11A, P18T, N35S, L79P, S87T, F91S | 1712 | 218660 | 5.00 | 273825 | 7.20 | 1269 | 1.56 | 69871 | 1.44 |
| V9D, V11M, Q12L, P18S, E37V, M55I, Q60R, K70Q, L79P, F91S, L104M, T107M | 1713 | 8693 | 0.20 | 790 | 0.02 | 852 | 1.04 | 1991 | 0.04 |
| T15S, P18S, Y30H, Q32L, Q62R, L79P, F91S, T107M | 1714 | 16213 | 0.37 | 2092 | 0.06 | 1056 | 1.29 | 6994 | 0.14 |
| CD155-ECD-Fc | 47 (ECD) | 43704 | 1.00 | 38032 | 1.00 | 816 | 1.00 | 48638 | 1.00 |
| CD 1 12-IgV | 761 | 1289 | | 824 | | 17819 | | 1172 | 0.02 |

**TABLE 15A: Variant CD112 selected against cognate binding partners. Molecule sequences, binding data, and costimulatory bioactivity data.**

| CD112 mutation(s) | SEQ ID NO (IgV) | TIGIT tfxn MFI (TIGIT MFI parental ratio) | CD112R tfxn MFI (CD112R MFI parental ratio) | CD226 MFI (CD226 MFI parental ratio) | Mock Expi293 MFI (Mock MFI parental ratio) | Anti-CD3 IFN-gamma (pg/mL) (Anti-CD3 IFN-gamma parental ratio) |
|---|---|---|---|---|---|---|
| WT CD112 | 761 | 210829 (1.00) | 1452 (1.00) | 265392 (1.00) | 1112 (1.00) | 676.6 (1.00) |
| Y33H, A112V, G117D | 762 | 12948 (0.06) | 1552 (1.07) | 1368 (0.01) | 1241 (1.12) | 164.8 (0.24) |
| V19A, Y33H, S64G, S80G, G98S, N106Y, A112V | 763 | 48356 (0.23) | 1709 (1.18) | 2831 (0.01) | 1098 (0.99) | |
| L32P, A112V | 764 | 191432 (0.91) | 1557 (1.07) | 11095 (0.04) | 1259 (1.13) | 390.4 (0.58) |
| A95V, A112I | 765 | 238418 (1.13) | 1706 (1.17) | 51944 (0.20) | 1215 (1.09) | 282.5 (0.42) |
| P28S, A112V | 766 | 251116 (1.19) | 1985 (1.37) | 153382 (0.58) | 1189 (1.07) | 503.4 (0.74) |
| P27A, T38N, V101A, A112V | 767 | 255803 (1.21) | 2138 (1.47) | 222822 (0.84) | 1399 (1.26) | 240.7 (0.36) |
| S118F | 768 | 11356 (0.05) | 5857 (4.03) | 6938 (0.03) | 1270 (1.14) | 271.7 (0.40) |
| R12W, H48Y, F54S, S118F | 769 | 10940 (0.05) | 3474 (2.39) | 5161 (0.02) | 1069 (0.96) | |
| R12W, Q79R, S118F | 770 | 2339 (0.01) | 7370 (5.08) | 1880 (0.01) | 1338 (1.20) | 447.4 (0.66) |
| T113S, S118Y | 771 | 6212 (0.03) | 6823 (4.70) | 1554 (0.01) | 1214 (1.09) | 225.1 (0.33) |
| S118Y | 772 | 2921 (0.01) | 6535 (4.50) | 2003 (0.01) | 1463 (1.32) | 190.4 (0.28) |
| N106I, S118Y | 773 | 2750 (0.01) | 7729 (5.32) | 1815 (0.01) | 1222 (1.10) | 265.8 (0.39) |
| N106I, S118F | 774 | 1841 (0.01) | 9944 (6.85) | 1529 (0.01) | 1308 (1.18) | 437.9 (0.65) |
| A95T, L96P, S118Y | 775 | 2352 (0.01) | 4493 (3.09) | 1412 (0.01) | 1329 (1.19) | 292.4 (0.43) |
| Y33H, P67S, N106Y, A112V | 776 | 225015 (1.07) | 3259 (2.24) | 204434 (0.77) | 1296 (1.17) | 618.8 (0.91) |
| N106Y, A112V | 777 | 6036 (0.03) | 1974 (1.36) | 15334 (0.06) | 1108 (1.00) | 409.9 (0.61) |
| T18S, Y33H, A112V | 778 | 252647 (1.20) | 1347 (0.93) | 183181 (0.69) | 1412 (1.27) | 601.8 (0.89) |
| P9S, Y33H, N47S, A112V | 779 | 240467 (1.14) | 1418 (0.98) | 203608 (0.77) | 1361 (1.22) | 449.1 (0.66) |

**TABLE 15A: Variant CD112 selected against cognate binding partners. Molecule sequences, binding data, and costimulatory bioactivity data.**

| CD112 mutation(s) | SEQ ID NO (IgV) | TIGIT tfxn MFI (TIGIT MFI parental ratio) | CD112R tfxn MFI (CD112R MFI parental ratio) | CD226 MFI (CD226 MFI parental ratio) | Mock Expi293 MFI (Mock MFI parental ratio) | Anti-CD3 IFN-gamma (pg/mL) (Anti-CD3 IFN-gamma parental ratio) |
|---|---|---|---|---|---|---|
| P42S, P67H, A112V | 780 | 204484 (0.97) | 1610 (1.11) | 188647 (0.71) | 1174 (1.06) | 530.6 (0.78) |
| P27L, L32P, P42S, A112V | 781 | 219883 (1.04) | 1963 (1.35) | 84319 (0.32) | 1900 (1.71) | 251.6 (0.37) |
| G98D, A112V | 782 | 4879 (0.02) | 2369 (1.63) | 6100 (0.02) | 1729 (1.55) | 387.0 (0.57) |
| Y33H, S35P, N106Y, A112V | 783 | 250724 (1.19) | 1715 (1.18) | 94373 (0.36) | 1495 (1.34) | 516.2 (0.76) |
| L32P, P42S, T100A, A112V | 784 | 242675 (1.15) | 1742 (1.20) | 202567 (0.76) | 1748 (1.57) | 435.3 (0.64) |
| P27S, P45S, N1061, A112V | 785 | 223557 (1.06) | 1799 (1.24) | 84836 (0.32) | 1574 (1.42) | 277.5 (0.41) |
| Y33H, N47K, A112V | 786 | 251339 (1.19) | 1525 (1.05) | 199601 (0.75) | 1325 (1.19) | 483.2 (0.71) |
| Y33H, N106Y, A112V | 787 | 297169 (1.41) | 1782 (1.23) | 258315 (0.97) | 1440 (1.30) | 485.4 (0.72) |
| K78R, D84G, A112V, F114S | 788 | 236662 (1.12) | 1638 (1.13) | 24850 (0.09) | 1345 (1.21) | 142.5 (0.21) |
| Y33H, N47K, F54L, A112V | 789 | 14483 (0.07) | 1617 (1.11) | 2371 (0.01) | 1353 (1.22) | 352.8 (0.52) |
| Y33H, A112V | 790 | 98954 (0.47) | 1216 (0.84) | 1726 (0.01) | 1298 (1.17) | |
| A95V, A112V | 791 | 168521 (0.80) | 2021 (1.39) | 200789 (0.76) | 1459 (1.31) | 412.9 (0.61) |
| R12W, A112V | 792 | 135635 (0.64) | 1582 (1.09) | 23378 (0.09) | 1412 (1.27) | 165.8 (0.24) |
| A112V | 798 | 213576 (1.01) | 1986 (1.37) | 151900 (0.57) | 1409 (1.27) | 211.4 (0.31) |
| Y33H, A112V | 790 | 250667 (1.19) | 1628 (1.12) | 230578 (0.87) | 1216 (1.09) | 612.7 (0.91) |
| R12W, P27S, A112V | 793 | 3653 (0.02) | 1308 (0.90) | 9105 (0.03) | 1051 (0.94) | |
| Y33H, V51M, A112V | 794 | 218698 (1.04) | 1384 (0.95) | 195450 (0.74) | 1170 (1.05) | 709.4 (1.05) |
| Y33H, A112V, S118T | 795 | 219384 (1.04) | 1566 (1.08) | 192645 (0.73) | 1313 (1.18) | 396.3 (0.59) |
| Y33H, | 796 | 5605 (0.03) | 1582 (1.09) | 5079 (0.02) | 1197 (1.08) | |

**TABLE 15A: Variant CD112 selected against cognate binding partners. Molecule sequences, binding data, and costimulatory bioactivity data.**

| CD112 mutation(s) | SEQ ID NO (IgV) | TIGIT tfxn MFI (TIGIT MFI parental ratio) | CD112R tfxn MFI (CD112R MFI parental ratio) | CD226 MFI (CD226 MFI parental ratio) | Mock Expi293 MFI (Mock MFI parental ratio) | Anti-CD3 IFN-gamma (pg/mL) (Anti-CD3 IFN-gamma parental ratio) |
|---|---|---|---|---|---|---|
| V101A, A112V, P115S | | | | | | |
| H24R, T38N, D43G, A112V | 797 | 227095 (1.08) | 1537 (1.06) | 229311 (0.86) | 1336 (1.20) | 858.6 (1.27) |
| A112V | 798 | 4056 (0.02) | 1356 (0.93) | 10365 (0.04) | 986 (0.89) | |
| P27A, A112V | 799 | 193537 (0.92) | 1531 (1.05) | 230708 (0.87) | 3084 (2.77) | 355.1 (0.52) |
| A112V, S118T | 800 | 233173 (1.11) | 1659 (1.14) | 121817 (0.46) | 845 (0.76) | 533.3 (0.79) |
| R12W, A112V, M122I | 801 | 235935 (1.12) | 1463 (1.01) | 217748 (0.82) | 1350 (1.21) | 528.0 (0.78) |
| Q83K, N106Y, A112V | 802 | 205948 (0.98) | 2042 (1.41) | 234958 (0.89) | 1551 (1.39) | 481.4 (0.71) |
| R12W, P27S, A112V, S118T | 803 | 11985 (0.06) | 2667 (1.84) | 12756 (0.05) | 1257 (1.13) | 334.4 (0.49) |
| P28S, Y33H, A112V | 804 | 4711 (0.02) | 1412 (0.97) | 3968 (0.01) | 955 (0.86) | |
| P27S, Q90R, A112V | 805 | 3295 (0.02) | 1338 (0.92) | 6755 (0.03) | 1048 (0.94) | |
| L15V, P27A, A112V, S118T | 806 | 209888 (1.00) | 1489 (1.03) | 84224 (0.32) | 1251 (1.13) | 512.3 0.76) |
| Y33H, N106Y, T108I, A112V | 807 | Not tested | | | | |
| Y33H, P56L, V75M, V101M, A112V | 808 | Not tested | | | | |

**TABLE 15B: Additional CD112 Variants and Binding Data.**

| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV |
| S118F | 768 | 1763 | 0.02 | 1645 | 0.08 | 2974 | 0.61 | 1659 | 0.19 |
| N47K, | 891 | | | | | | | | |

| TABLE 15B: Additional CD112 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV |
| Q79R, S118F | | 1738 | 0.02 | 1689 | 0.09 | 2637 | 0.54 | 1647 | 0.19 |
| Q40R, P60T, A112V, S118T | 892 | 4980 | 0.06 | 1608 | 0.08 | 2399 | 0.50 | 2724 | 0.32 |
| F114Y, S118F | 893 | 110506 | 1.34 | 7325 | 0.37 | 1502 | 0.31 | 1553 | 0.18 |
| N106I, S118Y | 773 | 1981 | 0.02 | 1700 | 0.09 | 2394 | 0.49 | 1582 | 0.19 |
| S118Y | 772 | 101296 | 1.23 | 9990 | 0.50 | 1429 | 0.30 | 1551 | 0.18 |
| Y33H, K78R, S118Y | 894 | 2276 | 0.03 | 2115 | 0.11 | 3429 | 0.71 | 2082 | 0.24 |
| N106I, S118F | 774 | 1875 | 0.02 | 1675 | 0.08 | 2365 | 0.49 | 1662 | 0.19 |
| R12W, A46T, K66M, Q79R, N106I, T113A, S118F | 895 | 3357 | 0.04 | 1808 | 0.09 | 1664 | 0.34 | 4057 | 0.48 |
| Y33H, A112V, S118F | 896 | 3376 | 0.04 | 2886 | 0.15 | 3574 | 0.74 | 3685 | 0.43 |
| R12W, Y33H, N106I, S118F | 897 | 100624 | 1.22 | 24513 | 1.24 | 1490 | 0.31 | 2060 | 0.24 |
| L15V, Q90R, S118F | 898 | 5791 | 0.07 | 4169 | 0.21 | 2752 | 0.57 | 4458 | 0.52 |
| N47K, D84G, N106I, S118Y | 899 | 3334 | 0.04 | 2819 | 0.14 | 2528 | 0.52 | 3498 | 0.41 |
| L32P, S118F | 900 | 3881 | 0.05 | 2506 | 0.13 | 2659 | 0.55 | 2518 | 0.29 |
| Y33H, Q79R, A112V, S118Y | 901 | Low to no protein produced | | | | | | | |
| T18A, | 902 | | | | | | | | |

| TABLE 15B: Additional CD112 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV |
| N106I, S118T | | 84035 | 1.02 | 10208 | 0.52 | 1585 | 0.33 | 1590 | 0.19 |
| L15V, Y33H, N106Y, A112V, S118F | 903 | Low to no protein produced | | | | | | | |
| V37M, S118F | 904 | 96986 | 1.18 | 2523 | 0.13 | 1985 | 0.41 | 1849 | 0.22 |
| N47K, A112V, S118Y | 905 | 1980 | 0.02 | 1859 | 0.09 | 2733 | 0.56 | 1825 | 0.21 |
| A46T, A112V | 906 | 4224 | 0.05 | 4685 | 0.24 | 3288 | 0.68 | 4273 | 0.50 |
| P28S, Y33H, N106I, S118Y | 907 | 6094 | 0.07 | 2181 | 0.11 | 1891 | 0.39 | 3021 | 0.35 |
| P30S, Y33H, N47K, V75M, Q79R, N106I, S118Y | 908 | 2247 | 0.03 | 2044 | 0.10 | 1796 | 0.37 | 2658 | 0.31 |
| V19A, N47K, N106Y, K116E, S118Y | 909 | 2504 | 0.03 | 2395 | 0.12 | 2174 | 0.45 | 2852 | 0.33 |
| Q79R, T85A, A112V, S118Y | 910 | 2192 | 0.03 | 1741 | 0.09 | 2367 | 0.49 | 1620 | 0.19 |
| Y33H, A112V | 790 | 20646 | 0.25 | 1465 | 0.07 | 1794 | 0.37 | 2589 | 0.30 |
| V101M, N106I, S118Y | 911 | 55274 | 0.67 | 6625 | 0.33 | 1357 | 0.28 | 1494 | 0.17 |
| Y33H, Q79R, N106I, A112V, S118T | 912 | 6095 | 0.07 | 1760 | 0.09 | 2393 | 0.49 | 3033 | 0.36 |

| TABLE 15B: Additional CD112 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV |
| Q79R, A112V | 913 | 1571 | 0.02 | 1490 | 0.08 | 2284 | 0.47 | 1326 | 0.16 |
| Y33H, A46T, Q79R, N106I, S118F | 914 | 90813 | 1.10 | 15626 | 0.79 | 1298 | 0.27 | 3571 | 0.42 |
| A112V, G121S | 915 | 95674 | 1.16 | 19992 | 1.01 | 1252 | 0.26 | 4005 | 0.47 |
| Y33H, Q79R, N106I, S118Y | 916 | 36246 | 0.44 | 2118 | 0.11 | 1970 | 0.41 | 3250 | 0.38 |
| Y33H, N106I, A112V | 917 | 47352 | 0.57 | 4217 | 0.21 | 2641 | 0.55 | 1488 | 0.17 |
| Y33H, A46T, V101M, A112V, S118T | 918 | 14413 | 0.17 | 1596 | 0.08 | 2335 | 0.48 | 1441 | 0.17 |
| L32P, L99M, N106I, S118F | 919 | 3056 | 0.04 | 1791 | 0.09 | 2210 | 0.46 | 2000 | 0.23 |
| L32P, T108A, S118F | 920 | 104685 | 1.27 | 4531 | 0.23 | 2308 | 0.48 | 1518 | 0.18 |
| A112V | 798 | 4937 | 0.06 | 1903 | 0.10 | 1646 | 0.34 | 3011 | 0.35 |
| R12W, Q79R, A112V | 921 | 55539 | 0.67 | 6918 | 0.35 | 1386 | 0.29 | 1740 | 0.20 |
| Y33H, N106Y, E110G, A112V | 922 | 2786 | 0.03 | 2517 | 0.13 | 1787 | 0.37 | 2023 | 0.24 |
| Y33H, N106I, S118Y | 923 | 1967 | 0.02 | 1579 | 0.08 | 2601 | 0.54 | 1517 | 0.18 |
| Q79R, S118F | 924 | 82055 | 1.00 | 7582 | 0.38 | 1298 | 0.27 | 1970 | 0.23 |
| Y33H, Q79R, G98D, V101M, | 925 | 21940 | 0.27 | 1632 | 0.08 | 1141 | 0.24 | 18423 | 2.16 |

**TABLE 15B: Additional CD112 Variants and Binding Data.**

| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV | MFI at 100nM | Fold Increase to WT IgV |
| A112V | | | | | | | | | |
| N47K, T81S, V101M, A112V, S118F | 926 | 6889 | 0.08 | 1311 | 0.07 | 1303 | 0.27 | 1145 | 0.13 |
| G82S, S118Y | 927 | 4267 | 0.05 | 1938 | 0.10 | 2140 | 0.44 | 2812 | 0.33 |
| Y33H, A112V, S118Y | 928 | 14450 | 0.18 | 1532 | 0.08 | 2353 | 0.49 | 3004 | 0.35 |
| Y33H, N47K, Q79R, N106Y, A112V | 929 | 70440 | 0.85 | 3557 | 0.18 | 1447 | 0.30 | 1679 | 0.20 |
| Y33H, S118T | 930 | 113896 | 1.38 | 17724 | 0.89 | 1252 | 0.26 | 5001 | 0.59 |
| R12W, Y33H, Q79R, V101M, A112V | 931 | 3376 | 0.04 | 2727 | 0.14 | 2047 | 0.42 | 2339 | 0.27 |
| S118F | 768 | 2685 | 0.03 | 1864 | 0.09 | 2520 | 0.52 | 1566 | 0.18 |
| Wildtype CD112-IgV Fc | 761 (IgV) | 82414 | 1.00 | 19803 | 1.00 | 4842 | 1.00 | 8541 | 1.00 |
| CD112 ECD-Fc | 48 (ECD) | 29157 | 0.35 | 8755 | 0.44 | 1107 | 0.23 | 1103 | 0.13 |
| Anti-hFc PE | - | 1383 | 0.02 | 1461 | 0.07 | 1358 | 0.28 | 1468 | 0.17 |

**TABLE 15C: Additional CD112 Variants and Binding Data.**

| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MFI 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV |
| N106I, S118Y | 773 | 1288 | 0.04 | 1334 | 0.12 | 6920 | 4.16 | 1102 | 0.44 |
| Y33H, Q83K, A112V, S118T | 1481 | 115690 | 3.31 | 10046 | 0.93 | 1128 | 0.68 | 2053 | 0.82 |
| R12W, | 779 | | | | | | | | |

| TABLE 15C: Additional CD112 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV |
| Q79R, S118F | | 1436 | 0.04 | 1296 | 0.12 | 6546 | 3.93 | 1046 | 0.42 |
| V29M, Y33H, N106I, S118F | 1482 | Not tested | | | | | | | |
| Y33H, A46T, A112V | 1483 | 111256 | 3.18 | 14974 | 1.39 | 1148 | 0.69 | 3333 | 1.34 |
| Y33H, Q79R, S118F | 1484 | 1483 | 0.04 | 1326 | 0.12 | 7425 | 4.46 | 1138 | 0.46 |
| Y33H, N47K, F74L, S118F | 1485 | 1338 | 0.04 | 1159 | 0.11 | 1516 | 0.91 | 1140 | 0.46 |
| R12W, V101M, N106I, S118Y | 1486 | 1378 | 0.04 | 1249 | 0.12 | 5980 | 3.59 | 1182 | 0.47 |
| A46T, V101A, N106I, S118Y | 1487 | 1359 | 0.04 | 1199 | 0.11 | 6729 | 4.04 | 1173 | 0.47 |
| Y33H, N106Y, A112V | 787 | 113580 | 3.25 | 17771 | 1.65 | 1207 | 0.72 | 2476 | 0.99 |
| N106Y, A112V, S118T | 1488 | Not tested | | | | | | | |
| S76P, T81I, V101M, N106Y, A112V, S118F | 1489 | Not tested | | | | | | | |
| N106Y, A112V | 777 | 29015 | 0.83 | 2760 | 0.26 | 1159 | 0.70 | 1639 | 0.66 |
| P9R, L21V, P22L, I34M, S69F, F74L, A87V, A112V, L125A | 1490 | 1920 | 0.05 | 1218 | 0.11 | 1107 | 0.66 | 1074 | 0.43 |
| Y33H, | 1491 | | | | | | | | |

| TABLE 15C: Additional CD112 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV |
| V101M, A112V | | 126266 | 3.61 | 24408 | 2.27 | 1150 | 0.69 | 4535 | 1.82 |
| N106I, S118F | 774 | 1776 | 0.05 | 1385 | 0.13 | 9058 | 5.44 | 1370 | 0.55 |
| V29A, L32P, S118F | 1492 | 1265 | 0.04 | 1148 | 0.11 | 5057 | 3.04 | 1194 | 0.48 |
| A112V | 798 | 69673 | 1.99 | 6387 | 0.59 | 1140 | 0.68 | 1214 | 0.49 |
| Y33H, V101M, A112V | 1491 | 133815 | 3.83 | 24992 | 2.32 | 1184 | 0.71 | 6338 | 2.54 |
| P28S, Y33H, N106I, S118Y | 907 | 2745 | 0.08 | 1689 | 0.16 | 6625 | 3.98 | 1978 | 0.79 |
| Y33H, V101M, N1061, A112V | 1493 | 118654 | 3.40 | 21828 | 2.03 | 1253 | 0.75 | 3871 | 1.55 |
| R12W, Y33H, N47K, Q79R, S118Y | 1494 | 171390 | 4.91 | 5077 | 0.47 | 1124 | 0.68 | 2636 | 1.06 |
| A112V, S118T | 800 | 103203 | 2.95 | 15076 | 1.40 | 1155 | 0.69 | 1426 | 0.57 |
| Y33H, A46T, A112V, S118T | 1495 | 141859 | 4.06 | 29436 | 2.74 | 1184 | 0.71 | 5760 | 2.31 |
| Y33H, A112V, F114L, S118T | 1496 | 5161 | 0.15 | 1734 | 0.16 | 1184 | 0.71 | 1249 | 0.50 |
| A112V | 798 | 78902 | 2.26 | 6224 | 0.58 | 1114 | 0.67 | 1181 | 0.47 |
| Y33H, T38A, A46T, V101M, A112V | 1497 | 111293 | 3.19 | 25702 | 2.39 | 1192 | 0.72 | 99015 | 39.69 |
| Q79R, A112V | 913 | 96674 | 2.77 | 7264 | 0.67 | 1130 | 0.68 | 1216 | 0.49 |
| Y33H, N106I, | 923 | 5720 | 0.16 | 1453 | 0.14 | 6543 | 3.93 | 1248 | 0.50 |

| TABLE 15C: Additional CD112 Variants and Binding Data. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CD112 Mutation(s) | SEQ ID NO (IgV) | TIGIT | | CD226 | | CD112R | | CD96 | |
| | | MFI 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV | MFI at 20nM | Fold Increase to WT IgV |
| S118Y | | | | | | | | | |
| P28S, Y33H, S69P, N106I, A112V, S118Y | 1498 | 22393 | 0.64 | 1378 | 0.13 | 1550 | 0.93 | 19174 | 7.68 |
| Y33H, P42L, N47K, V101M, A112V | 1499 | 214116 | 6.13 | 13878 | 1.29 | 1315 | 0.79 | 4753 | 1.91 |
| Y33H, N47K, F74S, Q83K, N106I, F111L, A112V, S118T | 1500 | 6719 | 0.19 | 1319 | 0.12 | 1305 | 0.78 | 1278 | 0.51 |
| Y33H, A112V, S118T, V119A | 1501 | 184794 | 5.29 | 10204 | 0.95 | 1269 | 0.76 | 4321 | 1.73 |
| Y33H, N106I, A112V, S118F | 1502 | 6872 | 0.20 | 1591 | 0.15 | 2308 | 1.39 | 2796 | 1.12 |
| Y33H, K66M, S118F, W124L | 1503 | 1724 | 0.05 | 1259 | 0.12 | 6782 | 4.07 | 1197 | 0.48 |
| S118F | 768 | 1325 | 0.04 | 1213 | 0.11 | 7029 | 4.22 | 1135 | 0.46 |
| N106I, A112V | 1504 | 111342 | 3.19 | 4241 | 0.39 | 1546 | 0.93 | 1178 | 0.47 |
| Y33H, A112V | 790 | 177926 | 5.09 | 13761 | 1.28 | 1152 | 0.69 | 3117 | 1.25 |
| WT CD112 IgV | 1367 | 34932 | 1.00 | 10762 | 1.00 | 1665 | 1.00 | 2495 | 1.00 |
| WT CD112-Fc ECD | 48 (ECD) | 28277 | 0.81 | 8023 | 0.75 | 1253 | 0.75 | 1064 | 0.43 |
| Anti-huFc PE | - | 1138 | 0.03 | 1006 | 0.09 | 1010 | 0.61 | 1062 | 0.43 |

| TABLE 16A: Variant PD-L2 selected against PD-1. Molecule sequence and binding data. | | | | |
|---|---|---|---|---|
| PD-L2 mutation(s) | SEQ ID NO (IgV) | Binding to Jurkat/PD-1 Cells | | Fortebio binding to PD-1-Fc Response Units |
| | | MFI at 50nM | Fold increase over wildtype PD-L2 IgV-Fc | |
| H15Q | 1357 | 15998 | 1.63 | 0.007 |
| N24D | 1358 | 1414 | 0.14 | -0.039 |
| E44D | 1359 | 2928 | 0.3 | -0.006 |
| V89D | 1360 | 3361 | 0.34 | 0.005 |
| Q82R,V89D | 1361 | 44977 | 4.57 | 1.111 |
| E59G,Q82R | 1362 | 12667 | 1.29 | -0.028 |
| S39I,V89D | 1363 | 26130 | 2.65 | 0.26 |
| S67L,V89D | 1364 | 15991 | 1.62 | 0.608 |
| S67L,I85F | 1365 | 529 | 0.05 | -0.005 |
| S67L,I86T | 1366 | 6833 | 0.69 | 0.141 |
| H15Q,K65R | 1367 | 13497 | 1.37 | -0.001 |
| H15Q,Q72H,V89D | 1368 | 12629 | 1.28 | 0.718 |
| H15Q,S67L,R76G | 1369 | 47201 | 4.8 | 0.418 |
| H15Q,R76G,I85F | 1370 | 2941 | 0.3 | -0.038 |
| H15Q,T47A,Q82R | 1371 | 65174 | 6.62 | 0.194 |
| H15Q,Q82R,V89D | 1372 | 49652 | 5.04 | 1.198 |
| H15Q,C23S,I86T | 1373 | 830 | 0.08 | -0.026 |
| H15Q,S39I,I86T | 1374 | 1027 | 0.1 | 0.309 |
| H15Q,R76G,I85F | 1375 | 1894 | 0.19 | -0.006 |
| E44D,V89D,W91R | 1376 | 614 | 0.06 | -0.048 |
| I13V,S67L,V89D | 1377 | 26200 | 2.66 | 1.42 |
| H15Q,S67L,I86T | 1378 | 15952 | 1.62 | 0.988 |
| I13V,H15Q,S67L,I86T | 1379 | 21570 | 2.19 | 1.391 |
| I13V,H15Q,E44D,V89D | 1380 | 23958 | 2.43 | 1.399 |
| I13V,S39I,E44D,Q82R,V89D | 1381 | 71423 | 7.26 | 0.697 |
| I13V,E44D,Q82R,V89D | 1382 | 45191 | 4.59 | 1.283 |
| I13V,Q72H,R76G,I86T | 1383 | 10429 | 1.06 | 0.733 |
| I13V,H15Q,R76G,I85F | 1384 | 4736 | 0.48 | -0.04 |
| H15Q,S67L,R76G,I85F | 1386 | 2869 | 0.29 | 0.025 |
| H15Q,S39I,R76G,V89D | 1386 | Little or no protein produced | | |
| H15Q,T47A,Q72H,R76G,I86T | 1387 | 32103 | 3.26 | 0.512 |
| H15Q,T47A,Q72H,R76G | 1388 | 16500 | 1.68 | 0.327 |
| I13V,H15Q,T47A,Q72H,R76G | 1389 | 73412 | 7.46 | 0.896 |

| TABLE 16A: Variant PD-L2 selected against PD-1. Molecule sequence and binding data. | | | | |
|---|---|---|---|---|
| PD-L2 mutation(s) | SEQ ID NO (IgV) | Binding to Jurkat/PD-1 Cells | | Fortebio binding to PD-1-Fc Response Units |
| | | MFI at 50nM | Fold increase over wildtype PD-L2 IgV-Fc | |
| H15Q,E44D,R76G,I85F | 1390 | 2885 | 0.29 | -0.013 |
| H15Q,S39I,S67L,V89D | 1391 | 45502 | 4.62 | 1.174 |
| H15Q,N32D,S67L,V89D | 1392 | 25880 | 2.63 | 1.407 |
| N32D,S67L,V89D | 1393 | 31753 | 3.23 | 1.155 |
| H15Q,S67L,Q72H,R76G,V89D | 1394 | 40180 | 4.08 | 1.464 |
| H15Q,Q72H,Q74R,R76G,I86T | 1395 | 4049 | 0.41 | 0.093 |
| G28V,Q72H,R76G,I86T | 1396 | 5563 | 0.57 | 0.003 |
| I13V,H15Q,S39I,E44D,S67L | 1397 | 63508 | 6.45 | 0.889 |
| E44D,S67L,Q72H,Q82R,V89D | 1398 | 51467 | 5.23 | 1.061 |
| H15Q,V89D | 1399 | 17672 | 1.8 | 0.31 |
| H15Q,T47A | 1400 | 26578 | 2.7 | 0.016 |
| I13V,H15Q,Q82R | 1401 | 76146 | 7.74 | 0.655 |
| I13V,H15Q,V89D | 1402 | 28745 | 2.92 | 1.331 |
| I13V,S67L,Q82R,V89D | 1403 | 58992 | 5.99 | 1.391 |
| I13V,H15Q,Q82R,V89D | 1404 | 49523 | 5.03 | 1.419 |
| H15Q,V31M,S67L,Q82R,V89D | 1405 | 67401 | 6.85 | 1.37 |
| I13V,H15Q,T47A,Q82R | 1406 | 89126 | 9.05 | 0.652 |
| I13V,H15Q,V31A,N45S,Q82R,V89D | 1407 | 68016 | 6.91 | 1.327 |
| H15Q,T47A,H69L,Q82R,V89D | 1409 | 65598 | 6.66 | 1.44 |
| I13V,H15Q,T47A,H69L,R76G,V89D | 1410 | 54340 | 5.52 | 1.719 |
| I12V,I13V,H15Q,T47A,Q82R,V89D | 1411 | 61207 | 6.22 | 1.453 |
| I13V,H15Q,R76G,D77N,Q82R,V89D | 1412 | 33079 | 3.36 | 0.065 |
| I13V,H15Q,T47A,R76G,V89D | 1413 | 53668 | 5.45 | 1.596 |
| I13V,H15Q,T47A,Q82R,V89D | 1414 | 63320 | 6.43 | 1.418 |
| I13V,H15Q,T47A,Q82R,V89D | 1414 | 60980 | 6.2 | 1.448 |
| I13V,H15Q,I36V,T47A,S67L,V89D | 1416 | 52835 | 5.37 | 1.627 |
| H15Q,T47A,K65R,S67L,Q82R,V89D | 1417 | 79692 | 8.1 | 1.453 |
| H15Q,L33P,T47A,S67L,P71S,V89D | 1418 | 45726 | 4.65 | 1.467 |
| I13V,H15Q,Q72H,R76G,I86T | 1419 | 24450 | 2.48 | 1.355 |
| H15Q,T47A,S67L,Q82R,V89D | 1420 | 67962 | 6.9 | 1.479 |
| F2L,H15Q,D46E,T47A,Q72H,R76G,Q82R,V89D | 1421 | 23039 | 2.34 | 1.045 |
| I13V,H15Q,L33F,T47A,Q82R,V89D | 1422 | 62254 | 6.32 | 1.379 |
| H15Q,N24S,T47A,Q72H,R76G,V89D | 1424 | 32077 | 3.26 | 0.4 |

**TABLE 16A: Variant PD-L2 selected against PD-1. Molecule sequence and binding data.**

| PD-L2 mutation(s) | SEQ ID NO (IgV) | Binding to Jurkat/PD-1 Cells | | Fortebio binding to PD-1-Fc Response Units |
|---|---|---|---|---|
| | | MFI at 50nM | Fold increase over wildtype PD-L2 IgV-Fc | |
| I13V,H15Q,E44V,T47A,Q82R,V89D | 1425 | 61005 | 6.2 | 1.329 |
| H15Q,N18D,T47A,Q72H,V73A,R76G,I86T,V89D | 1426 | 48317 | 4.91 | 0.475 |
| I13V,H15Q,T37A,E44D,S48C,S67L,Q82R,V89D | 1427 | 47605 | 4.84 | 1.255 |
| H15Q,L33H,S67L,R76G,Q82R,V89D | 1428 | 62326 | 6.33 | 1.507 |
| I13V,H15Q,T47A,Q72H,R76G,I86T | 1429 | 49016 | 4.98 | 1.477 |
| H15Q,S39I,E44D,Q72H,V75G,R76G,Q82R,V89D | 1430 | 43713 | 4.44 | 0.646 |
| H15Q,T47A,S67L,R76G,Q82R,V89D | 1431 | 71897 | 7.3 | 1.539 |
| I13V,H15Q,T47A,S67L,Q72H,R76G,Q82R,V89D | 1432 | 71755 | 7.29 | 1.536 |
| Wild Type PD-L2 IgV | 1263 | 9843 | 1 | -0.024 |
| Full length ECD of PD-L2 | 31 | 2145 | 0.22 | 0.071 |
| Full length ECD of PD-L1 (R&D Systems) | 30 | 23769 | 2.41 | 1.263 |
| Anti-PD-1 monoclonal antibody (nivolumab) | - | 87002 | 8.84 | 0.899 |

**TABLE 16B: Bioactivity Data of PD-L2 variants selected against PD-1 in MLR.**

| PD-L2 mutation(s) | SEQ ID NO (IgV) | IFN gamma levels pg/mL | Fold increase over wildtype PD-L2 IgV-Fc |
|---|---|---|---|
| H15Q | 1357 | 1817.1 | 1.32 |
| N24D | 1358 | 1976.3 | 1.44 |
| E44D | 1359 | 1499.4 | 1.09 |
| V89D | 1360 | 1168.1 | 0.85 |
| Q82R,V89D | 1361 | 1617 | 1.17 |
| E59G,Q82R | 1362 | 1511.3 | 1.1 |
| S39I,V89D | 1363 | 1314.5 | 0.95 |
| S67L,V89D | 1364 | 1230.1 | 0.89 |
| S67L,I85F | 1365 | 1281.9 | 0.93 |
| S67L,I86T | 1366 | 1020.4 | 0.74 |
| H15Q,K65R | 1367 | 1510.8 | 1.1 |
| H15Q,Q72H,V89D | 1368 | 1272.2 | 0.92 |
| H15Q,S67L,R76G | 1369 | 1426.2 | 1.04 |
| H15Q,R76G,I85F | 1370 | 1725.7 | 1.25 |
| H15Q,T47A,Q82R | 1371 | 1317.9 | 0.96 |
| H15Q,Q82R,V89D | 1372 | 1081.2 | 0.79 |
| H15Q,C23S,I86T | 1373 | 1847.2 | 1.34 |
| H15Q,S39I,I86T | 1374 | 1415.2 | 1.03 |

| TABLE 16B: Bioactivity Data of PD-L2 variants selected against PD-1 in MLR. | | | |
|---|---|---|---|
| PD-L2 mutation(s) | SEQ ID NO (IgV) | IFN gamma levels pg/mL | Fold increase over wildtype PD-L2 IgV-Fc |
| H15Q,R76G,I85F | 1375 | 1437.8 | 1.04 |
| E44D,V89D,W91R | 1376 | 1560.1 | 1.13 |
| I13V,S67L,V89D | 1377 | 867.5 | 0.63 |
| H15Q,S67L,I86T | 1378 | 1034.2 | 0.75 |
| I13V,H15Q,S67L,I86T | 1379 | 1014.4 | 0.74 |
| I13V,H15Q,E44D,V89D | 1380 | 1384.2 | 1.01 |
| I13V,S39I,E44D,Q82R,V89D | 1381 | 935.6 | 0.68 |
| I13V,E44D,Q82R,V89D | 1382 | 1009.5 | 0.73 |
| I13V,Q72H,R76G,I86T | 1383 | 1953 | 1.42 |
| I13V,H15Q,R76G,I85F | 1384 | 1528.5 | 1.11 |
| H15Q,S67L,R76G,I85F | 1386 | 1318.7 | 0.96 |
| H15Q,T47A,Q72H,R76G,I86T | 1387 | 1599.6 | 1.16 |
| H15Q,T47A,Q72H,R76G | 1388 | 1462.5 | 1.06 |
| I13V,H15Q,T47A,Q72H,R76G | 1389 | 1469.8 | 1.07 |
| H15Q,E44D,R76G,I85F | 1390 | 1391.6 | 1.01 |
| H15Q,S39I,S67L,V89D | 1391 | 1227 | 0.89 |
| H15Q,N32D,S67L,V89D | 1392 | 1285.7 | 0.93 |
| N32D,S67L,V89D | 1393 | 1194 | 0.87 |
| H15Q,S67L,Q72H,R76G,V89D | 1394 | 1061.2 | 0.77 |
| H15Q,Q72H,Q74R,R76G,I86T | 1395 | 933.8 | 0.68 |
| G28V,Q72H,R76G,I86T | 1396 | 1781.6 | 1.29 |
| I13V,H15Q,S39I,E44D,S67L | 1397 | 1256.9 | 0.91 |
| E44D,S67L,Q72H,Q82R,V89D | 1398 | 1281.4 | 0.93 |
| H15Q,V89D | 1399 | 1495.4 | 1.09 |
| H15Q,T47A | 1400 | 1637.2 | 1.19 |
| I13V,H15Q,Q82R | 1401 | 1432.9 | 1.04 |
| I13V,H15Q,V89D | 1402 | 1123 | 0.82 |
| I13V,S67L,Q82R,V89D | 1403 | 1372.8 | 1 |
| I13V,H15Q,Q82R,V89D | 1404 | 1596.6 | 1.16 |
| H15Q,V31M,S67L,Q82R,V89D | 1405 | 1206.5 | 0.88 |
| I13V,H15Q,T47A,Q82R | 1406 | 1703.3 | 1.24 |
| I13V,H15Q,V31A,N45S,Q82R,V89D | 1407 | 1723.1 | 1.25 |
| H15Q,T47A,H69L,Q82R,V89D | 1409 | 1732.5 | 1.26 |
| I13V,H15Q,T47A,H69L,R76G,V89D | 1410 | 1075.5 | 0.78 |
| I12V,I13V,H15Q,T47A,Q82R,V89D | 1411 | 1533.2 | 1.11 |
| I13V,H15Q,R76G,D77N,Q82R,V89D | 1412 | 1187.9 | 0.86 |

| TABLE 16B: Bioactivity Data of PD-L2 variants selected against PD-1 in MLR. | | | |
|---|---|---|---|
| PD-L2 mutation(s) | SEQ ID NO (IgV) | IFN gamma levels pg/mL | Fold increase over wildtype PD-L2 IgV-Fc |
| I13V,H15Q,T47A,R76G,V89D | 1413 | 1253.7 | 0.91 |
| I13V,H15Q,T47A,Q82R,V89D | 1414 | 1445.5 | 1.05 |
| I13V,H15Q,T47A,Q82R,V89D | 1414 | 1737 | 1.26 |
| I13V,H15Q,I36V,T47A,S67L,V89D | 1416 | 1357.4 | 0.99 |
| H15Q,T47A,K65R,S67L,Q82R,V89D | 1417 | 1335.3 | 0.97 |
| H15Q,L33P,T47A,S67L,P71S,V89D | 1418 | 1289.1 | 0.94 |
| I13V,H15Q,Q72H,R76G,I86T | 1419 | 1221 | 0.89 |
| H15Q,T47A,S67L,Q82R,V89D | 1420 | 1197.1 | 0.87 |
| F2L,H15Q,D46E,T47A,Q72H,R76G,Q82R,V89D | 1421 | 1170.7 | 0.85 |
| I13V,H15Q,L33F,T47A,Q82R,V89D | 1422 | 1468.4 | 1.07 |
| I13V,H15Q,T47A,E58G,S67L,Q82R,V89D | 1423 | 836.1 | 0.61 |
| H15Q,N24S,T47A,Q72H,R76G,V89D | 1424 | 1091.8 | 0.79 |
| I13V,H15Q,E44V,T47A,Q82R,V89D | 1425 | 1270.5 | 0.92 |
| H15Q,N18D,T47A,Q72H,V73A,R76G,I86T,V89D | 1426 | 1065.8 | 0.77 |
| I13V,H15Q,T37A,E44D,S48C,S67L,Q82R,V89D | 1427 | 1751.7 | 1.27 |
| H15Q,L33H,S67L,R76G,Q82R,V89D | 1428 | 1502 | 1.09 |
| I13V,H15Q,T47A,Q72H,R76G,I86T | 1429 | 1088.1 | 0.79 |
| H15Q,S39I,E44D,Q72H,V75G,R76G,Q82R,V89D | 1430 | 940.9 | 0.68 |
| H15Q,T47A,S67L,R76G,Q82R,V89D | 1431 | 1097.8 | 0.8 |
| I13V,H15Q,T47A,S67L,Q72H,R76G,Q82R,V89D | 1432 | 1559.6 | 1.13 |
| Wild Type PD-L2 IgV | 1263 | 1376.8 | 1 |
| Full length ECD of PD-L2 | 31 | 1173.2 | 0.85 |
| Full length ECD of PD-L1 | 30 | 2190.9 | 1.59 |
| Nivolumab (anti-PD-1) | - | 418.9 | 0.3 |

**EXAMPLE 9**

**Assessment of Bioactivity of Affinity-Matured IgSF Domain-Containing Molecules Using Mixed Lymphocyte Reaction (MLR)**

**[0469]** This Example describes Fc-fusion variant protein bioactivity characterization in human primary T cell in vitro assays.

**[0470]** Soluble variant PD-L1 IgV-Fc bioactivity was tested in a human Mixed Lymphocyte Reaction (MLR). Human primary dendritic cells (DC) were generated by culturing monocytes isolated from PBMC (BenTech Bio, USA) in vitro for 7 days with 50 ng/mL rIL-4 (R&D Systems, USA) and 80 ng/mL rGM-CSF (R&D

Systems, USA) in Ex-Vivo 15 media (Lonza, Switzerland). To fully induce DC maturation, lipopolysaccharide (LPS) (InvivoGen Corp., USA) was added to the DC cultures on day 6 and cells were incubated for an additional 24 hours. Approximately, 10,000 matured DC and 100,000 purified allogeneic CD3+ T cells (BenTech Bio, USA) were co-cultured with several concentrations of variant PD-L1 IgV-Fc fusion proteins in 96 well round-bottom plates in 200 µl final volume of Ex-Vivo 15 media. Irrelevant human IgG or media only (designated "No Add") were used as negative controls. As positive controls, PDL1-Fc (full PD-L1 extracellular domain), wildtype PD-L1 IgV-Fc and or positive control anti-PD-1 monoclonal antibody (nivolumab) was assessed. Variant PD-L1 IgV-Fc fusion proteins were tested at various concentrations as indicated in FIG. 8 and 9. On day 5, IFN-gamma secretion in culture supernatants was analyzed using the Human IFN-gamma Duoset ELISA kit (R&D Systems, USA). Optical density was measured on a BioTek Cytation Multimode Microplate Reader (BioTek Corp., USA) and quantitated against titrated rIFN-gamma standard included in the IFN-gamma Duo-set kit (R&D Systems, USA).

**[0471]** Results for the bioactivity studies for exemplary tested variant PD-L1 IgV-Fc are shown in FIG. 8 and FIG. 9, which sets forth the calculated levels of IFN-gamma in culture supernatants (pg/mL) at the indicated concentration of variant IgV-Fc fusion molecule. The variants are identified with reference to the amino acid substitutions in the IgV of PD-L1 with reference to positions corresponding to positions of the unmodified (wildtype) PD-L1 ECD sequence set forth in SEQ ID NO:30 or 1728. As shown in FIG. 8, exemplary variant PD-L1 IgV-Fc molecules exhibited improved activities to increase IFNg production in an MLR assay. As shown in FIG. 9, the exemplary variant PD-L1 IgV-Fc fusion molecules tested show comparable activity to nivolumab in an MLR assay.

**EXAMPLE 10**

**Generation of Stacked Molecules Containing Different Affinity-Modified Domains**

**[0472]** This Example describes immunomodulatory proteins that were generated as multi-domain stack constructs containing at least two different affinity modified IgV domains from identified variant PD-L1 polypeptides, identified variant CD112 polypeptides and identified variant CD155 polypeptides described above. Specifically, the exemplary variant PD-L1 IgV D43G/N45D/L56Q/V58A/ G101G-ins (G101GG) (SEQ ID NO: 303), CD112 IgV molecule S118F (SEQ ID NO: 768) and/or the exemplary variant CD155 IgV molecule P18S/S65W/S67A/L104Q/G111R (SEQ ID NO: 1576) were linked together and fused to an Fc in various configurations.

**[0473]** Homodimeric stacks were generated in various configurations as summarized in FIG. 5A and 5B and as follows. In the generated homodimeric stack constructs , the variant CD155 IgV variant, CD112 IgV and/or variant PD-L1 IgV were variously linked to the N- or C-terminus of a human IgG1 Fc region via a 2xGGGS (SEQ ID NO: 240) or 3x GGGGS (SEQ ID NO: 239) peptide linker. In this study, the exemplary IgG1 Fc region is set forth in SEQ ID NO:1155 and contained the mutations L234A, L235E, G237A, E356D and M358L by EU numbering (corresponding to L19A, L20E, G22A, E141D and M143L with reference to wild-type human IgG1 Fc set forth in SEQ ID NO:187). Further, the Fc region contained replacement of the cysteine residues to a serine residue at position 5 (C5S) compared to the wild-type or unmodified Fc set forth in SEQ ID NO: 187 (corresponding to C220S by EU numbering). In some examples, the Fc was further modified to remove the C-terminal lysine at position 232 of the wild-type or unmodified Fc set forth in SEQ ID NO: 197 (corresponding to K447del by EU numbering). The exemplary IgG1 Fc region comprising a lysine deletion is set forth in SEQ ID NO: 1715. Other Fc regions also are suitable for generation of stack molecules. Exemplary generated stacks are set forth below.

**[0474]** Expression constructs encoding Fc fusion proteins of interest were transiently expressed in Expi293 HEK293 cells from Invitrogen using the manufacturer's commercial Expifectamine reagents and media. Supernatants were harvested and protein was captured and eluted from a Protein A column using an AKTA protein purification system. The eluted material was then separated by an additional preparative SEC step to generate monomeric, highly purified material. The purified proteins were formulated in 15mM acetate, 200mM NaCl, 9% sucrose, pH 5.0 (ASU5). The protein was vialed in a sterile biosafety cabinet and frozen at -80 C. A vial was thawed and assessed by analytical SEC to demonstrate the material was stable and predominantly monomeric after thaw.

**[0475]** For each stack, the encoding nucleic acid molecule was designed to produce homodimeric stacks in various configurations with sequences in the order shown:

**A. Stack Constructs Containing PD-L1 and CD155**

**[0476]** The encoding nucleic acid molecule were designed to generate homodimeric stacks in various configurations of sequences in the order shown:

- PD-L1/CD155 Stack 1 (SEQ ID NO: 1716): CD155 variant (SEQ ID NO: 1576) - 2xGGGS (SEQ ID NO: 240) - Fc (SEQ ID NO: 1155) - 3x GGGGS (SEQ ID NO: 239) - PD-L1 (SEQ ID NO: 303)
- PD-L1/CD155 Stack 2 (SEQ ID NO: 1717): PD-L1 (SEQ ID NO: 303) - 2xGGGS (SEQ ID NO: 240) - Fc (SEQ ID NO: 1155) - 3x GGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576)
- PD-L1/CD155 Stack 3 (SEQ ID NO: 1718): CD155 variant (SEQ ID NO: 1576) -3x GGGGS (SEQ ID NO: 239) - PD-L1 (SEQ ID NO: 303) - 2xGGGS (SEQ ID NO: 240) -Fc (SEQ ID NO: 1155)
- PD-L1/CD155 Stack 4 (SEQ ID NO: 1719): PD-L1 (SEQ ID NO: 303) - 3x GGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576) - 2xGGGS (SEQ ID NO: 240) -Fc (SEQ ID NO: 1155)
- PD-L1/CD155 Stack 5 (SEQ ID NO: 1720): N-terminal HMSSVSAQ set forth in SEQ ID NO: 1156 - Fc (SEQ ID NO: 1155) - 3x GGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576) - 3xGGGS (SEQ ID NO: 239) - PD-L1 (SEQ ID NO: 303)
- PD-L1/CD155 Stack 6 (SEQ ID NO: 1721): N-terminal HMSSVSAQ set forth in SEQ ID NO: 1156 - Fc (SEQ ID NO: 1155) - 3x GGGGS (SEQ ID NO: 239) - PD-L1 (SEQ ID NO: 303) - 3xGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576)

**B. Stack Constructs Containing PD-L1, CD112 and CD155**

**[0477]** The encoding nucleic acid molecule were designed to generate homodimeric stacks in various configurations of sequences in the order shown:

- PD-L1/CD112/CD155 Stack 1 (SEQ ID NO: 1722): PD-L1 (SEQ ID NO: 303) - 3xGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576) - 2xGGGS (SEQ ID NO: 240) - Fc with lysine removed (SEQ ID NO: 1715) - 3x GGGGS (SEQ ID NO: 239) - CD112 (SEQ ID NO: 768)
- PD-L1/CD112/CD155 Stack 2 (SEQ ID NO: 1723): PD-L1 (SEQ ID NO: 303) - 3xGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576) - 3xGGGS (SEQ ID NO: 239) - CD112 (SEQ ID NO: 768) - 2xGGGS (SEQ ID NO: 240) - Fc (SEQ ID NO: 1155)
- PD-L1/CD112/CD155 Stack 3 (SEQ ID NO: 1724): PD-L1 (SEQ ID NO: 303) - 3xGGGS (SEQ ID NO: 239) - CD112 (SEQ ID NO: 768) - 3xGGGS (SEQ ID NO: 239) - CD155 variant (SEQ ID NO: 1576) -

2xGGGS (SEQ ID NO: 240) - Fc (SEQ ID NO: 1155)

**EXAMPLE 11**

**Assessment of Binding and Bioactivity of PD-L1/CD155 Stacked Affinity-Matured IgSF Domain-Containing Molecules**

**[0478]** This Example describes binding studies to assess specificity and affinity of exemplary PD-L1/CD155 variant IgV stack immunomodulatory proteins (PD-L1/CD155 stacked IgV-Fc), generated in Example 10, for binding to cognate binding partners. In addition, a Jurkat/Il,2/PD1/TIGIT reporter assay was used to assess PD-1 and TIGIT blocking activity of PD-L1/CD155 stacked IgV-Fc molecules. As a control, binding and blocking activity also was assessed of the non-stack variant PD-L1 IgV-Fc or CD155 IgV-Fc fusion molecules containing the same variant PD-L1 IgV (SEQ ID NO:303) or variant CD155 IgV (SEQ ID NO:1576), respectively, used in the stacks. Wild-type CD155-ECD-Fc and wild-type PD-L1-ECD-Fc containing the wildtype CD155 ECD (SEQ ID NO:20) or the wildtype PD-L1 ECD (SEQ ID NO:3), respectively, also were assessed.

**A. Binding to Cell-Expressed Counter Structure**

**[0479]** Binding studies were carried out using Jurkat/IL-2 reporter cells (purchased from Promega Corp. USA) that were transduced to stably express human PD-1 (Jurkat/PD-1 cells), human TIGIT (Jurkat/TIGIT cells) or both PD-1 and TIGIT (Jurkat/PD-1/TIGIT cells). For staining by flow cytometry, 100,000 Jurkat/PD-1, Jurkat/TIGIT, Jurkat/PD-1/TIGIT cells or negative control (Jurkat only) were plated in 96-well round-bottom plates. Cells were spun down and resuspended in staining buffer (PBS (phosphate buffered saline), 1% BSA (bovine serum albumin), and 0.1% sodium azide) for 20 minutes to block non-specific binding. Afterwards, cells were centrifuged again and resuspended in 50 µL staining buffer containing 100 nM to 6 pM of each candidate Fc fusion protein, either variant PD-L1 IgV-Fc or CD155 IgV-Fc fusion molecules or PD-L1/CD155 stacked IgV-Fc fusion molecules described above. Primary staining was performed on ice for 90 minutes, before washing cells twice in 200 µL staining buffer. PE-conjugated anti-human Fc (Jackson ImmunoResearch, USA) was diluted 1: 150 in 50 µL staining buffer and added to cells and incubated another 30 minutes on ice. Secondary antibody was washed out twice, cells were fixed in 4% formaldehyde/PBS, and samples were analyzed on Intellicyt flow cytometer (Intellicyt Corp., USA).

**[0480]** Mean Fluorescence Intensity (MFI) was calculated with FlowJo Version 10 software (FlowJo LLC, USA). Table 17 sets forth the binding activity as measured by the Mean Fluorescence Intensity (MFI) value for binding of 20 nM of each stack Fc-fusion molecule, non-stack variant PD-L1 IgV-Fc or CD155 IgV-Fc controls or wild-type ECD controls, to Jurkat/PD-1, Jurkat/TIGIT, and Jurkat/PD-1/TIGIT cells. As shown in Table 17, several stack proteins bound both PD-1 and TIGIT with high affinity. Studies were carried out substantially as described above to further assess binding of the stack proteins at various doses.

| TABLE 17: Binding of Stacks to Cell-Expressed Counter Structure | | | | |
|---|---|---|---|---|
| | | Flow Binding to Jurkat Cells Stably Expressing: | | |
| SEQ ID NO | Description | PD-1 | TIGIT | PD-1 + TIGIT |
| 1716 | (CD155 IgV) (G4S)2 Fc (G4S)3 (PD-L1 IgV) | 61805 | 80658 | 35128 |
| 1717 | (PD-L1 IgV) (G4S)2 Fc(G4S)3 (CD155 IgV) | 69813 | 36485 | 52538 |
| 1718 | (CD155 IgV) (G4S)3 (PD-L1 IgV) (G4S)2 Fc | 47261 | 81840 | 32188 |
| 1719 | (PD-L1 IgV) (G4S)3 (CD155 IgV) (G4S)2 | 77959 | 60515 | 51615 |
| 303 | non-stack variant PD-L1 IgV-Fc control | 111746 | 630 | 41390 |
| 1576 | non-stack variant CD155 IgV-Fc control | 460 | 79152 | 7910 |
| 20 (ECD) | CD155-ECD-Fc | 511 | 28790 | 1196 |
| 3 (ECD) | PD-L1-ECD-Fc (R&D Systems) | 35005 | 557 | 10358 |
| 1155 | Fc Control (homodimer) | 437 | 483 | 478 |
| | | MFI at 20nM | MFI at 20nM | MFI at 20nM |

**B. Assessment of Bioactivity of Affinity-Matured IgSF Domain-Containing Molecules**

**[0481]** Jurkat effector cells expressing an IL-2-luciferase reporter and cell-surface PD-1 and TIGIT were suspended at $2\times10^6$ cells/mL in Jurkat Assay buffer (RPMI1640 + 5%FBS) and anti-CD28 was added to a final concentration of 3 µg/mL. Jurkat cells were then plated at 50 µL/well for a total of 100,000 cells per well.

**[0482]** To each well, 25µL of PD-L1/CD155 stacked IgV-Fc test protein was added to the Jurkat cells. As a control, non-stack variant PD-L1 IgV-Fc or CD155 IgV-Fc fusion molecules, alone or in combination, also were assessed for comparison. Anti-TIGIT antibody (clone MBSA43), anti-PD-1 antibody (nivolumab) or an empty Fc molecule were also used as controls. All proteins were added at five concentrations: 400 nM, 100 nM, 25 nM, 6.25 nM, and 1.56 nM. The Jurkat cells with test or control proteins were incubated for 15 minutes at room temperature. CHO-derived artificial antigen presenting cells (aAPC) displaying transduced cell surface anti-CD3 single chain Fv (OKT3), PD-L1 and CD155 were brought to $0.8\times10^6$ cells/mL and 25 µL of cells was added to each well bringing the final volume of each well to 100 µL. Each well had a final ratio of 5:1 Jurkat:CHO cells and a test protein concentration of 100, 25, 6.25, 1.56 or 0.47 nM and an anti-CD28 concentration of 1.5 µg/mL. Jurkat cells and CHO cells were incubated for 5 hours at 37 degrees Celsius in a humidified 5% $CO_2$ incubation chamber. Plates are then removed from the incubator and acclimated to room temperature for 15 minutes. 100 µL of a cell lysis and luciferase substrate solution (BioGlo luciferase reagent, Promega) was added to each well and the plates were incubated on an orbital shaker for 10 minutes. Luminescence was measured with a 1 second per well integration time using a BioTek Cytation luminometer.

**[0483]** An average relative luminescence value was determined for each test sample and a fold increase (or decrease) in IL-2 reporter signal was calculated for each stack molecule compared to non-stack variant PD-L1 IgV-Fc and variant CD155 IgV-Fc proteins. Because the assay is a measure of blockade of inhibitory signals, an increase in luminescent signal compared to control indicates the presence of blocking activity.

**[0484]** As shown in Table 18, the luciferase activity of the Jurkat effector cells co-cultured with anti-CD3/PD-L1/CD155 aAPC and 100 nM PD-L1/CD155 stack Fc molecules was altered (increased) for each molecule tested compared to control. The differences in luminescence signals demonstrate the differences in binding of the PD-L1/CD155 stack-Fc molecules to PD-1 and TIGIT and the resulting co-blockade of inhibitory activity. In the Table, Column 1 sets forth the SEQ ID NO identifier for each PD-L1/CD155 stack-Fc variant tested. Studies were carried out substantially as described above to further assess binding of the stack proteins at various doses.

**TABLE 18: Jurkat/IL2/PD1/TIGIT + CHO/OKT3/PD-L1/CD155 Reporter Assay Results**

| SEQ ID NO | Description | RLU | Fold Increase Compared to non-stack variant PD-L1 IgV-Fc control | Fold Increase Compared to non-stack variant CD155 IgV-Fc control | Fold Increase Compared to variant PD-L1 IgV-Fc and variant CD155 IgV-Fc |
|---|---|---|---|---|---|
| 1716 | (CD155 IgV) (G4S)2 Fc (G4S)3 (PD-L1 IgV) | 573 | 0.6 | 1.7 | 0.8 |
| 1717 | (PD-L1 IgV) (G4S)2 Fc(G4S)3 (CD155 IgV) | 962 | 1.0 | 2.8 | 1.4 |
| 1718 | (CD155 IgV) (G4S)3 (PD-L1 IgV) (G4S)2 Fc | 434 | 0.5 | 1.3 | 0.6 |
| 1719 | (PD-L1 IgV) (G4S)3 (CD155 IgV) (G4S)2 Fc | 1923 | 2.0 | 5.6 | 2.7 |
| 303 | non-stack variant PD-L1 IgV-Fc control | 958 | 1.0 | 2.8 | 1.3 |
| 1576 | non-stack variant CD155 IgV-Fc control | 345 | 0.4 | 1.0 | 0.5 |
| - | Anti-TIGIT antibody (clone MBSA43), anti-PD-1 antibody (nivolumab) | 2192 | 2.3 | 6.4 | 3.1 |
| 303+1576 | PD-L1 IgV + CD155 IgV | 710 | 0.7 | 2.1 | 1.0 |
| 1715 | Fc Control (homodimer) | 235 | 0.2 | 0.7 | 0.3 |

**EXAMPLE 12**

**Assessment of Binding and Bioactivity of PD-L1/CD112/CD155 Stacked Affinity-Matured IgSF Domain-Containing Molecules**

**[0485]** This Example describes binding studies to assess specificity and affinity of PD-L1/CD112/CD155 stack immunomodulatory proteins (PD-L1/CD112/CD155 stacked IgV-Fc), generated in Example 10, for binding to cognate binding partners. In addition, a Jurkat/IL2/PD1/CD112R/TIGIT reporter assay was used to assess PD-1, CD112R, and TIGIT blocking activity of PD-L1/CD112/CD155 stacked IgV-Fc molecules. As a comparison, binding and blocking activity also was assessed of the non-stack variant PD-L1 IgV-Fc, CD112 IgV-Fc or CD155 IgV-Fc fusion molecules containing the same variant PD-L1 (SEQ ID NO:303), CD112 IgV (SEQ ID NO:768) or variant CD155 IgV (SEQ ID NO:1576), respectively, used in the stacks.

**A. Binding to Cell-Expressed Counter Structure**

**[0486]** Binding studies were carried out using Jurkat/IL-2 reporter cells which endogenously express CD112R (purchased from Promega Corp. USA) that were transduced to stably express human PD-1 (Jurkat/PD-1 cells), human TIGIT (Jurkat/TIGIT cells) or both PD-1 and TIGIT (Jurkat/PD-1/TIGIT cells). For staining by flow cytometry, 100,000 Jurkat parental (CD112R), Jurkat/PD-1, Jurkat/TIGIT, Jurkat/PD-1/TIGITcells were plated in 96-well round-bottom plates. Cells were spun down and resuspended in staining buffer (PBS (phosphate buffered saline), 1% BSA (bovine serum albumin), and 0.1% sodium azide) for 20 minutes to block non-specific binding. Afterwards, cells were centrifuged again and resuspended in 50 µL staining buffer containing 100 nM to 6 pM of each candidate Fc fusion protein. Primary staining was performed on ice for 90 minutes, before washing cells twice in 200 µL staining buffer. PE-conjugated anti-human Fc (Jackson ImmunoResearch, USA) was diluted 1: 150 in 50 µL staining buffer and added to cells and incubated another 30 minutes on ice. Secondary antibody was washed out twice, cells were fixed in 4% formaldehyde/PBS, and samples were analyzed on an LSRII flow cytometer (Becton Dickinson Corp., USA).

**[0487]** Binding values, expressed as Mean Fluorescence Intensity (MFI), were determined for a 33.3 nM concentration of each stack Fc fusion protein and non-stack variant PD-L1 IgV-Fc, CD112-IgV-Fc and CD155-IgV-Fc proteins. Data was analyzed using FlowJo Version 10 software (FlowJo LLC, USA). Results for the binding studies for exemplary tested PD-L1/CD112/CD155 stack Fc fusion molecules (tested at 33.3 nM) are shown in Table 19. As shown, several stack proteins bound PD-1, TIGIT and/or CD112R with high affinity.

**TABLE 19: Binding of Stacks to Cell-Expressed Counter Structure**

| Category | Description | SEQ ID NO | Binding to Jurkat Transfectants | | | |
|---|---|---|---|---|---|---|
| | | | TIGIT | CD112R | PD1 | TIGIT/ CD112R/ PD1 |
| Stacks | (PD-L1 IgV) (G4S)3 (CD155 IgV) (G4S)3 (CD112 IgV) (G4S)2 Fc | 1723 | 2457 | 969 | 16989 | 19041 |
| | (PD-L1 IgV) (G4S)3 (CD112 IgV) (G4S)3 (CD155 IgV) (G4S)2 Fc | 1724 | 1504 | 289 | 21968 | 18727 |
| | non-stack variant PD-L1 IgV-Fc control | 303 | 101 | 100 | 20713 | 18468 |

| TABLE 19: Binding of Stacks to Cell-Expressed Counter Structure | | | | | | |
|---|---|---|---|---|---|---|
| Category | Description | SEQ ID NO | Binding to Jurkat Transfectants | | | |
| | | | TIGIT | CD112R | PD1 | TIGIT/ CD112R/ PD1 |
| Controls | non-stack variant CD155 IgV-Fc control | 1576 | 6294 | 55 | 31 | 961 |
| | non-stack variant CD112 IgV-Fc control | 768 | 358 | 516 | 459 | 477 |
| | Fc Control | 1155 | 23.3 | 27 | 22 | 35 |
| | | | MFI at 33.3nM | | | |

**B. Assessment of Bioactivity of Affinity-Matured IgSF Domain-Containing Molecules**

**[0488]** Jurkat effector cells expressing an IL-2-luciferase reporter and cell-surface PD-1, CD112R, and TIGIT were suspended at $2\times10^6$ cells/mL in Jurkat Assay buffer (RPMI1640 + 5%FBS) and anti-CD28 was added to a final concentration of 3 µg/mL. Jurkat cells were then plated at 50 µL/well for a total of 100,000 cells per well.

**[0489]** To each well, 25µL of PD-L1/CD112/CD155 stacked IgV-Fc test protein was added to the Jurkat cells. As a control, non-stack variant PD-L1 IgV-Fc, CD112 IgV-Fc or CD155 IgV-Fc fusion molecules, alone or in combination, also were assessed for comparison. Anti-TIGIT antibody (clone MBSA43), anti-PD-1 antibody (nivolumab) or an empty Fc molecule are used as controls. All proteins were added at five concentrations: 400 nM, 100 nM, 25 nM, 6.25 nM, 1.56 nM, or 0.49 nM. The Jurkat cells with test or control proteins were incubated for 15 minutes at room temperature. CHO-derived artificial antigen presenting cells (aAPC) displaying transduced cell surface anti-CD3 single chain Fv (OKT3), PD-L1 and CD112 were brought to $0.8\times10^6$ cells/mL and 25 µL of cells was added to each well bringing the final volume of each well to 100 µL. Each well had a final ratio of 5:1 Jurkat:CHO cells and a test protein concentration of 100, 25, 6.25, 1.56, 0.47 or 0.12 nM and an anti-CD28 concentration of 1.5 µg/mL. Jurkat cells and CHO cells were incubated for 5 hours at 37 degrees Celsius in a humidified 5% $CO_2$ incubation chamber. Plates are then removed from the incubator and acclimated to room temperature for 15 minutes. 100 µL of a cell lysis and luciferase substrate solution (BioGlo luciferase reagent, Promega) was added to each well and the plates were incubated on an orbital shaker for 10 minutes. Luminescence was measured with a 1 second per well integration time using a BioTek Cytation luminometer.

**[0490]** An average relative luminescence value (RLU) was determined for each test sample and a fold increase (or decrease) in IL-2 reporter signal was calculated for each stack molecule compared to non-stack variant PD-L1 IgV-Fc, variant CD112 IgV-Fc and variant CD155 IgV-Fc proteins. Because the assay is a measure of blockade of inhibitory signals, an increase in luminescent signal compared to control indicates the presence of blocking activity.

**[0491]** As shown in Table 20, the luciferase activity of the Jurkat effector cells co-cultured with anti-CD3/PD-L1/CD112 aAPC and the PD-L1/CD112/CD155 stack Fc molecules was altered (increased) for each molecule tested. The differences in luminescence signals demonstrate the differences in binding of the PD-L1/CD112/CD155 stack-Fc molecules to PD-1, CD 112R and TIGIT and the resulting co-blockade of inhibitory activity. In the Table, Column 2 sets forth the SEQ ID NO identifier for each PD-L1/CD112/CD155

stack-Fc variant tested.

**Table 20: Jurkat/IL2/TIGIT/PD1 Reporter Assay**

| Description | SEQ ID NO | RLU | Fold Increase compared to Fc Control | Fold Increase compared to PD-L1 IgV-Fc | Fold Increase compared to CD155 IgV-Fc | Fold Increase compared to CD112-IgV - Fc |
|---|---|---|---|---|---|---|
| (PD-L1 IgV) (G4S)3 (CD155 IgV) (G4S)3 (CD112 IgV) (G4S)2 Fc | 1723 | 896 | 1.44 | 1.12 | 1.04 | 1.40 |
| (PD-L1 IgV) (G4S)3 (CD 112 IgV) (G4S)3 (CD155 IgV) (G4S)2 Fc | 1724 | 961 | 1.54 | 1.20 | 1.12 | 1.50 |
| non-stack variant PD-L1 IgV-Fc control | 303 | 800 | 1.28 | 1.00 | 0.93 | 1.25 |
| non-stack variant CD155 IgV-Fc control | 1576 | 859 | 1.38 | 1.07 | 1.00 | 1.34 |
| non-stack variant CD112 IgV-Fc control | 768 | 640 | 1.03 | 0.80 | 0.75 | 1.00 |
| Fc Control | 1155 | 624 | 1.00 | 0.78 | 0.73 | 0.98 |

**EXAMPLE 13**

**Assessment of Binding of Affinity-Matured IgSF Domain-Containing Molecules to Cell Expressed Counted Structure**

**[0492]** This Example describes flow cytometry binding studies to assess specificity and affinity of PD-L1-extracellular domain (ECD) variant immunomodulatory proteins for cognate binding partners PD-1 and CD80 using the selection scheme as described in Example 3 for second round selection against rPD-1.Fc or rCD80.Fc, except that mutant constructs were generated based on a wildtype human PD-L1 sequence set forth in SEQ ID NO: 1728 containing the full extracellular domain (IgV + IgC) as follows.

FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEEDLK

VQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGADYKRITVKVN

APYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTTTTNSKREE

KLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPPNERT (SEQ ID

NO:1728)

**[0493]** To measure binding to PD-1, studies were carried out using Jurkat/IL-2 reporter cells (purchased

from Promega Corp. USA) that were transduced to stably express human PD-1 (Jurkat/PD-1 cells). To measure binding to CD80, studies were carried out using Chinese Hamster Overy (CHO) cells that were transduced to stably express human CD80 (CHO/CD80). 100,000 cells per well of Jurkat/PD-1, CHO/CD80, Jurkat parental or CHO parental cells were plated into 96-well round-bottom plates. Cells were spun down and resuspended in staining buffer (PBS (phosphate buffered saline), 1% BSA (bovine serum albumin), and 0.1% sodium azide) for 20 minutes to block non-specific binding. Afterwards, cells were centrifuged again and resuspended in 50 µL staining buffer containing 100 nM to 3.7 nM of each candidate Fc fusion protein. Primary staining was performed on ice for 90 minutes, before washing cells twice in 200 µL staining buffer. APC-conjugated anti-human Fc (Jackson ImmunoResearch, USA) was diluted 1:150 in 50 µL staining buffer and added to cells and incubated another 30 minutes on ice. Secondary antibody was washed out twice, cells were fixed in 4% formaldehyde/PBS, and samples were analyzed on LSRII flow cytometer (Becton Dickinson Corp., USA).

**[0494]** Mean Fluorescence Intensity (MFI) was calculated at the 20 nM concentration and compared to wild type PD-L1 ECD-Fc protein with FlowJo Version 10 software (FlowJo LLC, USA). Results for the binding studies for exemplary tested PD-L1-ECD-Fc fusion molecules are shown in Table 21. As demonstrated, the selections resulted in the identification of variant PD-L1 proteins that bind either PD-1 alone, CD80 alone or both proteins with high affinity.

**Table 21: Flow Binding to Cells Expressing PD-1 or CD80**

| PD-L1 Mutation(s) | SEQ ID NO (ECD) | PD-1 | | CD80 | |
|---|---|---|---|---|---|
| | | MFI at 20nM | Fold Change Compared to WT PD-L1 | MFI at 20nM | Fold Change Compared to WT PD-L1 |
| K57R, S99G | 1729 | 2953 | 0.9 | 16253 | 121.3 |
| K57R, S99G, F189L | 1730 | 1930 | 0.6 | 12906 | 96.3 |
| M18V, M97L, F193S, R195G, E200K, H202Q | 1731 | 69 | 0.0 | 241 | 1.8 |
| I36S, M41K, M97L, K144Q, R195G, E200K, H202Q, L206F | 1732 | 3498 | 1.1 | 68715 | 512.8 |
| C22R, Q65L, L124S, K144Q, R195G, E200N, H202Q, T221L | 1733 | Little or no protein produced | | | |
| M18V, I98L, L124S, P198T, L206F | 1734 | 2187 | 0.7 | 143 | 1.1 |
| S99G, N117S, I148V, K171R, R180S | 1735 | Little or no protein produced | | | |
| I36T, M97L, A103V, Q155H | 1736 | 120 | 0.0 | 128 | 1.0 |
| K28I, S99G | 1737 | 830 | 0.3 | 693 | 5.2 |
| R195S | 1738 | 3191 | 1.0 | 138 | 1.0 |
| A79T, S99G, T185A, R195G, E200K, H202Q, L206F | 1739 | 1963 | 0.6 | 643 | 4.8 |
| K57R, S99G, L124S, K144Q | 1740 | 2081 | 0.7 | 14106 | 105.3 |
| K57R, S99G, R195G | 1741 | 2479 | 0.8 | 10955 | 81.8 |
| D55V, M97L, S99G | 1742 | 11907 | 3.8 | 71242 | 531.7 |

| Table 21: Flow Binding to Cells Expressing PD-1 or CD80 | | | | | |
|---|---|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (ECD) | PD-1 | | CD80 | |
| | | MFI at 20nM | Fold Change Compared to WT PD-L1 | MFI at 20nM | Fold Change Compared to WT PD-L1 |
| E27G, 136T, D55N, M97L, K111E | 1743 | 1904 | 0.6 | 88724 | 662.1 |
| E54G, M97L, S99G | 1744 | 8414 | 2.7 | 51905 | 387.4 |
| G15A, I36T, M97L, K111E, H202Q | 1745 | 112 | 0.0 | 13530 | 101.0 |
| G15A, I36T, V129D | 1746 | 114 | 0.0 | 136 | 1.0 |
| G15A, I36T, V129D, R195G | 1747 | 125 | 0.0 | 134 | 1.0 |
| G15A, V129D | 1748 | 2075 | 0.7 | 128 | 1.0 |
| I36S, M97L | 1749 | 3459 | 1.1 | 44551 | 332.5 |
| I36T, D55N, M97L, K111E, A204T | 1750 | 265 | 0.1 | 62697 | 467.9 |
| I36T, D55N, M97L, K111E, V129A, F173L | 1751 | 393 | 0.1 | 72641 | 542.1 |
| I36T, D55S, M97L, K111E, I148V, R180S | 1752 | 94 | 0.0 | 30704 | 229.1 |
| I36T, G52R, M97L, V112A, K144E, V175A, P198T | 1753 | 81 | 0.0 | 149 | 1.1 |
| I36T, 146V, D55G, M97L, K106E, K144E, T185A, R195G | 1754 | 69 | 0.0 | 190 | 1.4 |
| I36T, I83T, M97L, K144E, P198T | 1755 | 62 | 0.0 | 6216 | 46.4 |
| I36T, M97L, K111E | 1756 | Little or no protein produced | | | |
| I36T, M97L, K144E, P198T | 1757 | 197 | 0.1 | 40989 | 305.9 |
| I36T, M97L, Q155H, F193S, N201Y | 1758 | 69 | 0.0 | 1251 | 9.3 |
| I36T, M97L, V129D | 1759 | 523 | 0.2 | 50905 | 379.9 |
| L35P, I36S, M97L, K111E | 1760 | 190 | 0.1 | 155 | 1.2 |
| M18I, I36T, E53G, M97L, K144E, E199G, V207A | 1761 | 104 | 0.0 | 47358 | 353.4 |
| M18T, I36T, D55N, M97L, K111E | 1762 | 138 | 0.0 | 71440 | 533.1 |
| M18V, M97L, T176N, R195G | 1763 | 1301 | 0.4 | 45300 | 338.1 |
| M97L, S99G | 1764 | 12906 | 4.1 | 81630 | 609.2 |
| N17D, M97L, S99G | 1765 | 10079 | 3.2 | 73249 | 546.6 |
| S99G, T185A, R195G, | 1766 | 2606 | 0.8 | 22062 | 164.6 |

| Table 21: Flow Binding to Cells Expressing PD-1 or CD80 | | | | | |
|---|---|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (ECD) | PD-1 | | CD80 | |
| | | MFI at 20nM | Fold Change Compared to WT PD-L1 | MFI at 20nM | Fold Change Compared to WT PD-L1 |
| P198T | | | | | |
| V129D, H202Q | 1767 | 2001 | 0.6 | 219 | 1.6 |
| V129D, P198T | 1768 | 3245 | 1.0 | 152 | 1.1 |
| V129D, T150A | 1769 | 1941 | 0.6 | 142 | 1.1 |
| V93E, V129D | 1770 | 1221 | 0.4 | 150 | 1.1 |
| Y10F, M18V, S99G, Q138R, T203A | 1771 | 70 | 0.0 | 412 | 3.1 |
| WT PD-L1 (IgV+IgC) Fc | 1728 | 3121 | 1.0 | 134 | 1.0 |
| CTLA4-Fc | - | 59 | N/A | 199670 | N/A |
| Anti-PD1 mAb | - | 31482 | N/A | 134 | N/A |
| Fc Control | 1155 | 59 | N/A | 132 | N/A |

**[0495]** Additional variants identified in the screen as described in Example 3 are set forth in Table 22A.

| TABLE 22A. Additional Affinity-Matured IgSF Domain-Containing Molecules | | | |
|---|---|---|---|
| PD-L1 Mutation(s) | SEQ ID NO (ECD) | PD-L1 Mutation(s) | SEQ ID NO (ECD) |
| N45D | 1772 | N45D, G102D, R194W, R195G | 1796 |
| K160M, R195G | 1773 | N45D, G52V, Q121L, P198S | 1797 |
| N45D, K144E | 1774 | N45D, 1148V, R195G, N201D | 1798 |
| N45D, P198S | 1775 | N45D, K111T, T183A, I188V | 1799 |
| N45D, P198T | 1776 | N45D, Q89R, F189S, P198S | 1800 |
| N45D, R195G | 1777 | N45D, S99G, C137R, V207A | 1801 |
| N45D, R195S | 1778 | N45D, T163I, K167R, R195G | 1802 |
| N45D, S131F | 1779 | N45D, T183A, T192S, R194G | 1803 |
| N45D, V58D | 1780 | N45D, V50A, I119T, K144E | 1804 |
| V129D, R195S | 1781 | T19A, N45D, K144E, R195G | 1805 |
| I98T, F173Y, L196S | 1782 | V11E, N45D, T130A, P198T | 1806 |
| N45D, E134G, L213P | 1783 | V26A, N45D, T163I, T185A | 1807 |
| N45D, F173I, S177C | 1784 | K23N, N45D, L124S, K167T, R195G | 1808 |
| N45D, 1148V, R195G | 1785 | K23N, N45D, Q73R, T163I | 1809 |
| N45D, K111T, R195G | 1786 | K28E, N45D, W149R, S158G, P198T | 1810 |
| N45D, N113Y, R195S | 1787 | K28R, N45D, K57E, I98V, R195S | 1811 |
| N45D, N165Y, E170G | 1788 | K28R, N45D, V129D, T163N, R195T | 1812 |
| N45D, Q89R, 198V | 1789 | M41K, D43G, N45D, R64S, | 1813 |

| TABLE 22A. Additional Affinity-Matured IgSF Domain-Containing Molecules | | | |
|---|---|---|---|
| **PD-L1 Mutation(s)** | **SEQ ID NO (ECD)** | **PD-L1 Mutation(s)** | **SEQ ID NO (ECD)** |
| | | R195G | |
| N45D, S131F, P198S | 1790 | M41K, D43G, N45D, R64S, S99G | 1814 |
| N45D, S75P, P198S | 1791 | N45D, R68L, F173L, D197G, P198S | 1815 |
| N45D, V50A, R195T | 1792 | N45D, V50A, 1148V, R195G, N201D | 1816 |
| E27D, N45D, T183A, I188V | 1793 | M41K, D43G, K44E, N45D, R195G, N201D | 1817 |
| F173Y, T183I, L196S, T203A | 1794 | N45D, V50A, L124S, K144E, L179P, R195G | 1818 |
| K23N, N45D, S75P, N120S | 1795 | | |

**[0496]** Substantially as described above, flow cytometry binding studies were performed to assess specificity and affinity of PD-L1-extracellular domain (ECD) variant immunomodulatory proteins for cognate binding partner PD-1. To measure binding to PD-1, as described above Jurkat/IL-2 reporter cells transduced to stably express human PD-1 (Jurkat/PD-1 cells) were used. Mean Fluorescence Intensity (MFI) was calculated at the 20 nM concentration and compared to wild type PD-L1 ECD-Fc protein and an Fc only control. Results for the binding studies for exemplary tested PD-L1-ECD-Fc fusion molecules are shown in Table 22B. As demonstrated, the selections resulted in the identification of variant PD-L1 proteins that bind PD-1 alone with high affinity.

| TABLE 22B. Flow binding to Jurkat cells expressing PD-1 | | | |
|---|---|---|---|
| **PD-L1 Mutation(s)** | **SEQ ID NO (ECD)** | **MFI at 20nM cone.** | **Fold Increase over WT PD-L1 ECD-Fc** |
| N45D | 1772 | 95635 | 19.1 |
| R195S | 1827 | 5365 | 1.1 |
| K160M, R195G | 1773 | 3931 | 0.8 |
| N45D, K144E | 1774 | 99704 | 19.9 |
| N45D, P198S | 1775 | 117460 | 23.5 |
| N45D, P198T | 1776 | 122118 | 24.4 |
| N45D, R195G | 1777 | 121779 | 24.3 |
| N45D, R195S | 1778 | 128736 | 25.7 |
| N45D, S131F | 1779 | 122458 | 24.5 |
| N45D, V58D | 1780 | 152085 | 30.4 |
| V129D, R195S | 1781 | 7699 | 1.5 |
| I98T, F173Y, L196S | 1782 | 236 | 0.0 |
| N45D, E134G, L213P | 1783 | 2255 | 0.5 |
| N45D, F173I, S177C | 1784 | 2199 | 0.4 |
| N45D, 1148V, R195G | 1785 | 109276 | 21.8 |
| N45D, K111T, R195G | 1786 | 65728 | 13.1 |

| TABLE 22B. Flow binding to Jurkat cells expressing PD-1 | | | |
|---|---|---|---|
| **PD-L1 Mutation(s)** | **SEQ ID NO (ECD)** | **MFI at 20nM cone.** | **Fold Increase over WT PD-L1 ECD-Fc** |
| N45D, N113Y, R195S | 1787 | 112042 | 22.4 |
| N45D, N165Y, E170G | 1788 | 88971 | 17.8 |
| N45D, Q89R, I98V | 1789 | 90467 | 18.1 |
| N45D, S131F, P198S | 1790 | 116162 | 23.2 |
| N45D, S75P, P198S | 1791 | 129814 | 25.9 |
| N45D, V50A, R195T | 1792 | 141881 | 28.3 |
| E27D, N45D, T183A, 1188V | 1793 | 133100 | 26.6 |
| K23N, N45D, S75P, N120S | 1795 | 131995 | 26.4 |
| N45D, G102D, R194W, R195G | 1796 | 13381 | 2.7 |
| N45D, G52V, Q121L, P198S | 1797 | 296 | 0.1 |
| N45D, 1148V, R195G, N201D | 1798 | 130537 | 26.1 |
| N45D, K111T, T183A, 1188V | 1799 | 108670 | 21.7 |
| N45D, S99G, C137R, V207A | 1801 | 834 | 0.2 |
| N45D, T163I, K167R, R195G | 1802 | 133842 | 26.7 |
| N45D, T183A, T192S, R194G | 1803 | 1349 | 0.3 |
| N45D, V50A, I119T, K144E | 1804 | 34217 | 6.8 |
| V11E, N45D, T130A, P198T | 1806 | 137230 | 27.4 |
| K23N, N45D, Q73R, T163I | 1809 | 139149 | 27.8 |
| K28E, N45D, W149R, S158G, P198T | 1810 | 691 | 0.1 |
| K28R, N45D, V129D, T163N, R195T | 1812 | 137230 | 27.4 |
| M41K, D43G, N45D, R64S, R195G | 1813 | 137230 | 27.4 |
| M41K, D43G, N45D, R64S, S99G | 1814 | 134587 | 26.9 |
| N45D, R68L, F173L, D197G, P198S | 1815 | 59308 | 11.8 |
| N45D, V50A, 1148V, R195G, N201D | 1816 | 131264 | 26.2 |
| M41K, D43G, K44E, N45D, R195G, N201D | 1817 | 144667 | 28.9 |
| N45D, V50A, L124S, K144E, L179P, R195G | 1818 | 18163 | 3.6 |
| WT PD-L1 ECD-Fc N10653 | 1728 | 5005 | 1.0 |
| Fc only control | 1155 | 46 | 0.0 |

**[0497]** Bioactivity of the additional affinity-matured PD-L1 domain-containing molecules was further assessed using Jurkat effector cells expressing an IL-2-luciferase reporter and cell-surface PD-1substantially as described in Example 12B. As shown in Table 22C, increased luciferase activity of the

Jurkat effector cells co-cultured with anti-CD3 and K562/CD80 cells was observed for some Fc fusion molecules with the indicated PD-L1 variant. An average relative luminescence value (RLU) was determined for each test sample and a fold change in IL-2 reporter signal was calculated for each tested molecule compared to wild-type PD-L1 or the Fc only control protein. In the Table, Column 2 sets forth the SEQ ID NO identifier for each PD-L1 variant tested. The differences in luminescence signals demonstrate the differences in binding of the PD-L1variant Fc molecules to PD-1 and the resulting co-blockade of inhibitory activity.

**TABLE 22C: Jurkat/IL2/PD-1 + K562/CD80 + soluble anti-CD3 Reporter Assay**

| PD-L1 Mutation(s) | SEQ ID NO (ECD) | Relative Luciferase Units (RLU) | Fold Increase over WT PD-L1 ECD-Fc |
|---|---|---|---|
| | | PD-L1 vECD-Fc Cone 50.0nM | |
| N45D | 1772 | 460 | 2.3 |
| R195S | 1827 | 215 | 1.1 |
| K160M, R195G | 1773 | 215 | 1.1 |
| N45D, K144E | 1774 | 385 | 1.9 |
| N45D, P198S | 1775 | 457 | 2.3 |
| N45D, P198T | 1776 | 476 | 2.3 |
| N45D, R195G | 1777 | 405 | 2.0 |
| N45D, R195S | 1778 | 417 | 2.1 |
| N45D, S131F | 1779 | 429 | 2.1 |
| N45D, V58D | 1780 | 484 | 2.4 |
| V129D, R195S | 1781 | 223 | 1.1 |
| I98T, F173Y, L196S | 1782 | 209 | 1.0 |
| N45D, E134G, L213P | 1783 | 276 | 1.4 |
| N45D, F173I, S177C | 1784 | 265 | 1.3 |
| N45D, 1148V, R195G | 1785 | 496 | 2.4 |
| N45D, K111T, R195G | 1786 | 338 | 1.7 |
| N45D, N113Y, R195S | 1787 | 492 | 2.4 |
| N45D, N165Y, E170G | 1788 | 446 | 2.2 |
| N45D, Q89R, I98V | 1789 | 550 | 2.7 |
| N45D, S131F, P198S | 1790 | 422 | 2.1 |
| N45D, S75P, P198S | 1791 | 549 | 2.7 |
| N45D, V50A, R195T | 1792 | 594 | 2.9 |
| E27D, N45D, T183A, 1188V | 1793 | 554 | 2.7 |
| K23N, N45D, S75P, N120S | 1795 | 504 | 2.5 |
| N45D, G102D, R194W, R195G | 1796 | 211 | 1.0 |
| N45D, G52V, Q121L, P198S | 1797 | 225 | 1.1 |
| N45D, 1148V, R195G, N201D | 1798 | 401 | 2.0 |

| TABLE 22C: Jurkat/IL2/PD-1 + K562/CD80 + soluble anti-CD3 Reporter Assay | | | |
|---|---|---|---|
| **PD-L1 Mutation(s)** | **SEQ ID NO (ECD)** | **Relative Luciferase Units (RLU)** | **Fold Increase over WT PD-L1 ECD-Fc** |
| | | **PD-L1 vECD-Fc Cone 50.0nM** | |
| N45D, K111T, T183A, 1188V | 1799 | 354 | 1.7 |
| N45D, S99G, C137R, V207A | 1801 | 226 | 1.1 |
| N45D, T163I, K167R, R195G | 1802 | 440 | 2.2 |
| N45D, T183A, T192S, R194G | 1803 | 250 | 1.2 |
| N45D, V50A, I119T, K144E | 1804 | 292 | 1.4 |
| V11E, N45D, T130A, P198T | 1806 | 470 | 2.3 |
| K23N, N45D, Q73R, T163I | 1809 | 449 | 2.2 |
| K28E, N45D, W149R, S158G, P198T | 1810 | 197 | 1.0 |
| K28R, N45D, V129D, T163N, R195T | 1812 | 437 | 2.2 |
| M41K, D43G, N45D, R64S, R195G | 1813 | 590 | 2.9 |
| M41K, D43G, N45D, R64S, S99G | 1814 | 597 | 2.9 |
| N45D, R68L, F173L, D197G, P198S | 1815 | 327 | 1.6 |
| N45D, V50A, 1148V, R195G, N201D | 1816 | 494 | 2.4 |
| M41K, D43G, K44E, N45D, R195G, N201D | 1817 | 464 | 2.3 |
| N45D, V50A, L124S, K144E, L179P, R195G | 1818 | 308 | 1.5 |
| WT PD-L1 ECD-Fc | 1728 | 203 | 1.0 |
| Fc only control | 1155 | 205 | 1.0 |

**EXAMPLE 14**

**Generation of Secreted Immunomodulatory Protein and Assessment of Proliferation of Pan T cells transduced with PD-L1 SIP**

**[0498]** To generate a PD-L1 secreted immunomodulatory protein (SIP), DNA encoding exemplary SIPs was obtained as gene blocks from Integrated DNA Technologies (Coralville, USA) and then cloned by

Gibson assembly (New England Biolabs Gibson assembly kit) into a modified version of pRRL vector (Dull et al., (1998) J Virol, 72(11): 8463-8471) between restriction sites downstream of MND promoter to remove GFP. Exemplary SIP constructs were generated to encode a protein set forth in SEQ ID NO: 2012-2013, including the signal peptide. In this exemplary Example, the constructs were generated to additionally include a tag moiety. The gene blocks had the following structure in order: 39 base pair overlap with pRRL prior to first restriction site-first restriction site-GCCGCCACC (Kozak); complete ORF encoding PD-L1 IgV wildtype amino acid sequence set forth in SEQ ID NO: 309 or variant PD-L1 IgV set forth in SEQ ID NO: 303 (D43G/N45D/L56Q/V58A/G101G-ins (G101GG), also including in all cases the signal peptide MGSTAILALLLAVLQGVSA as set forth in SEQ ID NO: 2009; DNA encoding Flag-tag as set forth in SEQ ID NO:2010 (DYKDDDDK); DNA encoding His tag as set forth in SEQ ID NO: 2011 (HHHHHH); TAA stop codon; second restriction site- 41 base pair overlap with pRRL beyond second restriction site. For comparison, a SIP encoding wild-type PD-L1 also was assessed.

**[0499]** To prepare lentiviral vectors, $3\times10^6$ HEK293 cells were plated per 100mm dish. On the next day, 4.5µg of P-Mix (3 µg of PAX2 and 1.5µg of pMD2G) was added to 6µg of DNA encoding the SIPs constructs in a 5mL polypropylene tube. Diluent buffer (10mM HEPES/150mM NaCl pH7.05/1L TC grade H20) was added to the tube to bring up the total volume of 500µL. To the diluent DNA(PEI:total DNA 4:1), 42µL of PEI (1µg/µL) was added and mixed by vortexing. The mixture was incubated at room temperature for 10 minutes and cells were prepared by aspirating medium from the dish gently without disturbing the adherent cells, then replaced with 6mL of Opti-MEM(1X). DNA/PEI mixture was then added to the dish and incubated at 37°C for 24 hours. After 24 hours, media was aspirated from the dishes and replaced with 10mL of fresh DMEM media and then incubated at 37°C. Viral supernatant was collected after 48 hours using a syringe attached to a 0.45µm filter PES to remove cells and debris from the culture (Thermo Scientific Nalgene Syringe Filter). A separate lentiviral vector stock also was prepared encoding an anti-CD19 CAR (containing an anti-CD19 scFv, a hinge and transmembrane domain derived from CD8 and a CD3zeta signaling domain) substantially as described. The exemplary anti-CD19 CAR used is set forth in SEQ ID NO: 2016 (encoded by the sequence in set forth in SEQ ID NO: 2017) containing the scFv set forth in SEQ ID NO:1163, the CD8-derived hinge and transmembrane domain set forth in SEQ ID NO: 242, and the CD3zeta set forth in SEQ ID NO:243

**[0500]** T-cells were thawed and activated with anti-CD3/anti-CD28 beads (Dynal) at a 1:1 ratio. The T-cells ($1 \times 10^6$ cells) were mixed with 1 mL total lentiviral vector supernatant containing equal volume (0.5 mL each) of the lentiviral vector supernatant encoding the indicated PD-L1 SIP (D43G/N45D/L56Q/V58A/G101GG or wildtype) and a lentiviral vector supernatant encoding the anti-CD19 CAR. As a control, cells were transduced only with the lentiviral vector encoding the anti-CD19 CAR or were transduced with mock vector control. Transduction was performed in the presence of 10 µg/mL polybrene and 50 IU/mL IL-2. Cells were spun down at 2500 rpm for 60 min at 30°C. After 24 hours, 3mL of Xvivo15 plus media and IL2 was added to each well. The cells were fed every two days with fresh media and cytokines.

**[0501]** At 14 days after activation, cells were re-stimulated with Nalm6 cells that had been transduced with a lenti-viral vector to provide expression of PD-L1 (Nalm6 PDL1+). Transduced T cells were labeled with Cell Trace Far Red and proliferation was measured at day 3 by determining the fraction of the cells that showed dilution of the dye. Results for the proliferation studies for T cells transduced with exemplary tested variant PD-L1 SIP are shown in FIG. 10.

**EXAMPLE 15**

**Assessment of PD-L1 Multi-Domain Stack Molecules on Activity of Primary Exhausted T cells**

**[0502]** This Example describes studies to assess specificity and affinity of exemplary PD-L1/CD155 and PD-L1/CD112/CD155 variant IgV stack immunomodulatory proteins (stacked IgV-Fc), generated as described in Example 10, for modulating the activity of exhausted T cells via blocking interactions involving cognate binding partners PD-1, TIGIT and/or CD112R.

**A. Generation of Exhausted T cells**

**[0503]** To generate exhausted T cells, primary human T cells were stimulated for 7 days with mitomycin C-treated K562 cells that stably expressed anti-CD3 single-chain Fc (OKT3) at a ratio of 10 T cells to 1 K562/OKT3 cell. On day 3, 50 IU/mL of recombinant IL-2 was added to the culture media to promote T cell expansion and survival. On day 7, T cells were harvested, counted and then set up for two additional cycles of restimulation with K562/OKT3 plus IL-2. At the end of 21 days (3 rounds of stimulation), T cells were counted, characterized for expression of inhibitory receptors PD-L1, TIGIT and Lag-3 and then frozen for subsequent assays. Exhausted T cells were detected based on elevated expression of the inhibitory receptors PD-1, TIGIT and Lag-3.

**B. Binding to Exhausted T cells**

**[0504]** Exhausted T cells were generated as described above by three rounds of stimulation and expansion with K562/OKT3 + IL-2. Binding studies were performed essentially as described in Example 12 except with exhausted T cells. Exemplary stack molecules tested included PD-L1/CD155 Stack 4 (SEQ ID NO: 1719) and PD-L1/CD112/CD155 Stack 2 (SEQ ID NO: 1724) generated as described above in Example 10. As a control, binding activity of non-stack formats of each variant IgV fused to an Fc (set forth in SEQ ID NO: 1157, containing the mutations C220S, R292C, N297G and V302C by EU numbering (corresponding to C5S, R77C, N82G and V87C with reference to wild-type human IgG1 Fc set forth in SEQ ID NO: 187), were assessed, i.e., variant PD-L1 IgV-Fc (containing variant PD-L1 IgV set forth in SEQ ID NO:303), CD155 IgV-Fc (containing variant CD155 IgV set forth in SEQ ID NO:1576), or CD112 IgV-Fc (containing variant CD112 IgV set forth in SEQ ID NO: 768).

**[0505]** Results for the binding studies for exemplary tested PD-L1/CD155 and PD-L1/CD112/CD155 stack Fc fusion molecules tested at various concentrations are shown in FIG. 11. As shown, the exemplary tested PD-L1/CD155 stack Fc fusion molecule demonstrated superior binding to exhausted T cells than either domain alone.

**C. Stimulation and Assessment of Bioactivity**

**[0506]** Previously derived exhausted T cells, generated as described above, were thawed and plated at 100,000 cells per well in a flat-bottom 96-well plate in assay buffer (X-vivo 15 media (Lonza, Corp., USA) supplemented with Glutamax (ThermoFisher, Inc. USA)). An exemplary stack Fc fusion molecule PD-L1/CD155 Stack 4 SEQ ID NO: 1719, or control non-stack variant PD-L1 IgV-Fc or CD155 IgV-Fc were added to the T cells at final concentrations of 100nM, 10nM and 1nM. The control non-stack variant PD-L1 IgV-Fc or CD155 IgV-Fc fusion molecules contained the same variant PD-L1 IgV (SEQ ID NO:303) or

variant CD155 IgV (SEQ ID NO:1576), used in the stacks. An anti-TIGIT antibody (eBioscience, Inc., USA) and anti-PD-1 antibody (Bristol-Myers Squibb, Inc., USA) or human IgG1 antibody (BioLegend, Inc., USA) were also used as controls and added to the T cells at the indicated concentrations.

**[0507]** K562 cells previously transduced to express anti-CD3 single-chain Fv (OKT3) and human PD-L1 were treated for 20 minutes with 50 µg/mL mitomycin C (Millipore Sigma, Corp., USA) to arrest proliferation, washed and then added to the assay plate at 10,000 cells per well. As a further control, T cells were incubated with K562-OKT3-PD-L1 cells in the absence of added molecule or T cells were incubated without stimulation with K562-OKT3-PD-L1 cells. The assay plate was then incubated for three days at 37°C in a humidified, 5% $CO_2$ incubator. After three days, IL-2, IFNγ and T cell proliferation were measured using standard ELISA and flow cytometry techniques.

**[0508]** Results for the bioactivity studies are shown in FIG. 12, which sets forth the levels of IFN-gamma in culture supernatants (pg/mL) at the indicated concentrations of the tested molecules. As shown, the exemplary tested PD-L1/CD155 stack Fc fusion molecule exhibited improved activities to increase IFNg production compared to non-stack controls, demonstrating blockade of both PD-1 and TIGIT pathways resulting in increased signal compared to only individual pathway blockade.

**[0509]** The present invention is not intended to be limited in scope to the particular disclosed embodiments, which are provided, for example, to illustrate various aspects of the invention. Various modifications to the compositions and methods described will become apparent from the description and teachings herein.

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Patentkrav

1. Variant PD-L1-polypeptid, som omfatter et IgV-domæne eller både et IgV-domæne og et IgC-domæne, hvor det variante PD-L1-polypeptid omfatter en aminosyresubstitution i en modificeret human PD-L1 svarende til aminosyresubstitution N45D med henvisning til nummereringen i SEQ ID NO: 30, hvor det variante PD-L1-polypeptid binder specifikt til ektodomænet af PD-1 med forøget affinitet sammenlignet med bindingen af den umodificerede humane PD-L1 til ektodomænet af PD-1;
hvor den umodificerede PD-L1 omfatter sekvensen af aminosyrer som vist i SEQ ID NO: 30 eller en del deraf, der omfatter IgV-domænet eller IgV-domænet og IgC-domænet; og
hvor det variante PD-L1-polypeptid omfatter en sekvens af aminosyrer, der fremviser mindst 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 % eller 99 % sekvensidentitet med SEQ ID NO: 30, 55, 309 eller 1728.

2. Variant PD-L1-polypeptid ifølge krav 1, hvor den variante PD-L1 omfatter en eller flere aminosyresubstitutioner valgt blandt I20L/N45D, I36T/N45D, D43G/N45D, N45D/V58A, N45D/S75P, N45D/N78I, I20L/I36T/N45D, D43G/N45D/V58A, I20L/E27G/D43G/N45D/V58A/N78I, I20L/D43G/N45D/V58A/N78I, I20L/A33D/D43G/N45D/V58A/N78I, I20L/D43G/N45D/N78I, V11A/I20L/E27G/D43G/N45D/H51Y/S99G, I20L/K28E/D43G/N45D/V58A/Q89R, I20L/I36T/N45D, A33D/D43G/N45D/V58A/S75P, K23R/D43G/N45D, D43G/N45D/L56Q/V58A/G101G-ins (G101GG), I20L/K23E/D43G/N45D/V58A/N78I, I20L/K23E/D43G/N45D/V50A/N78I, I20L/K28E/D43G/N45D/V58A/Q89R/G101G-ins (G101GG), N45D, N45D/K144E, N45D/P198S, N45D/P198T, N45D/R195G, N45D/R195S, N45D/S131F, N45D/V58D, N45D/I148V/R195G, N45D/K111T/R195G, N45D/N113Y/R195S, N45D/N165Y/E170G, N45D/Q89R/I98V, N45D/S131F/P198S, N45D/S75P/P198S, N45D/V50A/R195T, E27D/N45D/T183A/I188V, K23N/N45D/S75P/N120S, N45D/I148V/R195G/N201D, N45D/K111T/T183A/I188V, N45D/Q89R/F189S/P198S, N45D/T163I/K167R/R195G, N45D/V50A/I119T/K144E, T19A/N45D/K144E/R195G,

V11E/N45D/T130A/P198T, V26A/N45D/T163I/T185A,
K23N/N45D/L124S/K167T/R195G, K23N/N45D/Q73R/T163I,
K28R/N45D/K57E/I98V/R195S, K28R/N45D/V129D/T163N/R195T,
M41K/D43G/N45D/R64S/R195G, M41K/D43G/N45D/R64S/S99G,
N45D/R68L/F173L/D197G/P198S, N45D/V50A/I148V/R195G/N201D,
M41K/D43G/K44E/N45D/R195G/N201D,
N45D/V50A/L124S/K144E/L179P/R195G.

3. Variant PD-L1-polypeptid ifølge krav 1 eller krav 2, hvor:
det variante PD-L1-polypeptid omfatter det ekstracellulære domæne (ECD) af PD-L1; eller
det variante PD-L1-polypeptid omfatter IgV-domænet og IgC-domænet; eller
det variante PD-L1-polypeptid omfatter IgV-domænet, eventuelt hvor IgV-domænet eller et specifikt bindingsfragment deraf er den eneste PD-L1-del af det variante PD-L1-polypeptid.

4. Variant PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-3, hvor det variante PD-L1-polypeptid omfatter sekvensen af aminosyrer som vist i en hvilken som helst af SEQ ID NO: 66, 82, 101-104, 107, 109-110, 112-113, 115-117, 131, 147, 166-169, 172, 174-175, 177-178, 180-182, 254, 270, 289-292, 295, 297-298, 300-301, 303-305, 1725-1727, 1774-1780, 1785-1793, 1795, 1798-1800, 1802, 1804-1809, 1811-1818, 1863-1869, 1874-1882, 1884, 1887-1889, 1891, 1893-1898, 1900-1907, 1920-1922, 1935-1937, 1953, 1969, 1988-1991, 1994, 1996-1997, 1999-2000, 2002-2004, 2008 eller et specifikt bindingsfragment deraf eller en sekvens af aminosyrer, der fremviser mindst 95 % sekvensidentitet med en hvilken som helst af SEQ ID NO: 66, 82, 101-104, 107, 109-110, 112-113, 115-117, 131, 147, 166-169, 172, 174-175, 177-178, 180-182, 254, 270, 289-292, 295, 297-298, 300-301, 303-305, 1725-1727, 1774-1780, 1785-1793, 1795, 1798-1800, 1802, 1804-1809, 1811-1818, 1863-1869, 1874-1882, 1884, 1887-1889, 1891, 1893-1898, 1900-1907, 1920-1922, 1935-1937, 1953, 1969, 1988-1991, 1994, 1996-1997, 1999-2000, 2002-2004, 2008.

5. Variant PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-4, hvor:

det variante PD-L1-polypeptid er et opløseligt protein, eventuelt hvor det variante PD-L1-polypeptid er koblet til et multimeriseringsdomæne.

6. Immunmodulatorisk Fc-fusionsprotein, som omfatter det variante PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-5 koblet til et Fc-domæne eller en variant deraf med reduceret effektorfunktion.

7. Variant PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-5, som er et transmembrant immunmodulatorisk protein, der yderligere omfatter et transmembrandomæne, eventuelt hvor transmembrandomænet er koblet direkte eller indirekte til det ekstracellulære domæne (ECD) eller et specifikt bindingsfragment af det variante PD-L1-polypeptid, eventuelt hvor PD-L1-polypeptidet yderligere omfatter et cytoplasmatisk signalleringsdomæne.

8. Immunmodulatorisk protein, som omfatter det variante PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-7, der er koblet direkte eller indirekte via en linker til et andet polypeptid, der omfatter et immunoglobulin-superfamilie (IgSF)-domæne fra et IgSF-familiemedlem; eventuelt hvor IgSF-domænet er et affinitetsmodificeret IgSF-domæne, hvilket affinitetsmodificerede IgSF-domæne omfatter en eller flere aminosyremodifikationer sammenlignet med det umodificerede eller vildtype-IgSF-domænet fra IgSF-familiemedlemmet og udviser ændret binding til en eller flere af dets tilhørende bindingspartnere sammenlignet med det umodificerede eller vildtype-IgSF-domænet.

9. Immunmodulatorisk protein ifølge krav 8, hvor det immunmodulatoriske protein yderligere omfatter et multimeriseringsdomæne, der er koblet til mindst ét af det variante PD-L1-polypeptid eller det andet polypeptid, eventuelt hvor multimeriseringsdomænet er et Fc-domæne.

10. Konjugat, som er et fusionsprotein, der omfatter et variant

PD-L1-polypeptid ifølge et hvilken som helst af kravene 1-5 eller et immunmodulatorisk protein ifølge et hvilket som helst af kravene 6, 8 og 9, der er koblet til en målsøgningsdel, der binder specifikt til et molekyle på overfladen af en celle, eventuelt hvor cellen er en immuncelle eller en celle i en tumor, og eventuelt hvor delen er et antistof eller et antigenbindende fragment.

11. Nukleinsyremolekyle, som koder for et variant PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-5, et immunmodulatorisk protein ifølge et hvilket som helst af kravene 6, 8 og 9 eller konjugatet, som er et fusionsprotein ifølge krav 10.

12. Vektor, som omfatter nukleinsyremolekylet ifølge krav 11, eventuelt hvor vektoren er en ekspressionsvektor.

13. Farmaceutisk sammensætning, som omfatter det variante PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-7, et immunmodulatorisk protein ifølge et hvilket som helst af kravene 6, 8 og 9 eller et konjugat ifølge krav 10, eventuelt hvor den farmaceutiske sammensætning omfatter et farmaceutisk acceptabelt hjælpestof.

14. Farmaceutisk sammensætning til anvendelse til modulering af et immunrespons hos et individ, hvilken farmaceutiske sammensætning omfatter det variante PD-L1-polypeptid ifølge et hvilket som helst af kravene 1-7, et immunmodulatorisk protein ifølge et hvilket som helst af kravene 6, 8 og 9 eller et konjugat ifølge krav 10, eventuelt hvor den farmaceutiske sammensætning omfatter et farmaceutisk acceptabelt hjælpestof, hvor modulering af immunresponset behandler en sygdom eller tilstand hos individet.

# DRAWINGS

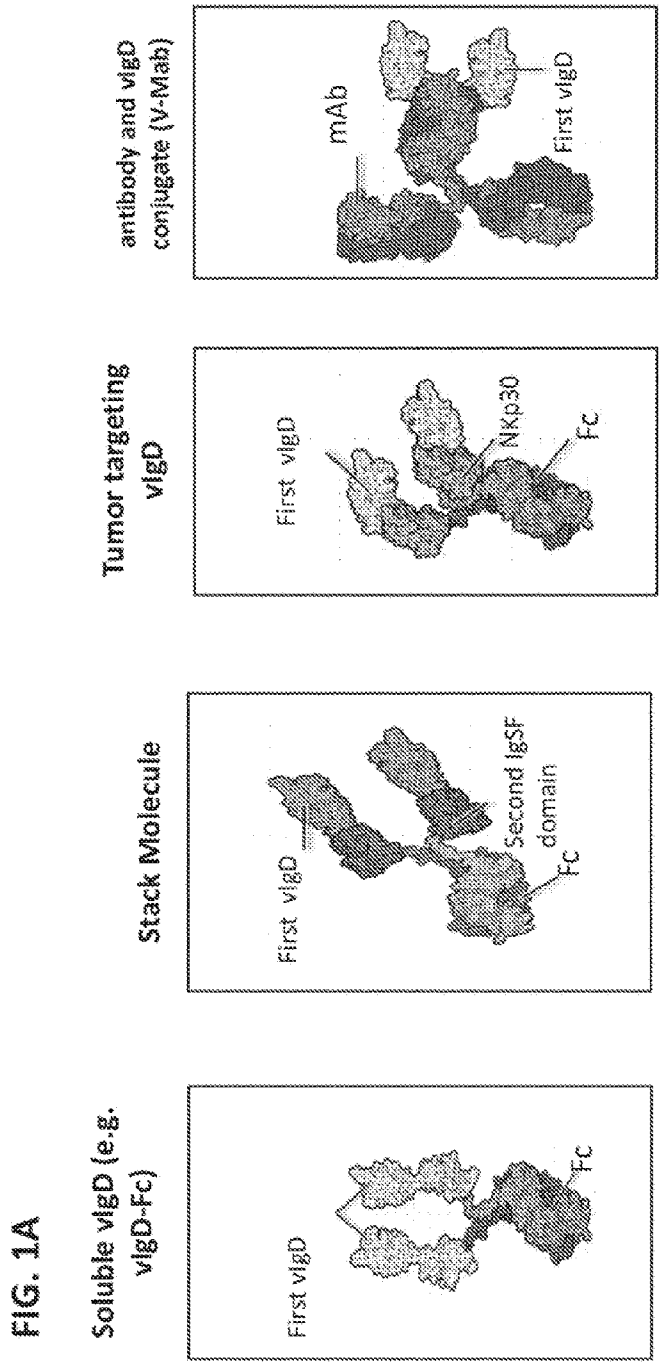


FIG. 1A

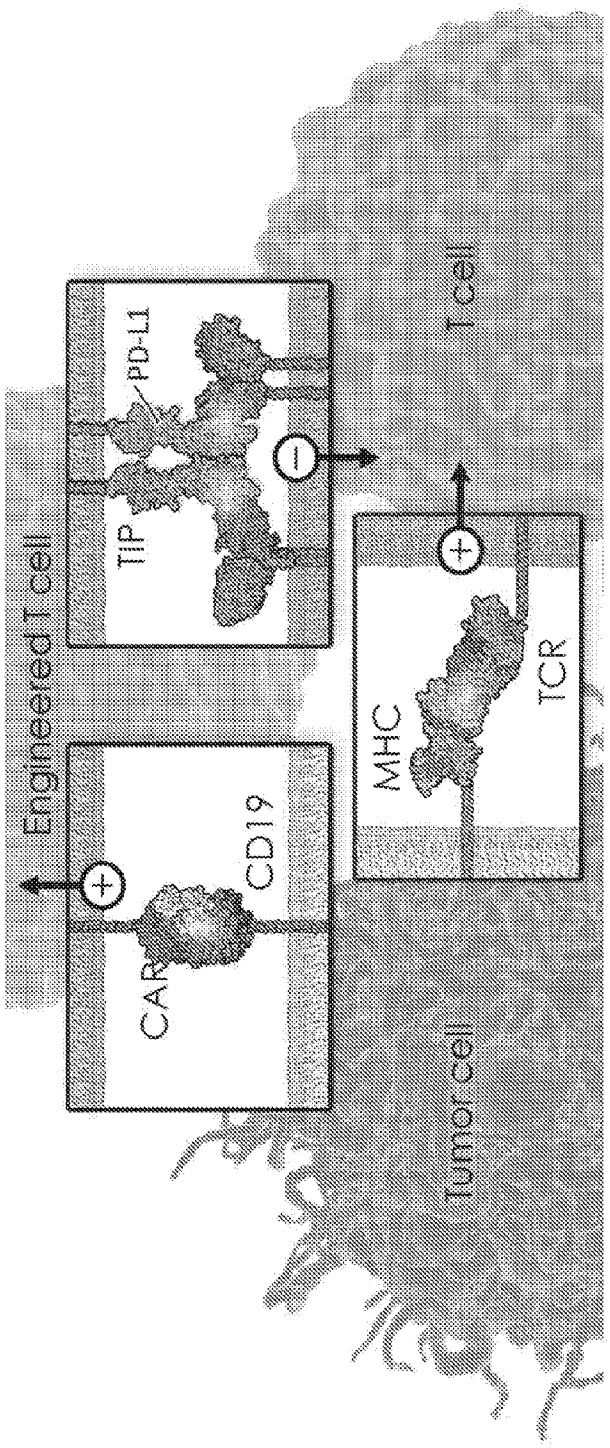


FIG. 1B

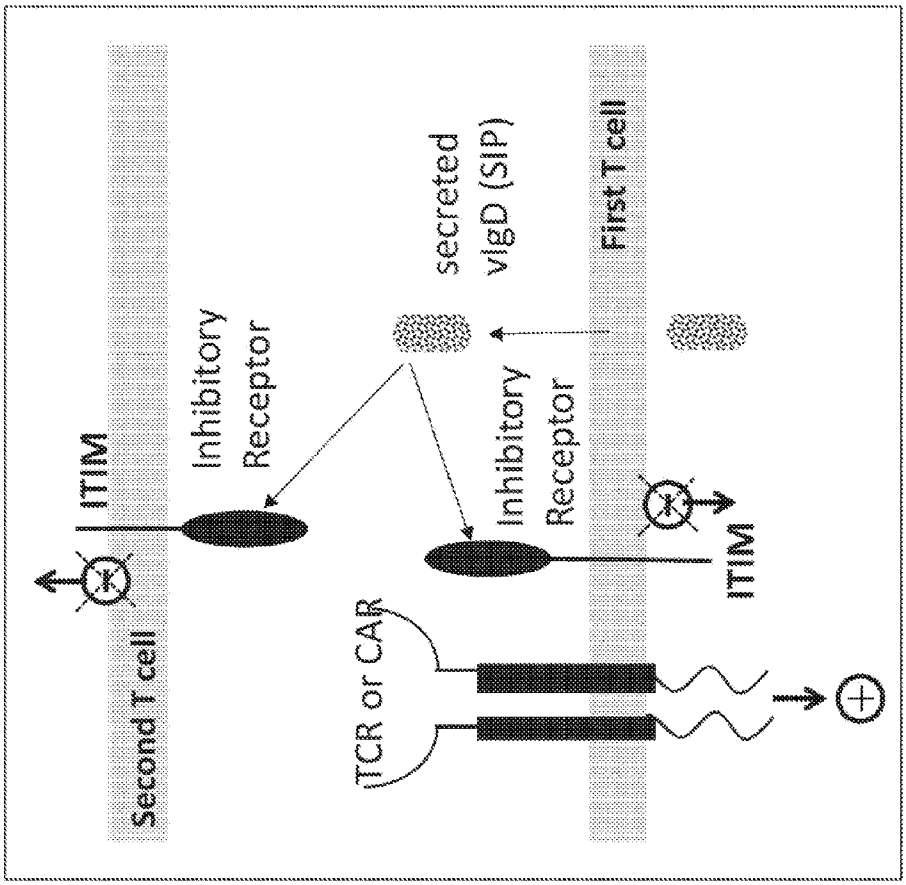
Second T cell
ITIM
Inhibitory Receptor
secreted vIgD (SIP)
First T cell
Inhibitory Receptor
ITIM
TCR or CAR

FIG. 1C

FIG. 2

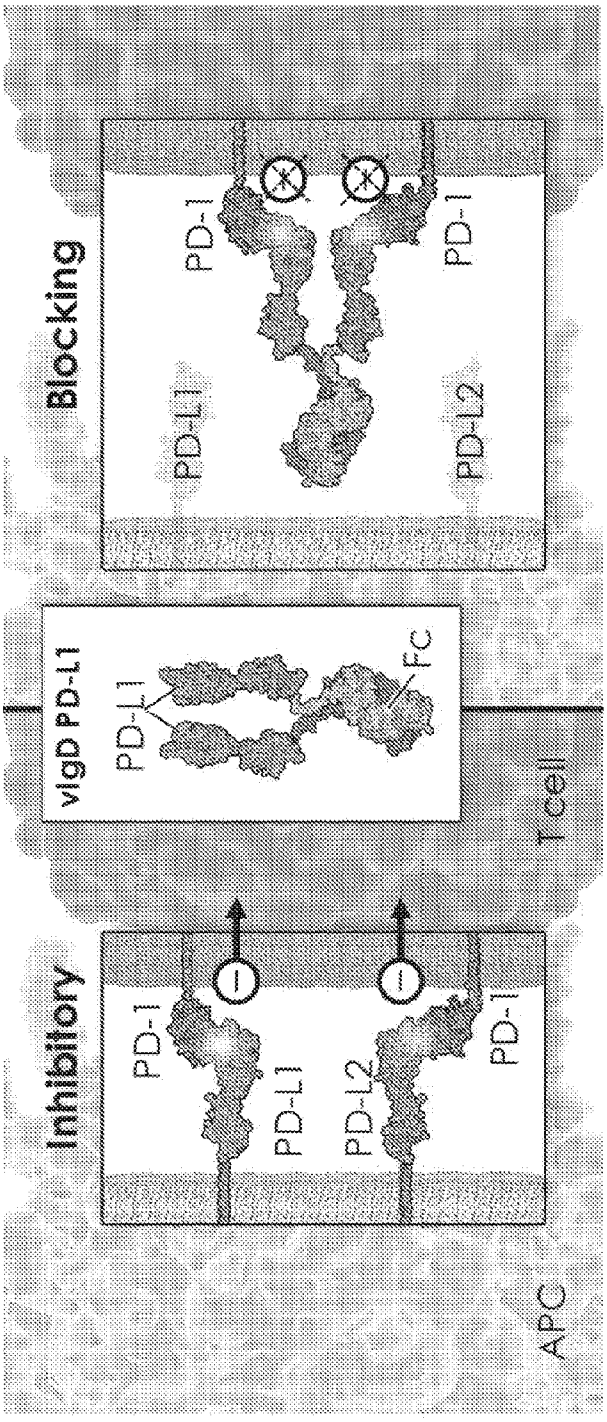
Inhibitory
PD-1
PD-L1
PD-L2
PD-1
APC
vIgD PD-L1
PD-L1
Fc
T cell
Blocking
PD-1
PD-1
PD-L1
PD-L2

FIG. 3

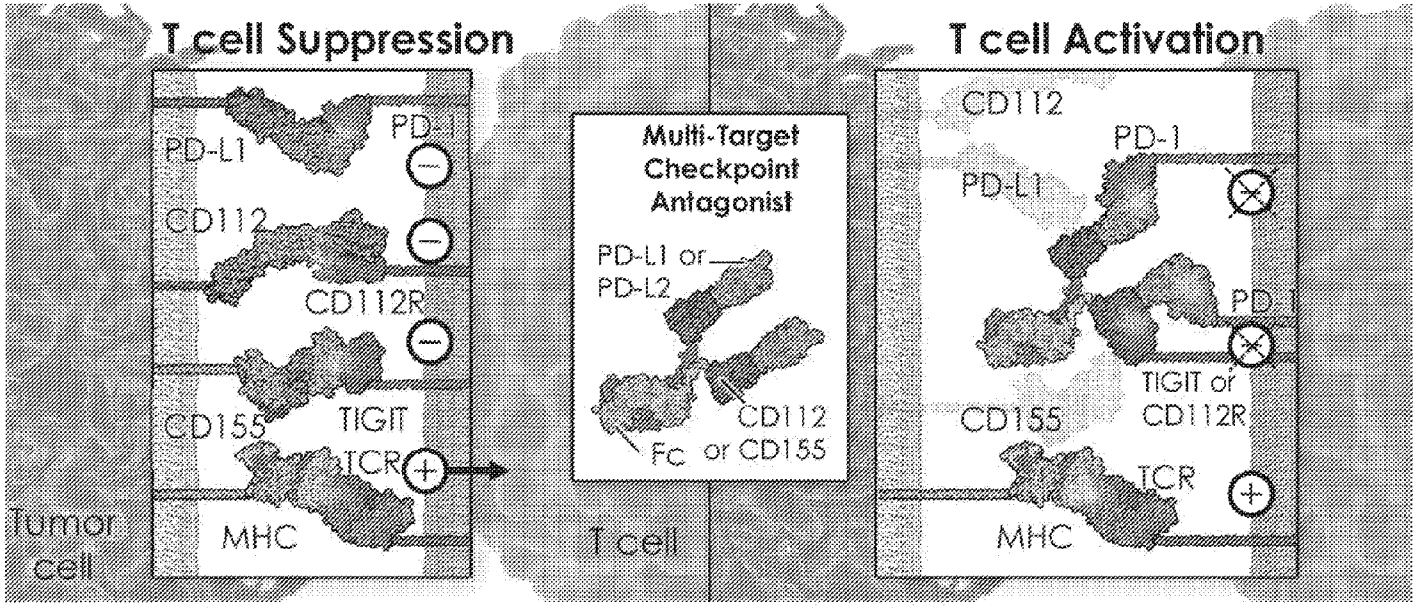


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FIG. 4

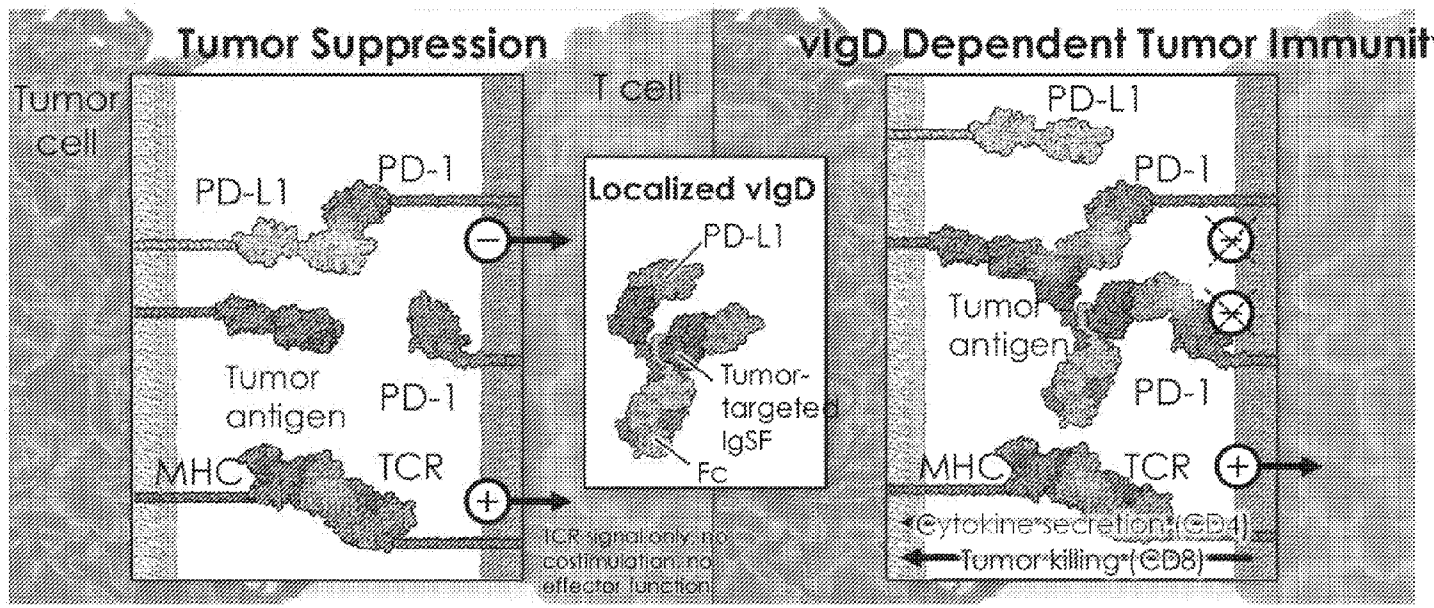


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FIG. 5A

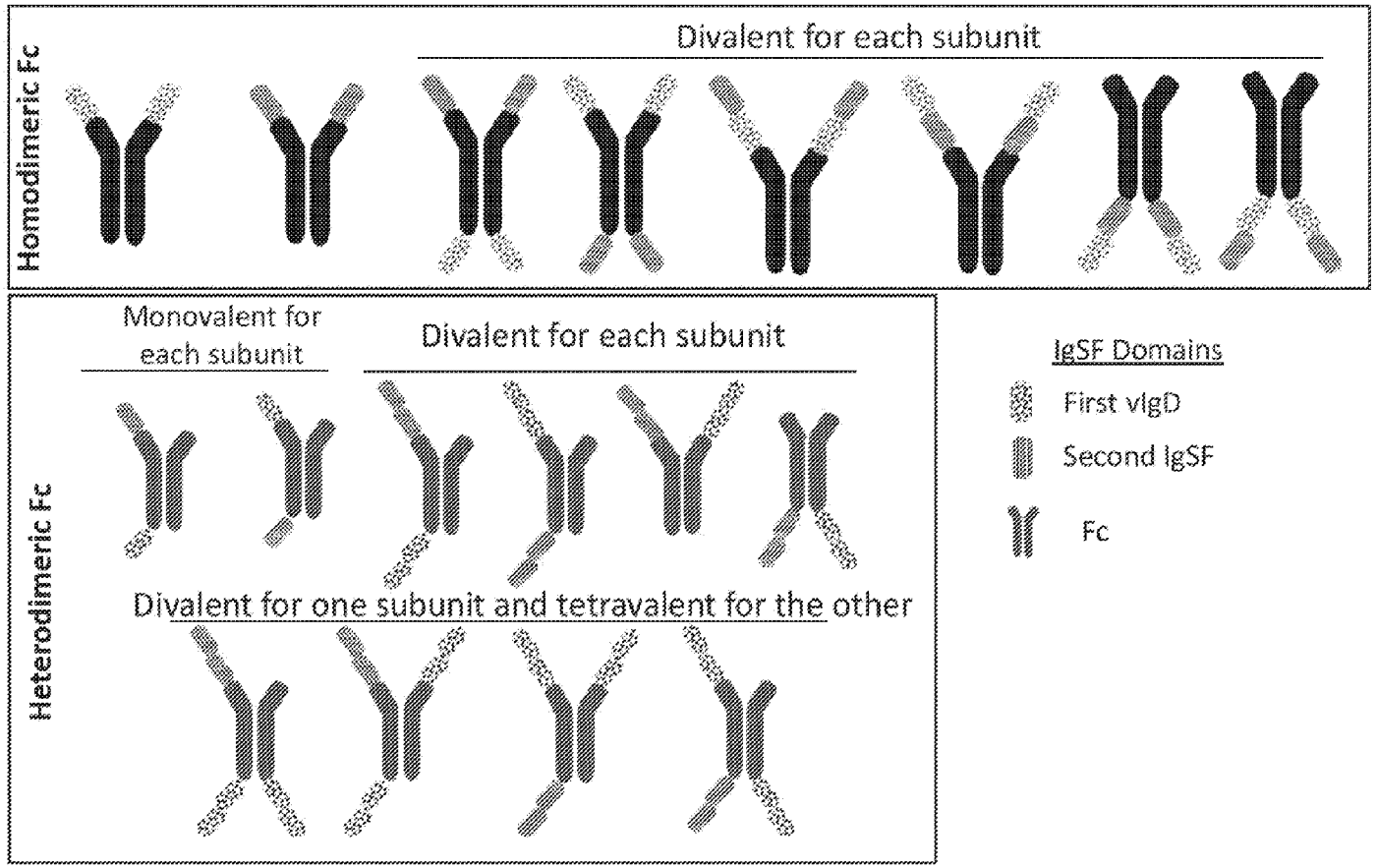

FIG. 5B

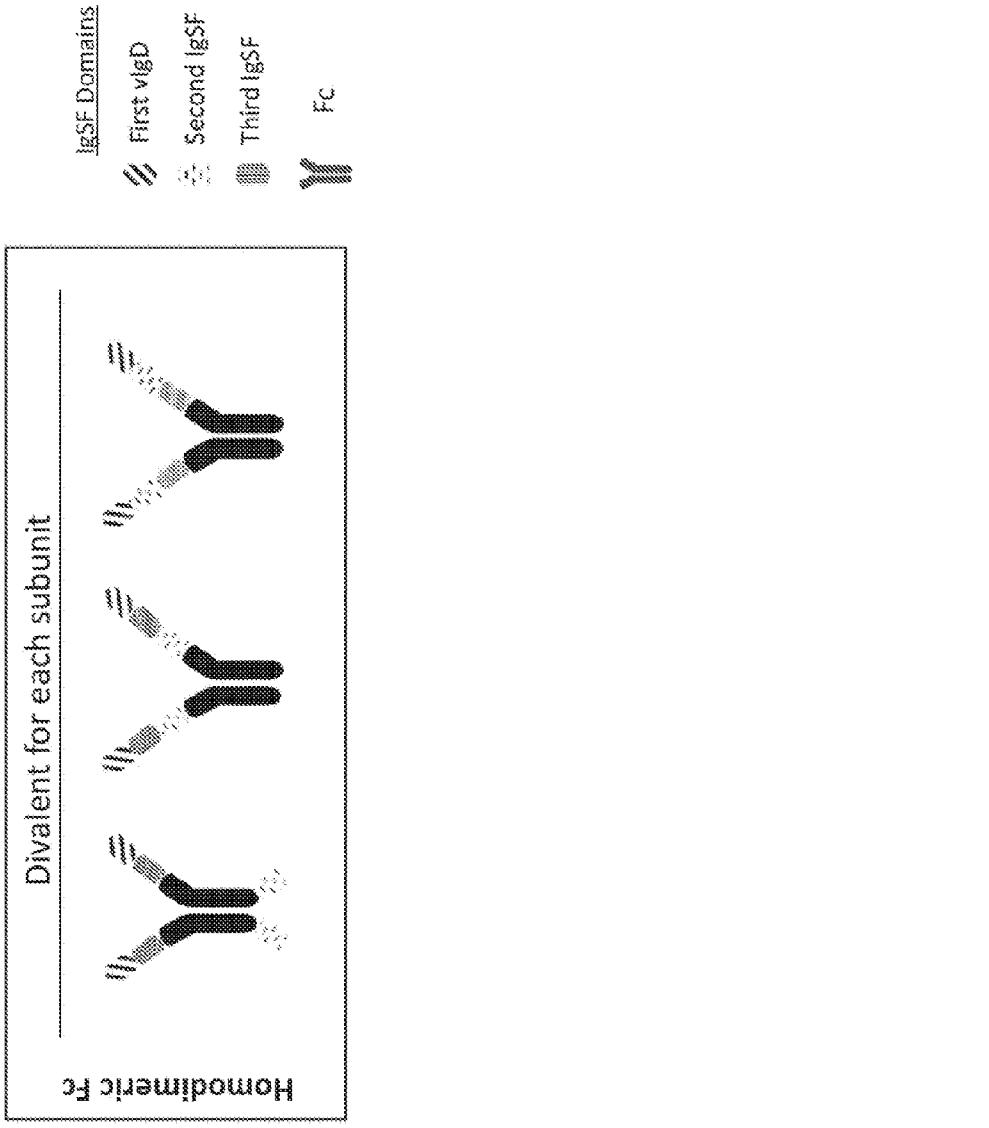
IgSF Domains
First vIgD
Second IgSF
Third IgSF
Fc
Homodimeric Fc
Divalent for each subunit

FIG. 6

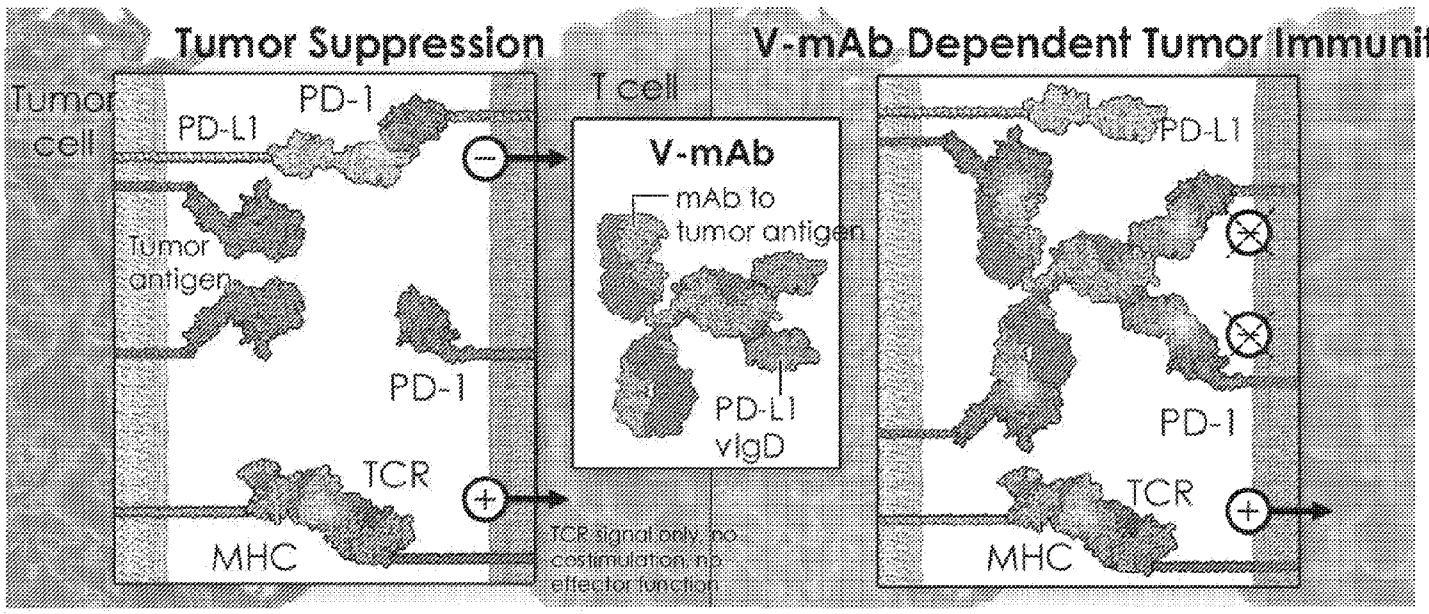


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FIG. 7A

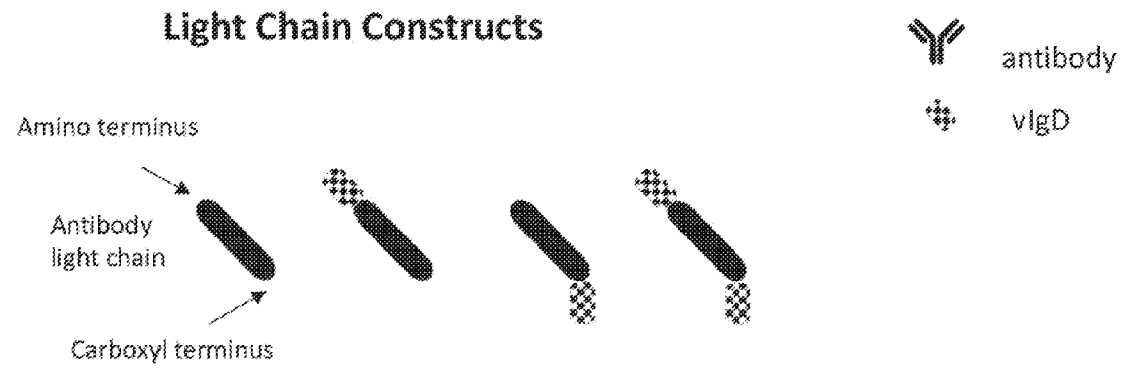
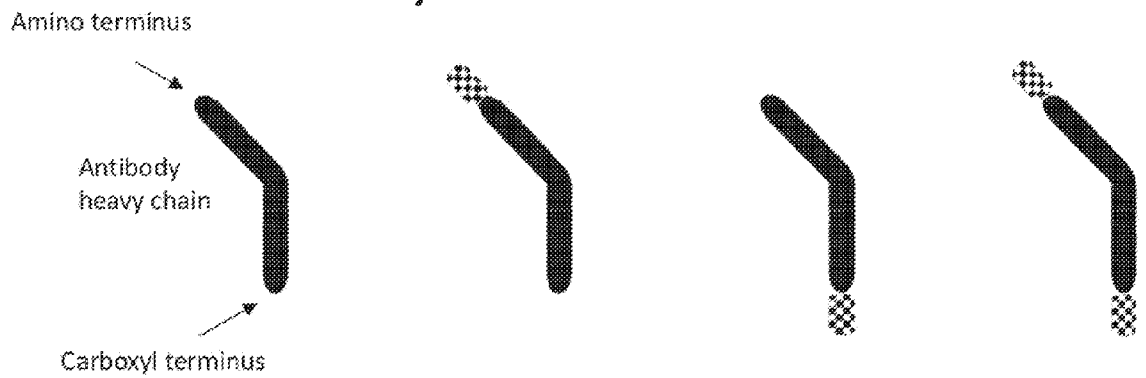

Light Chain Constructs
antibody
vIgD
Amino terminus
Antibody
light chain
Carboxyl terminus

FIG. 7B


Heavy Chain Constructs
Amino terminus
Antibody
heavy chain
Carboxyl terminus

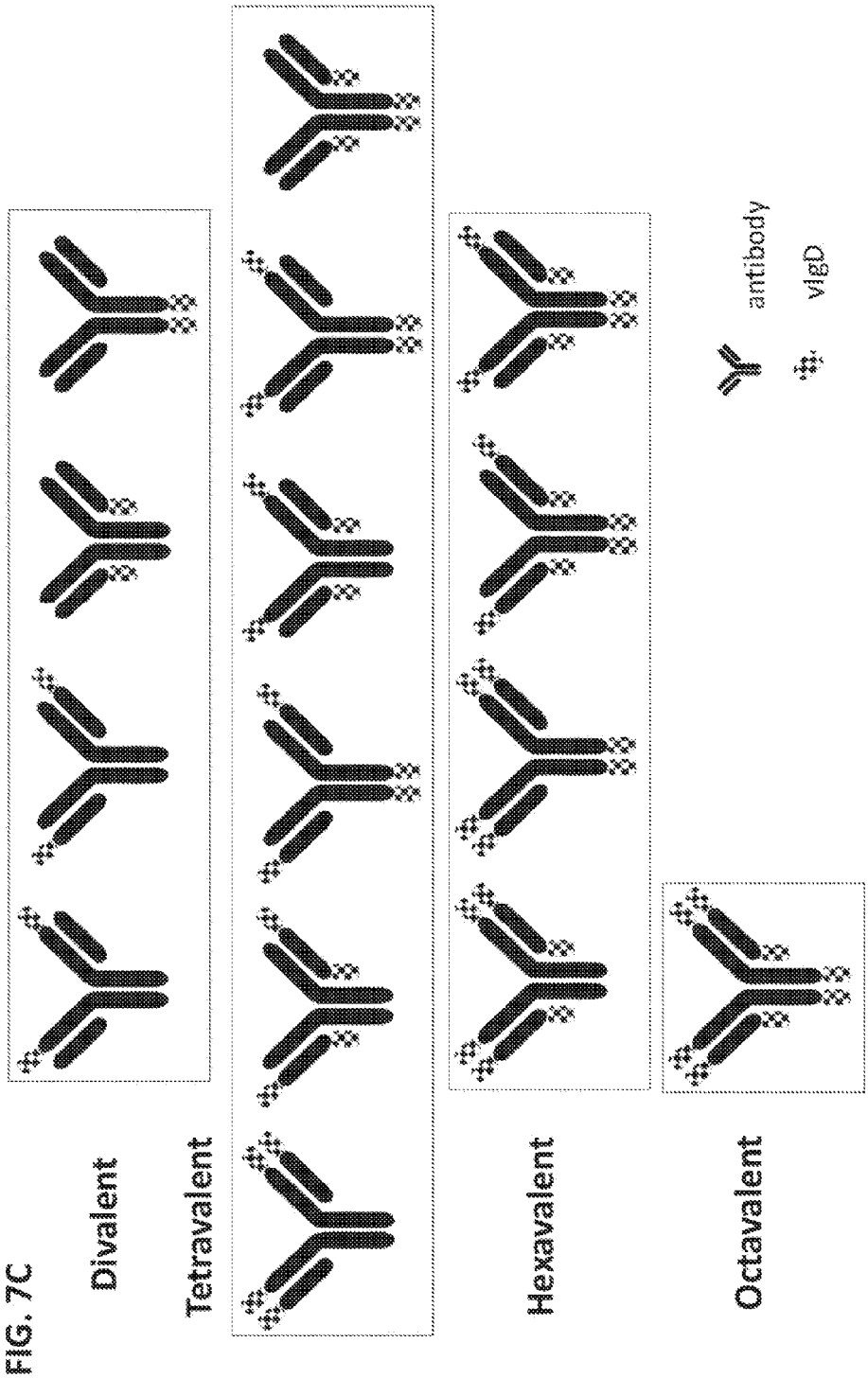

Divalent
Tetravalent
Hexavalent
Octavalent
antibody
vIgD

FIG. 7C

FIG. 8

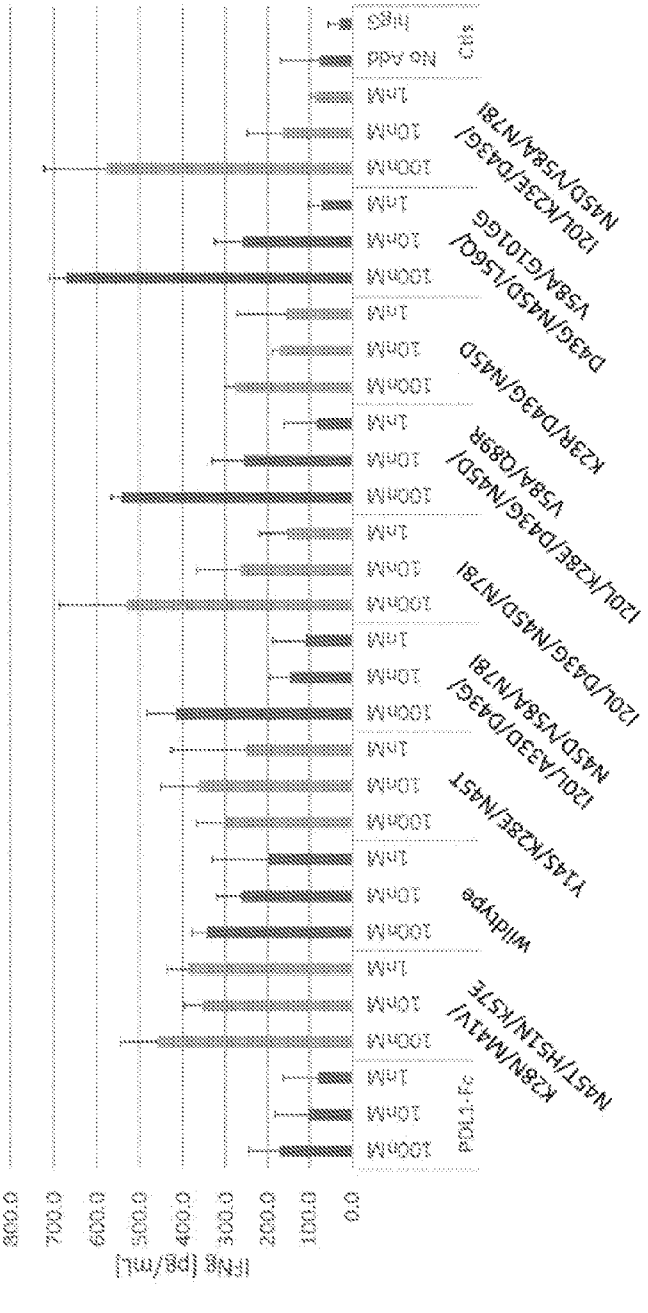
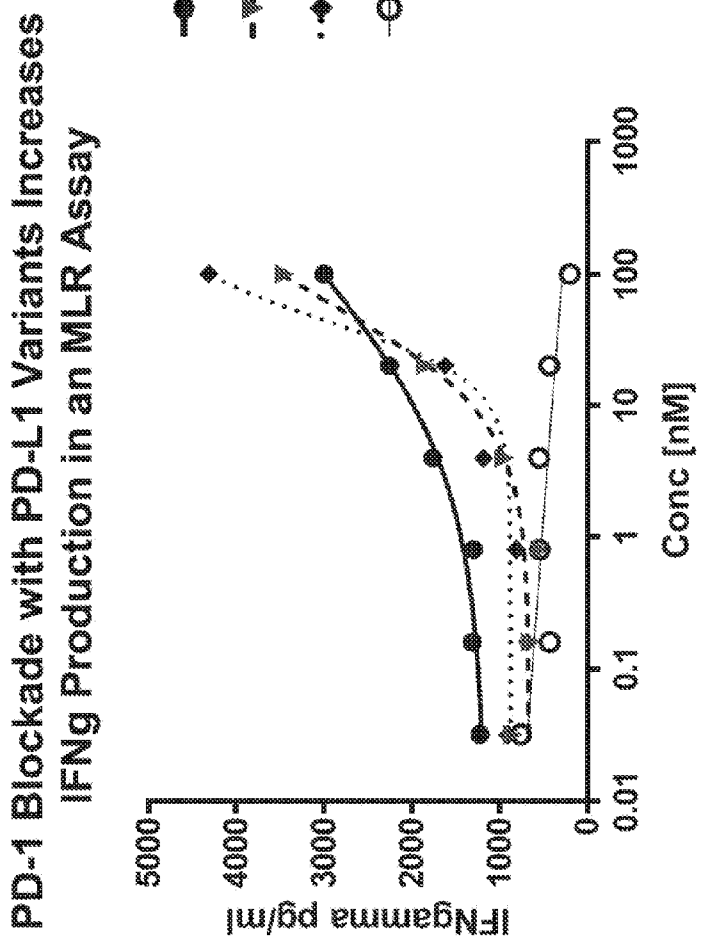

IFNg [pg/mL]
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
CTRL
No Add
hIgG1
PGLALA
100nM
10nM
1nM
K28N/M41V/
N45T/H51N/K57E
wildtype
Y14S/K28E/N45T
I20L/A33D/D43G/
N45D/V58A/N78I
I20L/D43G/N45D/N78I
I20L/K28E/D43G/N45D/
V58A/Q89R
K28R/D43G/N45D
D43G/N45D/L56Q/
V58A/G101GG
I20L/K28E/D43G/
N45D/V58A/N78I

FIG. 9


PD-1 Blockade with PD-L1 Variants Increases
IFNg Production in an MLR Assay
Nivolumab
I20L/K28E/D43G/
N45D/V58A/Q89R
D43G/N45D/L56Q/
V58A/G101G-ins
hIgG
IFNgamma pg/ml
5000
4000
3000
2000
1000
0
0.01
0.1
1
10
100
1000
Conc [nM]

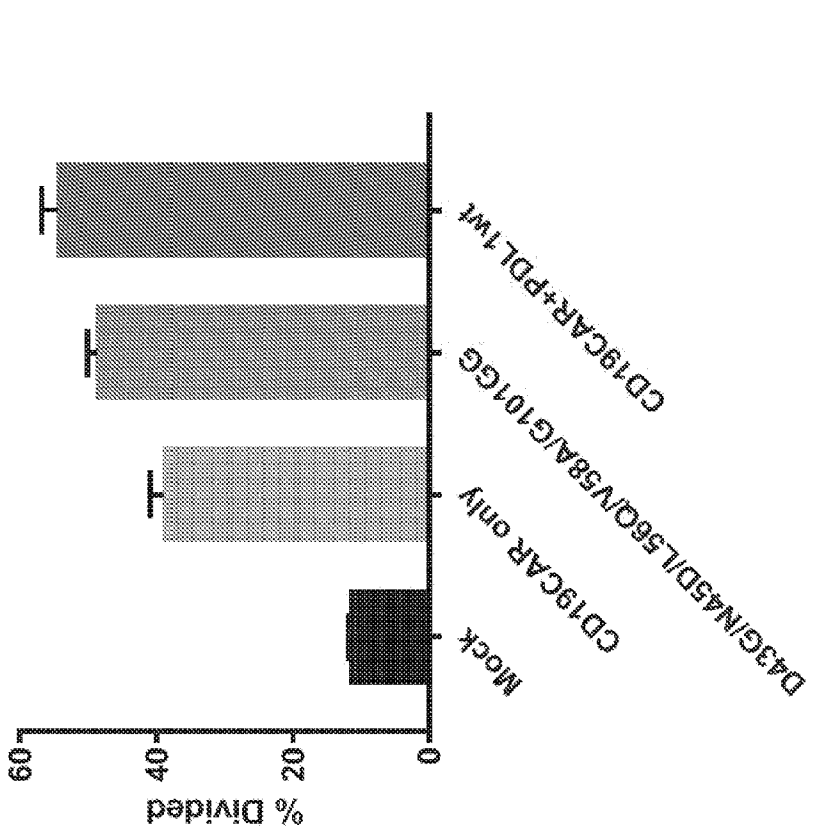

Proliferation of Transduced T cells on Day 3
% Divided
0
20
40
60
Mock
CD19CAR only
D43G/N45D/L56Q/V58A/G101GG
CD19CAR+PDL1wt

FIG. 10

FIG. 11

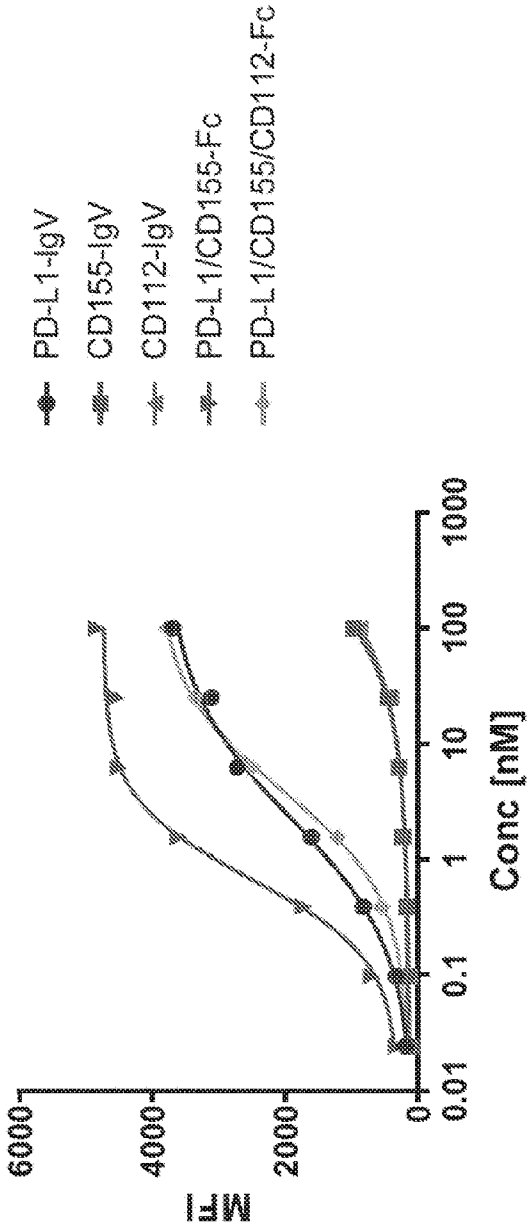
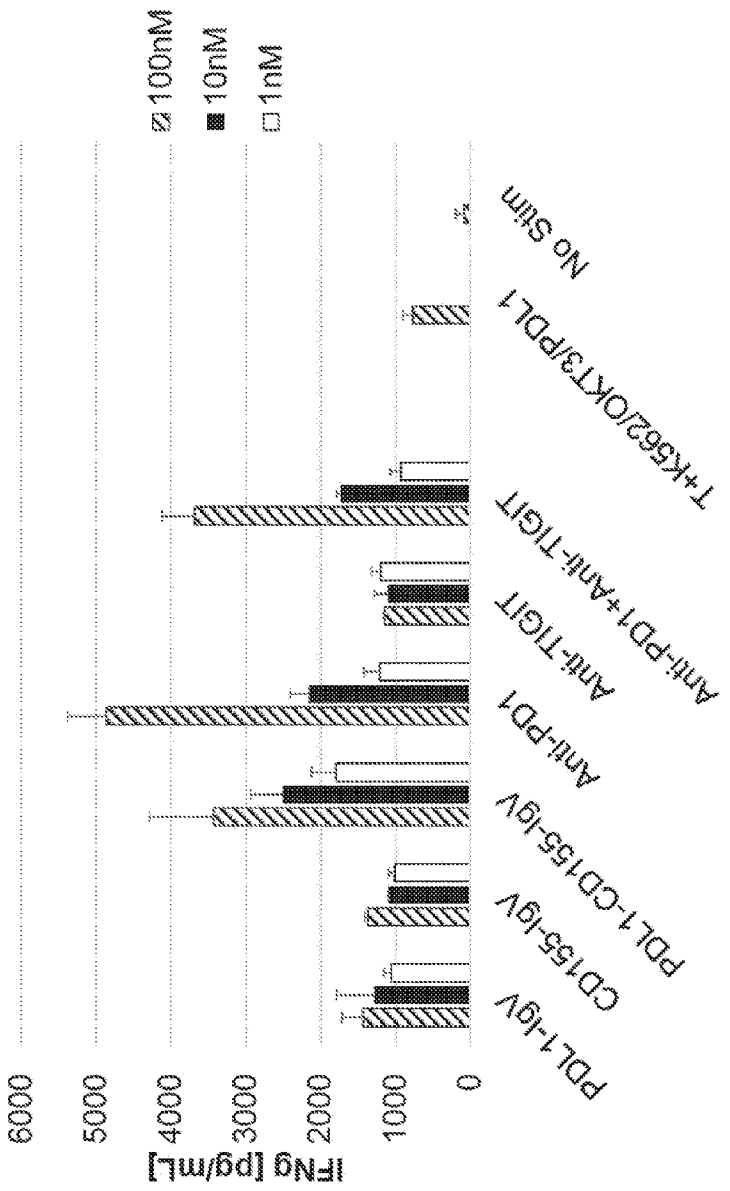

Binding to Exhausted CD4+ T Cells
PD-L1-IgV
CD155-IgV
CD112-IgV
PD-L1/CD155-Fc
PD-L1/CD155/CD112-Fc
MFI
6000
4000
2000
0
0.01
0.1
1
10
100
1000
Conc [nM]

FIG. 12


Multi-Domain vIgDs Increased IFNg Production by Exhausted T Cells
100nM
10nM
1nM
IFNg [pg/mL]
6000
5000
4000
3000
2000
1000
0
PDL1-IgV
CD155-IgV
PDL1-CD155-IgV
Anti-PD1
Anti-TIGIT
Anti-PD1+Anti-TIGIT
T+K562/OKT3/PDL1
No Stim

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.