



(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2018/08/08
(87) Date publication PCT/PCT Publication Date: 2019/02/14
(85) Entrée phase nationale/National Entry: 2020/01/14
(86) N° demande PCT/PCT Application No.: US 2018/045743
(87) N° publication PCT/PCT Publication No.: 2019/032663
(30) Priorité/Priority: 2017/08/09 (US62/542,921)

(51) Cl.Int./Int.Cl. *A61K 38/00* (2006.01),
A61K 38/19 (2006.01), *A61K 39/00* (2006.01),
C07K 14/52 (2006.01), *C07K 16/28* (2006.01)

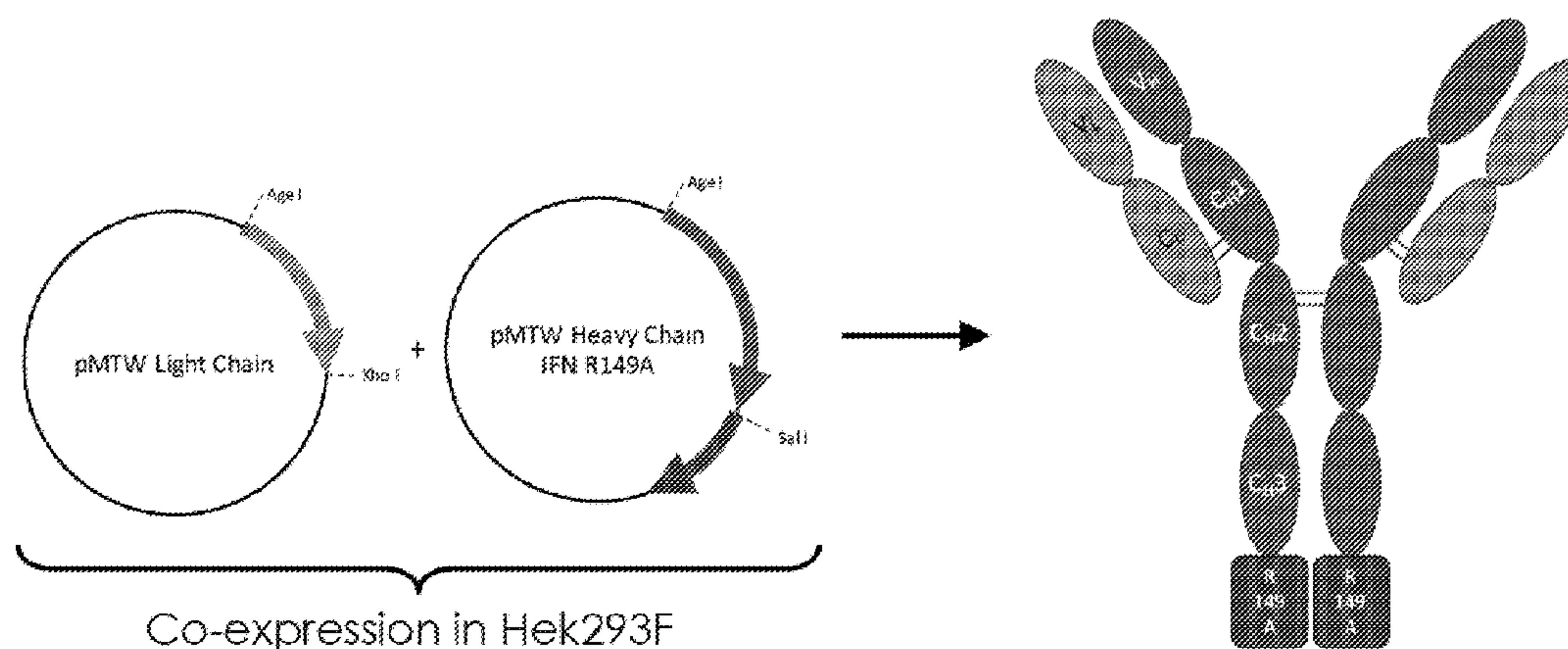
(71) Demandeurs/Applicants:
ORIONIS BIOSCIENCES INC., US;
ORIONIS BIOSCIENCES NV, BE

(72) Inventeurs/Inventors:
KLEY, NIKOLAI, US;
TAVERNIER, JAN, BE;
ZABEAU, LENNART, BE;
DEPLA, ERIK, BE

(74) Agent: DEETH WILLIAMS WALL LLP

(54) Titre : AGENTS DE LIAISON A PD-1 ET PD-L1
(54) Title: PD-1 AND PD-L1 BINDING AGENTS

Figure 1



(57) **Abrégé/Abstract:**

The present invention relates, in part, to agents that bind PD-1 or PD-L1 and their use as diagnostic and therapeutic agents. The present invention further relates to pharmaceutical compositions comprising the PD-1 or PD-L1 binding agents and their use in the treatment of various diseases.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
14 February 2019 (14.02.2019)



(10) International Publication Number
WO 2019/032663 A1

(51) International Patent Classification:

A61K 38/00 (2006.01) *C07K 14/52* (2006.01)
A61K 38/19 (2006.01) *C07K 16/28* (2006.01)
A61K 39/00 (2006.01)

(21) International Application Number:

PCT/US2018/045743

(22) International Filing Date:

08 August 2018 (08.08.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/542,921 09 August 2017 (09.08.2017) US

(71) Applicants: **ORIONIS BIOSCIENCES INC.** [US/US];
275 Grove Street, Newton, MA 02466 (US). **ORIONIS**
BIOSCIENCES NV [BE/BE]; Rijnvisschestraat 120,
B-9052 Zwinjnaarde (BE).

(72) Inventors: **KLEY, Nikolai**; c/o Orionis Biosciences Inc.,
275 Grove Street, Newton, MA 02466 (US). **TAV-**
ERNIER, Jan; Bottelweg 2, B-9860 Balegem (BE).
ZABEAU, Lennart; c/o Orionis Biosciences NV, Rijnviss-
chestraat 120, B-9052 Zwinjnaarde (BE). **DEPLA, Erik**;

C/o Orionis Biosciences NV, Rijnvisschestraat 120, B-9052
Zwinjnaarde (BE).

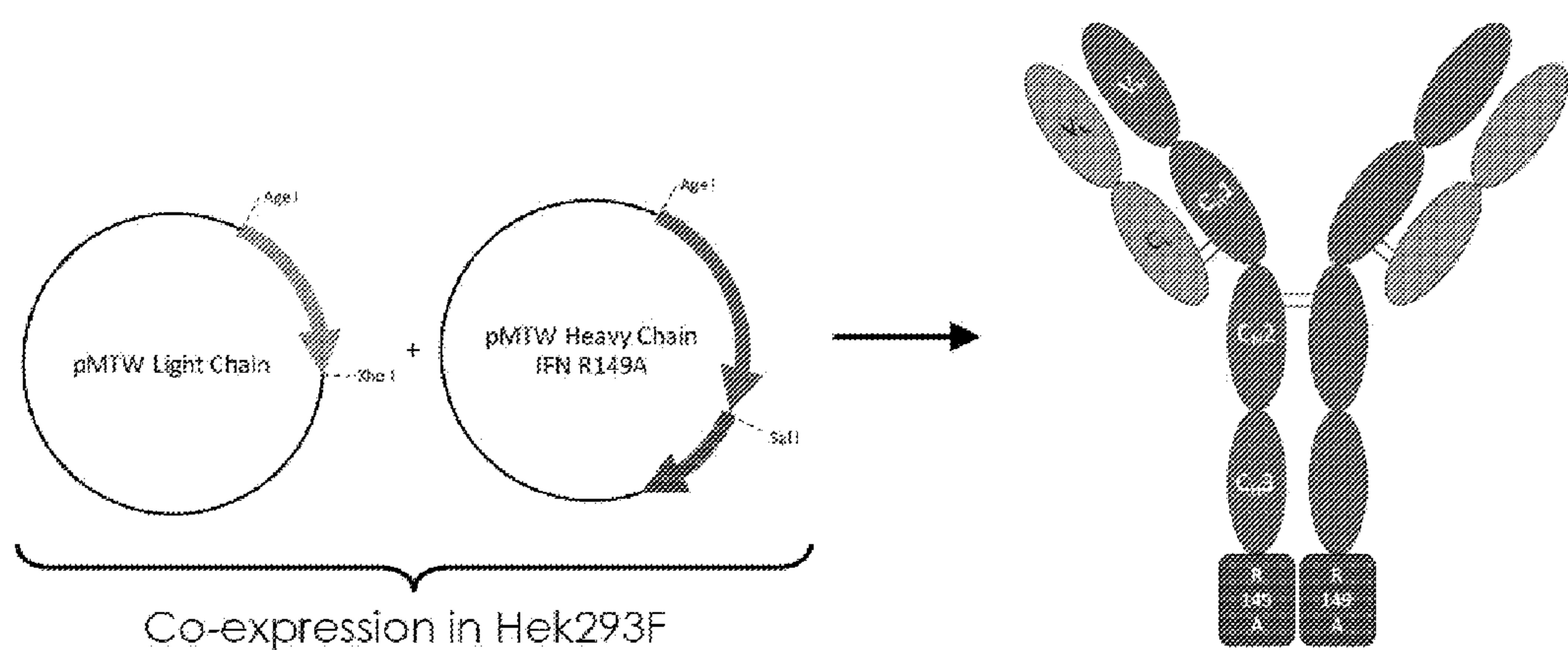
(74) Agent: **ALTIERI, Stephen L.** et al.; Morgan, Lewis &
Bockius LLP, 1111 Pennsylvania Avenue, NW, Washing-
ton, DC 20004 (US).

(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: PD-1 AND PD-L1 BINDING AGENTS

Figure 1



(57) Abstract: The present invention relates, in part, to agents that bind PD-1 or PD-L1 and their use as diagnostic and therapeutic agents. The present invention further relates to pharmaceutical compositions comprising the PD-1 or PD-L1 binding agents and their use in the treatment of various diseases.

[Continued on next page]

WO 2019/032663 A1

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

PD-1 AND PD-L1 BINDING AGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of and priority to U.S. Provisional Patent Application No. 62/542,921, filed August 9, 2017, the content of which is hereby incorporated by reference in its entirety.

FIELD

The present invention relates, in part, to binding agents which bind PD-1 or PD-L1 and their use as therapeutic and diagnostic agents.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: ORN-034PC_ST25, date created: August 8, 2018; file size: 827 KB).

BACKGROUND

Immunotherapies have been developed to re-direct the body's immune system towards cancers. Immunotherapy provides the advantage of cell specificity that other treatment modalities, such as chemotherapy and radiation, lack. As such, methods for enhancing the efficacy of immune based therapies can be clinically beneficial. For example, immune checkpoint molecules that provide costimulatory or coinhibitory signals play a central role in the regulation of T cell immune responses against tumor cells.

However, despite impressive patient responses to agents targeting the checkpoint molecules, including, for example, clinical trials that led to the approval of YERVOY, KEYTRUDA, and OPDIVO, immunotherapies such as checkpoint inhibition therapy still fails in the overwhelming majority of patients. Further still, many immunotherapies are complicated by side effects that significantly narrows a patient's therapeutic window for treatment and makes the patient more susceptible to other diseases.

Accordingly, there remains a need for improved immunotherapeutic agents that can provide targeted therapy against cancers while causing minimal side effects.

SUMMARY

In various aspects, the present invention relates to binding agents having at least one targeting moiety that specifically binds to PD-1 or PD-L1. In various embodiments, these binding agents bind to, and functionally modulate (e.g. partially or fully neutralize) PD-1 or PD-L1. In various embodiments, these binding agents bind to, but do not functionally modulate (e.g. partially or fully neutralize) PD-1 or PD-L1. Therefore, in various embodiments, the present binding agents have use in, for instance, directly or indirectly recruiting a PD-1-expressing cell or a PD-L1-expressing cell to a site of interest while still allowing the cell to signal via either PD-1

or PD-L1 (*i.e.* the binding of the PD-1 or PD-L1 binding agent does not reduce or eliminate PD-1 or PD-L1 signaling at the site of interest). In an embodiment, the targeting moiety is a single domain antibody (VHH).

In various embodiments, the binding agents of the invention further comprises a signaling agent, *e.g.*, without limitation, an interferon, an interleukin, and a tumor necrosis factor, that may be modified to attenuate activity. In various embodiments, the binding agent comprises additional targeting moieties that bind to other targets (*e.g.* antigens, receptor) of interest. In an embodiment, the other targets (*e.g.* antigens, receptor) of interest are present on tumor cells. In another embodiment, the other targets (*e.g.* antigens, receptor) of interest are present on immune cells. In some embodiments, the present binding agent may directly or indirectly recruit an immune cell (*e.g.* a dendritic cell) to a site of action (such as, by way of non-limiting example, the tumor microenvironment). In some embodiments, the present binding agent facilitates the presentation of antigens (*e.g.*, tumor antigens) by dendritic cells.

In various embodiments, the present binding agents find use in the treatment of various diseases or disorders such as cancer, infections, immune disorders, and other diseases and disorders, and the present invention encompasses various methods of treatment.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 provides a schematic of the cloning strategy of the PD-1 or PD-L1 Actaferons described in Example 1.

Figure 2 shows binding of PD-L1 (top panel) or PD-1 (bottom panel) Actaferons to transfected Hek293T cells.

Figure 3 shows PD-1-targeting as assessed by pSTAT1 status in peripheral blood mononuclear cells (PBMC's). Data are plotted as percentage of unstimulated pSTAT1 mean fluorescence intensity (MFI)

Figure 4 shows PD-1-targeting as assessed by pSTAT1 status in transfected Hek293T cells. Percentage of pSTAT1 cells is plotted.

Figure 5 shows PD-L1-targeting as assessed by pSTAT1 status in MDA-MB-231 cells. Data are plotted mean fluorescence intensities (MFI's).

Figure 6 shows binding of PD-L1 VHHs to transfected Hek293T cells. Data are plotted mean fluorescence intensities (MFI's).

Figures 7A-G are graphs showing the biological activity of PD-L1 VHH Actaferons (AFNs). Biological activity was measured on parental HL116 cells (an IFN responsive cell-line stably transfected with a p6-16 luciferase reporter). Luciferase activity was induced by serial dilutions of PD-L1 VHH AFNs in the presence or absence of an excess (20 μ g/ml) of the corresponding PD-L1 VHH. The PD-L1 VHHS tested were 2LIG3 (Figure 7A), 2LIG27 (Figure 7B), 2LIG97 (Figure 7C), 2LIG99 (Figure 7D), 2LIG176 (Figure 7E), 2LIG189 (Figure 7F), and 3LIG8 (Figure 7G).

Figure 8 is a graph showing the biological activity of an atezolizumab-Actaferon (ate-AFN; *i.e.*, Ate-IFNa2mut) on parental HL116 cells (an IFN responsive cell-line stably transfected with a p6-16 luciferase reporter). Biological

activity was measured by luciferase activity induced by a serial dilution of ate-AFN in the presence or absence of an excess (50 µg/ml) of atezolizumab (ate).

Figure 9 is a graph showing the *in vivo* anti-tumoral activity of 2LIG99 based-AFN in a RL tumor model in mice with a humanized immune system.

Figures 10A-C are graphs showing pSTAT1 in Hek293T cells transfected with human PD-1 or an empty vector that were stimulated with serial dilutions of 102C3 AFN (Figure 10A), 102C12 AFN (Figure 10B), or wild type IFNα2 (Figure 10C). Percentage of pSTAT1 positive cells are plotted in the graphs.

DETAILED DESCRIPTION

The present invention is based, in part, on the discovery of binding agents (e.g. antibodies such as, by way of non-limiting example, VHHs) that recognize and bind to PD-1 or PD-L1. In some embodiments, the present binding agents are part of a chimeric or fusion protein with one or more targeting moieties and/or one or more signaling agents. In various embodiments, these binding agents bind to, and functionally modulate (e.g. partially or fully neutralize) PD-1 or PD-L1. In some embodiments, these binding agents bind to, but do not functionally modulate PD-1 or PD-L1.

The present invention further provides pharmaceutical compositions comprising the binding agents and their use in the treatment of various diseases, including cancer, autoimmune, and/or neurodegenerative diseases.

PD-1 or PD-L1 Binding Agents

In various embodiments, the present PD-1 or PD-L1 binding agent is a protein-based agent capable of specific binding to PD-1 or PD-L1. In various embodiments, the present PD-1 or PD-L1 binding agent is a protein-based agent capable of specific binding to PD-1 or PD-L1 without functional modulation (e.g., partial or full neutralization) of PD-1 or PD-L1.

Programmed cell death protein 1, also known as PD-1 and cluster of differentiation 279 (CD279), is a cell surface receptor that is primarily expressed on activated T cells, B cells, and macrophages. PD-1 has been shown to negatively regulate antigen receptor signaling upon engagement of its ligands (*i.e.*, PD-L1 and/or PD-L2). PD-1 plays an important role in down-regulating the immune system and promoting self tolerance by suppressing T cell inflammatory activity. PD-1 is a type I transmembrane glycoprotein containing an Ig Variable-type (V-type) domain responsible for ligand binding and a cytoplasmic tail that is responsible for the binding of signaling molecules. The cytoplasmic tail of PD-1 contains two tyrosine-based signaling motifs, an ITIM (immunoreceptor tyrosine-based inhibition motif) and an ITSM (immunoreceptor tyrosine-based switch motif).

In various embodiments, the PD-1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that recognizes an epitope present on PD-1. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes present on PD-1. As used herein, a linear epitope refers to any continuous sequence of amino acids present on PD-1. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on PD-1. As used herein, a conformation epitope refers to one or

more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the PD-1 binding agent of the present invention may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of human PD-1. In various embodiments, the PD-1 binding agent of the invention may bind to any forms of the human PD-1. In an embodiment, the PD-1 binding agent binds to a phosphorylated form of PD-1.

In an embodiment, the present PD-1 binding agent comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on human PD-1. In an embodiment, the human PD-1 comprises the amino acid sequence of SEQ ID NO: 1.

In another embodiment, the human PD-1 comprises the amino acid sequence of SEQ ID NO:1 without the amino-terminal signal peptide.

In various embodiments, the present PD-1 binding agent comprises a targeting moiety capable of specific binding. In various embodiments, the PD-1 binding agent comprises a targeting moiety having an antigen recognition domain such as an antibody or derivatives thereof. In an embodiment, the PD-1 binding agent comprises a targeting moiety which is an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains. Each heavy chain includes one variable region (e.g., V_H) and at least three constant regions (e.g., CH₁, CH₂ and CH₃), and each light chain includes one variable region (V_L) and one constant region (C_L). The variable regions determine the specificity of the antibody. Each variable region comprises three hypervariable regions also known as complementarity determining regions (CDRs) flanked by four relatively conserved framework regions (FRs). The three CDRs, referred to as CDR1, CDR2, and CDR3, contribute to the antibody binding specificity. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the PD-1 binding agent comprises a targeting moiety which is an antibody derivative or format. In some embodiments, the present PD-1 binding agent comprises a targeting moiety which is a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; an aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab', a F(ab')₂, a peptide mimetic molecule, or a synthetic molecule, as described in US Patent Nos. or Patent Publication Nos. US 7,417,130, US 2004/132094, US 5,831,012, US 2004/023334, US 7,250,297, US 6,818,418, US 2004/209243, US 7,838,629, US 7,186,524, US 6,004,746, US 5,475,096, US 2004/146938, US 2004/157209, US 6,994,982, US 6,794,144, US 2010/239633, US 7,803,907, US

2010/119446, and/or US 7,166,697, the contents of which are hereby incorporated by reference in their entireties. See also, Storz MABs. 2011 May-Jun; 3(3): 310–317.

In some embodiments, the PD-1 binding agent comprises a targeting moiety which is a single-domain antibody, such as a VHH. The VHH may be derived from, for example, an organism that produces VHH antibody such as a camelid, a shark, or the VHH may be a designed VHH. VHHs are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (V_H) and two constant domains (CH2 and CH3).

In an embodiment, the PD-1 binding agent comprises a VHH. In some embodiments, the VHH is a humanized VHH or camelized VHH.

In some embodiments, the VHH comprises a fully human V_H domain, e.g. a HUMABODY (Crescendo Biologics, Cambridge, UK). In some embodiments, fully human V_H domain, e.g. a HUMABODY is monovalent, bivalent, or trivalent. In some embodiments, the fully human V_H domain, e.g. a HUMABODY is mono- or multi-specific such as monospecific, bispecific, or trispecific. Illustrative fully human V_H domains, e.g. a HUMABODIES are described in, for example, WO2016/113555 and WO2016/113557, the entire disclosure of which is incorporated by reference.

In some embodiments, the PD-1 binding agent comprises a targeting moiety which is a VHH comprising a single amino acid chain having four “framework regions” or FRs and three “complementary determining regions” or CDRs. As used herein, “framework region” or “FR” refers to a region in the variable domain which is located between the CDRs. As used herein, “complementary determining region” or “CDR” refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the PD-1 binding agent comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences. In various embodiments, the PD-1 binding agent comprises a VHH having a variable region comprising at least one FR1, FR2, FR3, and FR4 sequences.

In some embodiments, the PD-1 CDR1 sequence is selected from: GFSMDYYAIA (SEQ ID NO: 2); GFSVDYYAIA (SEQ ID NO: 3); GGFNRVSYMG (SEQ ID NO: 4); GIIKSINFMG (SEQ ID NO: 5); GFILDYYGIG (SEQ ID NO: 6); GLSLDYDGVG (SEQ ID NO: 7); GRTFSSLGMG (SEQ ID NO: 8); GFAFGSYDMG (SEQ ID NO: 9); GFSFGNNDMS (SEQ ID NO: 10); IHAMG (SEQ ID NO: 11); INAMA (SEQ ID NO: 12); SGTMG (SEQ ID NO: 13); GSIASIHAM (SEQ ID NO: 14); GSIASIHAMG (SEQ ID NO: 15); FYGMG (SEQ ID NO: 16); GGTFSFYGMG (SEQ ID NO: 17); YYAIA (SEQ ID NO: 18); VSYMG (SEQ ID NO: 19); INFMG (SEQ ID NO: 20); SLGMG (SEQ ID NO: 21); SYDMG (SEQ ID NO: 22); and NNDMS (SEQ ID NO: 23).

In some embodiments, the PD-1 CDR2 sequence is selected from: CITGSDFMVD (SEQ ID NO: 24); SVTSGGEI (SEQ ID NO: 25); STTSDGRT (SEQ ID NO: 26); CISSSDGST (SEQ ID NO: 27); AIAWNGAST (SEQ ID NO: 28); GINSGGRT (SEQ ID NO: 29); AINSGGGST (SEQ ID NO: 30); AITWSGGITYYEDSVKG (SEQ ID NO: 31); VITWSGGITYYADSVKG (SEQ ID NO: 32); VITVSGGITYYADSVKG (SEQ ID NO: 33); AITWSGGITYYADSLKG

(SEQ ID NO: 34); LISWSGGSTYYEDSVKG (SEQ ID NO: 35); SIPWSGGRIYYADSVKG (SEQ ID NO: 36); VITWSGGITY (SEQ ID NO: 37); VITVSGGITY (SEQ ID NO: 38); DIRTSAGRITYYADSVKG (SEQ ID NO: 39); DIRTSAGRITY (SEQ ID NO: 40); CITGSDFMVDITY (SEQ ID NO: 41); CITGSDFMVDITYYVASVKG (SEQ ID NO: 42); SVTSGGEIT (SEQ ID NO: 43); SVTSGGEITIADSVKG (SEQ ID NO: 44); SVTSGGEITVADSVKG (SEQ ID NO: 45); STTSDGRTT (SEQ ID NO: 46); STTSDGRTTVADSVKG (SEQ ID NO: 47); CISSSDGSTY (SEQ ID NO: 48); AIAWNGASTY (SEQ ID NO: 49); AIAWNGASTYYTESVKG (SEQ ID NO: 50); GINSGGRITD (SEQ ID NO: 51); GINSGGRITDYADSVTG (SEQ ID NO: 52); AINSGGGSTY (SEQ ID NO: 53); and AINSGGGSTYYADSVKG (SEQ ID NO: 54).

In some embodiments, the PD-1 CDR3 sequence is selected from: AVRSTANTLCPSHYVMDY (SEQ ID NO: 55); AVRSTANTLCPSHYIMDY (SEQ ID NO: 56); NADIWVSDARMYNY (SEQ ID NO: 57); NADIWLPSDRMYNY (SEQ ID NO: 58); ATATLCDGGIWGY (SEQ ID NO: 59); AASGLGSVVVTANEYDY (SEQ ID NO: 60); AQGDRSSWHYYGMDY (SEQ ID NO: 61); ATKSDPMTNEYDL (SEQ ID NO: 62); DRAESSWYDY (SEQ ID NO: 63); DKHQSSWYDY (SEQ ID NO: 64); DKHQSSFYDY (SEQ ID NO: 65); DRAQSSWYDY (SEQ ID NO: 66); DRVDSNWYDY (SEQ ID NO: 67); KERSTGWDFAS (SEQ ID NO: 68); and EMSGISGWDY (SEQ ID NO: 69).

In various exemplary embodiments, the PD-1 binding agent comprises an amino acid sequence selected from the following sequences:

2PD23

QVQLQESGGGLVQPGGSLRLSCAASGFSMDYYAIAWFRQAPGKEREEISCITGSDFMVDITYYVASVKGRFTISR
DNAENTAYLQMNNLKPEDTGVYFCAVRSTANTLCPSHYVMDYWGKGTQVTVSSAAAYPYDVPDYGSHHHHH
H (SEQ ID NO: 70);

2PD26

QVQLQESGGGLVQAGGSLRLSCAASGFSMDYYAIAWFRQAPGKEREEISCITGSDFMVDITYYVASVKGRFTISR
DNAENTAYLQMNNLKPEDTGVYFCAVRSTANTLCPSHYVMDYWGKGTQVTVSSAAAYPYDVPDYGSHHHHH
H (SEQ ID NO: 71);

2PD90

QVQLQESGGGLVQPGGSLRLSCSASGFSVDYYAIAWFRQAPGKEREEISCITGSDFMVDITYYVASVKGRFTISR
DNAKNTAYLQMNSLKPEDTGVYFCAVRSTANTLCPSHYIMDYWGKGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 72);

2PD-106

QVQLQESGGGLVQPGGSLRLSCSASGFSMDYYAIAWFRQAPGKEREEISCITGSDFMVDITYYVASVKGRFTISR
DNAKNTAHLQMNSLKPEDTGVYFCAVRSTANTLCPSHYVMDYWGKGTQVTVSSAAAYPYDVPDYGSHHHHH
H (SEQ ID NO: 73);

2PD-16

QVQLQESGGGLVQAGGSLRLSCAASGGFNRSYMGWYRQAPGTKRELVASVTSGGEITIADSVKGRFTVSRDN
SKNTLYLQMNGLKPEDGATYWCNADIWVSDARMYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 74);

2PD71

QVQLQESGGGLVQTGESLRLSCAASGGFNRSYMGWYRQAPGSKRELVASVTSGGEITVADSVKGRFTVSRD
NNKNTLYLQMNGLKPEDGATYWCNADIWVSDARMYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 75);

2PD-152

QVQLQESGGGLVQTGESLRLSCAASGLIKSINFMGWYRQPPGTKRELVASTTSDGRITVADSVKGRFTISRDN
KNTIYLEMSSLKPEDTATYWCNADIWLPSDRMYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 76);

2PD-12

QVQLQESGGGLVQAGGSLRLSCAVSGFILDYYGIGWFRQAPGKEREAVSCISSSDGSTYYADSVKGRFTISRDN
ALNTLYLQMNSLKPEDTAVYHCATATLCDGGIWGYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 77);

3PD55

QVQLQESGGGLAQAGGSLRLSCEGSGLSLDYDGVGWFRQAPGKEREAVSCISSSDGSTYYADSVKGRFTISRG
NALNTLYLQMNSLKPEDTAVYYCATATLCDGGIWGYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 78);

3PD82

QVQLQESGGGSVQPGSLRLSCAVSGFILDYYGIGWFRQAPGKEREAVSCISSSDGSTYYADSVKGRFTISRDN
ALNTLYLQMNSLKPEDTAVYYCATATLCDGGIWGYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 79);

2PD8

QVQLQESGGGSVQAGDSLRLSCTASGRTFSSLGMGWFRQAPGKEREFVSAIAWNGASTYYTESVKGRFTISRDN
DAKNTVYLQMNSLKPTDTAVYFCAASGLGSVVTANEYDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 80);

2PD27

QVQLQESGGGSVQPGKSLRLSCAASGRTFSSLGMGWFRQAPGKEREFVSAIAWNGASTYYTESVKGRFTISRDN
DAKNTVYLQMNSLKPTDTAVYFCAASGLGSVVTANEYDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 81);

2PD82

QVQLQESGGGLVQPGGSLRLSCTTSGFAFGSYDMGWVRQAPGKGPEWWSGINSGRITDYADSVTGRFTISR
DNAKNTLYLQMNSLKPEDTAVYYCAQGDRSSWHYYGMDYWGKGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 82); or

3PD36

QVQLQESGGGLVQPGGSLRLSCAASGFSFGNNDMSWVRQAPGKGPEWWSAINSGGGSTYYADSVKGRFTISR
DNAKNTLYLQMNSLKPEDTAVYYCATKSDPMTNEYDLWGXGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 83).

In various exemplary embodiments, the PD-1 binding agent comprises an amino acid sequence selected from SEQ ID NO: 70 to SEQ ID NO: 83 without the terminal histidine tag sequence (*i.e.*, HHHHHH; SEQ ID NO: 84).

In some embodiments, the PD-1 binding agent comprises an amino acid sequence selected from SEQ ID Nos: 70-83 (provided above) without the HA tag (*i.e.*, YPYDVPDYGS; SEQ ID NO: 85).

In some embodiments, the PD-1 binding agent comprises an amino acid sequence selected from SEQ ID Nos: 70-83 (provided above) without the AAA linker.

In some embodiments, the PD-1 binding agent comprises an amino acid sequence selected from SEQ ID Nos: 70-83 (provided above) without the AAA linker, HA tag, and terminal histidine tag sequence (*i.e.*, AAAYPYDVPDYGSHHHHHH; SEQ ID NO: 86).

In various exemplary embodiments, the PD-1 binding agent comprises an amino acid sequence selected from the following sequences:

102C3:

QVQLQESGGGLVQAGKSLRLSCAASGSIFSIIHAMGWFRQAPGKEREFVAAITWSGGITYYEDSVKGRFTISRDN
AKNTVYLYQMNSLKPEDTAIYYCAADRAESSWYDYWGQGTQVTVSS (SEQ ID NO: 1246); or

102C12:

QVQLQESGGGLVQAGKSLRLSCAASGSIIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
AKNTVYLYQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 1247).

In various embodiments, the PD-1 binding agent comprises an amino acid sequence described in U.S. Publication No. 2017/0137517, the entire contents of which are incorporated by reference. By way of example, in some embodiments the PD-1 binding agent comprises one of the following sequences in U.S. Publication No. 2017/0137517:

EVQLVESGGGLVQAGGSLRLSCAASGSIIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
AKNTVYLYQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 87);

EVQLVESGGGLVQAGGSLRLSCAASGSIIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
AKNTVYLYQMNSLKPEDTATYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 88);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVYQLQMNSLKPEDTATYYCAGDKHQSSWYDYWGQGTLVKVSS (SEQ ID NO: 89);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTQVQVSS (SEQ ID NO: 90);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTTLVTVKS (SEQ ID NO: 91);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTTLVTVQS (SEQ ID NO: 92);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRDN
AKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVKVSS (SEQ ID NO: 93);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVQVSS (SEQ ID NO: 94);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTTLVTVKS (SEQ ID NO: 95);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTTLVTVQS (SEQ ID NO: 96);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTTLTVSS (SEQ ID NO: 97);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVKVSS (SEQ ID NO: 98);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVQVSS (SEQ ID NO: 99);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NAKNTVYQLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTTLVTVKS (SEQ ID NO: 100);

EVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NSKNTVYQLQMNSLRPEDTALYYCAAEMSGISGWDYWGQGTTLVTVQS (SEQ ID NO: 101);

EVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREFVADIRTSAGRITYYADSVKGRFTISRD
NSKNTVYQLQMNSLRPEDTALYYCAAEMSGISGWDYWGQGTTLTVSS (SEQ ID NO: 102);

DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRITYYADSVKGRFTISRD
NSKNTVYQLQMNSLRPEDTALYYCAAEMSGISGWDYWGQGTTLTVSS (SEQ ID NO: 103);

DVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEREFVADIRTSAGRITYYADSVKGRFTISRD
NSKNTVYQLQMNSLRPEDTALYYCAAEMSGISGWDYWGQGTTLTSSA (SEQ ID NO: 104);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTATYYCAAEMSGISGWDYWGQGTLVKVSSA (SEQ ID NO: 105);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTLVQVSSA (SEQ ID NO: 106);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTLVTVKSA (SEQ ID NO: 107);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTLVTVQSA (SEQ ID NO: 108);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTLVKVSSA (SEQ ID NO: 109);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVQVSSA (SEQ ID NO: 110);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVTVKSA (SEQ ID NO: 111);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVTVQSA (SEQ ID NO: 112);

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVTVSSA (SEQ ID NO: 113);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVKVSSA (SEQ ID NO: 114);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVQVSSA (SEQ ID NO: 115);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVTVKSA (SEQ ID NO: 116);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVTVQSA (SEQ ID NO: 117);

EVQLVESGGGVVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NAKNTVYLQMNSLKPEDTALYYCAAEMSGISGWDYWGQGTLVTVSSA (SEQ ID NO: 118);

EVQLVESGGGVVQPGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRD
NSKNTVYLQMNSLRPEDTALYYCAAEMSGISGWDYWGQGTLVTVSSA (SEQ ID NO: 119);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
SKNTVYLQMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 120);

EVQLVESGGGLVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITWSGGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTAIYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 121);

EVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITWSGGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 122);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITWSGGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGG
SGGGGSGGGGSEVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLY
ADSVKGRFTISRDNAKTTLYLMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA (SEQ ID NO: 123);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITWSGGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGG
SGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYAD
SVKGRFTISRDNSKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGG
SGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVS
SISGSGSDTLYADSVKGRFTISRDNAKTTLYLMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA (SEQ ID NO:
124);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITVSGGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 125);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDQ
SKNTVYLMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 126);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDPS
KNTVYLMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 127);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDPS
KNTVYLMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 128);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDQ
SKNTVYLMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 129);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDSS
KNTVYLMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 130);

EVQLVESGGGLVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITWSGGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTAIYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGS
GGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGSIASIHAMGWERQAPGKEREEVAVITWSGGITYYADS
VKGRFTISRDNSKNTVYLMNSLRPEDTAIYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGGS
GGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS
ISGSGSDTLYADSVKGRFTISRDNAKTTLYLMNSLRPEDTAVYYCTIGGSLSRSSQGTLVTVSS (SEQ ID NO:
131); and

MQIPQAPWPVWVAVLQLGWRPGWFLDSPDRPWNPTFSPALLVTEGDNATFTCSFSNTSESFVLNWYRMSP
 SNQTDKLAAPEDRSQPGQDCRFRTQLPNRDFHMSVVRARRNDSTYLCGAISLAPKAQIKESLRAELRVTE
 RRAEVPTAHPSPSPRPAGQFQTLVVGWVGLLGSLVLLVWVLAVICSRAARGTIGARRTGQPLKEDPSAVPVFV
 DYGELDFQWREKTPEPPVPCVPEQTEYATIVFSPGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL (SEQ ID
 NO: 132).

In some embodiments, the PD-1 binding agent comprises an amino acid sequence selected from SEQ ID NOs: 87-132 having one or more substitutions at positions 1, 11, 14, 52a, 73, 74, 83, 89, 100a, 110, and 112 (according to Kabat numbering). In some embodiments, the amino acid at position 1 is E or D. In some embodiments, the amino acid at position 11 is L or V. In some embodiments, the amino acid at position 14 is A or P. In some embodiments, the amino acid at position 52a is W or V. In some embodiments, the amino acid at position 73 is N, S, P, or Q. In some embodiments, the amino acid at position 74 is A or S. In some embodiments, the amino acid at position 83 is K or R. In some embodiments, the amino acid at position 89 is T, V, I, or L. In some embodiments, the amino acid at position 100a is W or F. In some embodiments, the amino acid at position 110 is T, K, or Q. In some embodiments, the amino acid at position 112 is S, K, or Q.

In various embodiments, the PD-1 binding agent comprises an amino acid sequence described in PCT Publication No. WO 2017/087587, the entire contents of which are incorporated by reference. By way of example, in some embodiments the PD-1 binding agent comprises one of the following sequences in PCT Publication No. WO 2017/087587:

EVQLVESGGGLVQAGGSLRLSCAASGGTFSFYGMGWFRQAPGKEQEFVADIRTSAGRTYYADSVKGRFTISRDN
 NAKNTVYLYQMNSLKPEDTAVYYCAAEMSGISGWDYWGQGTQVTVSS (SEQ ID NO: 133);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTATYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 134);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 135);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 136);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVKS (SEQ ID NO: 137);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVQS (SEQ ID NO: 138);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 139);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
 AKNTVYLYQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 140);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVKS (SEQ ID NO: 141);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVQS (SEQ ID NO: 142);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 143);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVKVSS (SEQ ID NO: 144);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVQVSS (SEQ ID NO: 145);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVKS (SEQ ID NO: 146);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVQS (SEQ ID NO: 147);

EVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
SKNTVY LQMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 148);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
SKNTVY LQMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 149);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTATYYCAGDKHQSSWYDYWGQGTLVTVSSA (SEQ ID NO: 150);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTLVKVSSA (SEQ ID NO: 151);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTLVQVSSA (SEQ ID NO: 152);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTLVTVKSA (SEQ ID NO: 153);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMCWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTLVTVQSA (SEQ ID NO: 154);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMCWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVKVSSA (SEQ ID NO: 155);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGGITYYADSVKGRFTISRDN
AKNTVY LQMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVQVSSA (SEQ ID NO: 156);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVKSA (SEQ ID NO: 157);

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVQSA (SEQ ID NO: 158);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSA (SEQ ID NO: 159);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVKVSSA (SEQ ID NO: 160);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVQVSSA (SEQ ID NO: 161);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVKSA (SEQ ID NO: 162);

EVQLVESGGGVVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTALYYCAGDKHQSSWYDYWGQGTLVTVQSA (SEQ ID NO: 163);

EVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSA (SEQ ID NO: 164);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSA (SEQ ID NO: 165);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAITWSSGITYYADSVKGRFTISRDN
KNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 166);

EVQLVESGGGLVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 167);

EVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSS (SEQ ID NO: 168);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGG
SGGGGSGGGGSEVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSDTLY
ADSVKGRFTISRDNKTTLYLMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA (SEQ ID NO: 169);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYADSVKGRFTISRDN
SKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGG
SGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITWSSGITYYAD
SVKGRFTISRDNKNTVYLMNSLRPEDTALYYCAGDKHQSSWYDYWGQGTLVTVSSGGGGSGGGGSGGGG
SGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGNSLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVS

SISGSGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLSRSSQGTLVTVSSA (SEQ ID NO: 170);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDNKNTVYLQMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 171);

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDNQSKNTVYLQMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 172); and

DVQLVESGGGVVQPGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAVITVSGGITYYADSVKGRFTISRDNPSKNTVYLQMNSLRPEDTALYYCAGDKHQSSFYDYWGQGTLVTVSS (SEQ ID NO: 173).

In some embodiments, the PD-1 binding agent comprises an amino acid sequence selected from SEQ ID NOs: 133-173 having one or more substitutions at positions 1, 11, 14, 52a, 73, 74, 83, 89, 100a, 110, and 112 (according to Kabat numbering). In some embodiments, the amino acid at position 1 is E or D. In some embodiments, the amino acid at position 11 is L or V. In some embodiments, the amino acid at position 14 is A or P. In some embodiments, the amino acid at position 52a is W or V. In some embodiments, the amino acid at position 73 is N, S, P, or Q. In some embodiments, the amino acid at position 74 is A or S. In some embodiments, the amino acid at position 83 is K or R. In some embodiments, the amino acid at position 89 is T, V, I, or L. In some embodiments, the amino acid at position 100a is W or F. In some embodiments, the amino acid at position 110 is T, K, or Q. In some embodiments, the amino acid at position 112 is S, K, or Q.

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analogs") of the PD-1 binding agent of the invention as described herein. In various embodiments, the amino acid sequence of the PD-1 binding agent further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In various embodiments, the present further provides PD-L1 binding agents. Programmed death-ligand 1 (PD-L1) also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1) is a type 1 transmembrane protein that has been speculated to play a major role in suppressing the immune system. PD-L1 is upregulated on macrophages and dendritic cells (DC) in response to LPS and GM-CSF treatment, and on T cells and B cells upon TCR and B cell receptor signaling.

In various embodiments, the PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that recognizes an epitope present on PD-L1. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes present on PD-L1. As used herein, a linear epitope refers to any continuous sequence of amino acids present on PD-L1. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on PD-L1. As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the PD-L1 binding agent of the present invention may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of human PD-L1. In various embodiments, the PD-L1 binding agent of the invention may bind to any forms of the human PD-L1. In an embodiment, the PD-L1 binding agent binds to a phosphorylated form of PD-L1. In an embodiment, the PD-L1 binding agent binds to an acetylated form of PD-L1.

In an embodiment, the present PD-L1 binding agent comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on human PD-L1. In an embodiment, the human PD-L1 comprises the amino acid sequence of (signal peptide underlined):

Isoform 1:

MRIFAVFIFMTYWHLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAA
LIVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDV
KLQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVDPVTSEHELTC
QAEGYPKAEVIWTSSDHQVLSGKTTTTNSKREEKLFNVTSTLRINTTTNEIF
YCTFRRLDPEENHTAELVIPELPLAHPNERTHLVILGAILLCLGVALTFIFRL
RKGRMMDVKKCGIQDTNSKKQSDTHLEET (SEQ ID NO: 174);

Isoform 2:

MRIFAVFIFMTYWHLNAPYNKINQRILVDPVTSEHELTCQAEGYPKAEVIW
TSSDHQVLSGKTTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENH
TAEVIPELPLAHPNERTHLVILGAILLCLGVALTFIFRLRKGRMMDVKKCGI
QDTNSKKQSDTHLEET (SEQ ID NO: 175); or

Isoform 3:

MRIFAVFIFMTYWHLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAAL
IVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVK
LQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVDPVTSEHELTCQA
EGYPKAEVIWTSSDHQVLSGD (SEQ ID NO: 176).

In various embodiments, the present PD-L1 binding agent comprises a targeting moiety capable of specific binding. In various embodiments, the PD-L1 binding agent comprises a targeting moiety having an antigen recognition domain such as an antibody or derivatives thereof. In an embodiment, the PD-L1 binding agent comprises a targeting moiety which is an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains. Each heavy chain includes one variable region (e.g., V_H) and at least three constant regions (e.g., CH₁, CH₂ and CH₃), and each light chain includes one variable region (V_L) and one constant region (C_L). The variable regions determine the specificity of the antibody. Each variable region comprises three hypervariable regions also known as complementarity determining regions (CDRs) flanked by four relatively conserved framework regions (FRs). The three CDRs, referred to as CDR1, CDR2, and CDR3, contribute

to the antibody binding specificity. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the PD-L1 binding agent comprises a targeting moiety which is an antibody derivative or format. In some embodiments, the present PD-L1 binding agent comprises a targeting moiety which is a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; an aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab', a F(ab')₂, a peptide mimetic molecule, or a synthetic molecule, as described in US Patent Nos. or Patent Publication Nos. US 7,417,130, US 2004/132094, US 5,831,012, US 2004/023334, US 7,250,297, US 6,818,418, US 2004/209243, US 7,838,629, US 7,186,524, US 6,004,746, US 5,475,096, US 2004/146938, US 2004/157209, US 6,994,982, US 6,794,144, US 2010/239633, US 7,803,907, US 2010/119446, and/or US 7,166,697, the contents of which are hereby incorporated by reference in their entireties. See also, Storz MABs. 2011 May-Jun; 3(3): 310–317.

In some embodiments, the PD-L1 binding agent comprises a targeting moiety which is a single-domain antibody, such as a VHH. The VHH may be derived from, for example, an organism that produces VHH antibody such as a camelid, a shark, or the VHH may be a designed VHH. VHHs are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (V_HH) and two constant domains (CH2 and CH3).

In an embodiment, the PD-L1 binding agent comprises a VHH. In some embodiments, the VHH is a humanized VHH or camelized VHH.

In some embodiments, the VHH comprises a fully human V_H domain, e.g. a HUMABODY (Crescendo Biologics, Cambridge, UK). In some embodiments, fully human V_H domain, e.g. a HUMABODY is monovalent, bivalent, or trivalent. In some embodiments, the fully human V_H domain, e.g. a HUMABODY is mono- or multi-specific such as monospecific, bispecific, or trispecific. Illustrative fully human V_H domains, e.g. a HUMABODIES are described in, for example, WO2016/113555 and WO2016/113557, the entire disclosure of which is incorporated by reference.

In some embodiments, the PD-L1 binding agent comprises a targeting moiety which is a VHH comprising a single amino acid chain having four “framework regions” or FRs and three “complementary determining regions” or CDRs. As used herein, “framework region” or “FR” refers to a region in the variable domain which is located between the CDRs. As used herein, “complementary determining region” or “CDR” refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the PD-L1 binding agent comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences. In various embodiments, the PD-L1 binding agent comprises a VHH having a variable region comprising at least one FR1, FR2, FR3, and FR4 sequences.

In some embodiments, the PD-L1 CDR1 sequence is selected from: GFTLDYYAIG (SEQ ID NO: 177); GTIFSINHMD (SEQ ID NO: 178); GFTFDDYGMS (SEQ ID NO: 179); GFTLDYYAIN (SEQ ID NO: 180); GTIFSINRMD (SEQ ID NO: 181); GFTFSSYGMS (SEQ ID NO: 182); GKIFSGNDMG (SEQ ID NO: 183); GFTFNDYAMS (SEQ ID NO: 184); GFNLDPYAIA (SEQ ID NO: 185); GFTFTAYAMS (SEQ ID NO: 186); GFTFDYYAIG (SEQ ID NO: 187); GFNLDPYAIG (SEQ ID NO: 188); ESIFSIEAMG (SEQ ID NO: 189); GRTFSISAMG (SEQ ID NO: 190); YYAIG (SEQ ID NO: 191); -YYAKC (SEQ ID NO: 192); QYDVG (SEQ ID NO: 193); NSAMG (SEQ ID NO: 194); DSIVS (SEQ ID NO: 195); INHMD (SEQ ID NO: 196); DYGMS (SEQ ID NO: 197); YYAIN (SEQ ID NO: 198); INRMD (SEQ ID NO: 199); SYGMS (SEQ ID NO: 200); GNDMG (SEQ ID NO: 201); DYAMS (SEQ ID NO: 202); PYAIA (SEQ ID NO: 203); AYAMS (SEQ ID NO: 204); PYAIG (SEQ ID NO: 205); IEAMG (SEQ ID NO: 206); and ISAMG (SEQ ID NO: 207).

In some embodiments, the PD-L1 CDR2 sequence is selected from: ISSSDGSTY (SEQ ID NO: 208); ITSDGFPT (SEQ ID NO: 209); IRWNGGSTN (SEQ ID NO: 210); ITSDGTPT (SEQ ID NO: 211); IDSGGGSTS (SEQ ID NO: 212); ITSGGITD (SEQ ID NO: 213); ITSDGTPT (SEQ ID NO: 214); IDSGGGSTS (SEQ ID NO: 215); IRSNGGYTN (SEQ ID NO: 216); ISSSDVGTY (SEQ ID NO: 217); INSSDGSTY (SEQ ID NO: 218); ISGSDSSTY (SEQ ID NO: 219); ISSSDVGTY (SEQ ID NO: 220); ITSDGTPT (SEQ ID NO: 221); ITSDGTPA (SEQ ID NO: 222); IDSGGGSTS (SEQ ID NO: 223); ISSGDGSKY (SEQ ID NO: 224); ISSSDVGTY (SEQ ID NO: 225); IFGGGFTN (SEQ ID NO: 226); ITSGGITD (SEQ ID NO: 227); IDSGGGSTS (SEQ ID NO: 228); ITSDGTPT (SEQ ID NO: 229); IDSGGGSTS (SEQ ID NO: 230); ISSSDVGTY (SEQ ID NO: 231); ITWSGGSTS (SEQ ID NO: 232); IDSGGGSTS (SEQ ID NO: 233); IRSNGGYTN (SEQ ID NO: 234); SISSSDGSTYYADSVKG (SEQ ID NO: 235); CISSSDGSTYYADSVKG (SEQ ID NO: 236); CISGGDNSTYYADSVKG (SEQ ID NO: 237); FSSSGGRTIYPDSVKG (SEQ ID NO: 238); RITGGGLIAYTDSVKG (SEQ ID NO: 239); GISNGGTIKYAESVLG (SEQ ID NO: 240); LITSDGFPT (SEQ ID NO: 241); LITSDGFPTYADSAKG (SEQ ID NO: 242); AIRWNGGSTN (SEQ ID NO: 243); AIRWNGGSTNYADSVKG (SEQ ID NO: 244); LITSDGTPT (SEQ ID NO: 245); LITSDGTPTYADSAKG (SEQ ID NO: 246); AIDSGGGSTS (SEQ ID NO: 247); AIDSGGGSTSYADSVKG (SEQ ID NO: 248); IITSGGITD (SEQ ID NO: 249); IITSGGITDYADAVKG (SEQ ID NO: 250); GIRSNGGYTN (SEQ ID NO: 251); GIRSNGGYTNADSVKG (SEQ ID NO: 252); CISSSDVGTY (SEQ ID NO: 253); CISSSDVGTYADSVKG (SEQ ID NO: 254); CINSSDGSTY (SEQ ID NO: 255); CINSSDGSTYYADSVKG (SEQ ID NO: 256); CISGSDSSTY (SEQ ID NO: 257); CISGSDSSTYYADSVKG (SEQ ID NO: 258); LITSDGTPA (SEQ ID NO: 259); LITSDGTPAYADSAKG (SEQ ID NO: 260); CISSGDGSKY (SEQ ID NO: 261); CISSGDGSKYYADSVKG (SEQ ID NO: 262); AIFGGGFTN (SEQ ID NO: 263); AIFGGGFTNYADSVKG (SEQ ID NO: 264); AITWSGGSTS (SEQ ID NO: 265); and AITWSGGSTSYTDSVKG (SEQ ID NO: 266).

In some embodiments, the CDR3 sequence is selected from: DGWSSCRHGINEYLYW (SEQ ID NO: 267); SSGVYNYW (SEQ ID NO: 268); QGYCSCGYGCPR (SEQ ID NO: 269); SGWRLCRPTDEYDYSYW (SEQ ID NO: 270); and SGWRLCRPTDEYDYSYW (SEQ ID NO: 271).

270); QGYCYSGYGCSYDW (SEQ ID NO: 271); RDRTIWW (SEQ ID NO: 272); QGYCYSGYGCYP (SEQ ID NO: 273); DGYCYCSDYPHPLYW (SEQ ID NO: 274); DGWRDCTWSNEYAYW (SEQ ID NO: 275); TGWRCTCRGLNEYDYW (SEQ ID NO: 276); DLVSGSSRLYDYW (SEQ ID NO: 277); MGRTNYGVIYDPNMYNYW (SEQ ID NO: 278); SGWRLCRPTDEYDYLYW (SEQ ID NO: 279); SQAPITIATMMKPFYDY (SEQ ID NO: 280); RHGGPLTVEYFFDY (SEQ ID NO: 281); GGWKYCSGYDPEIY (SEQ ID NO: 282); DWYLSY (SEQ ID NO: 283); INSRDG (SEQ ID NO: 284); and RQY (SEQ ID NO: 285).

In various exemplary embodiments, the PD-L1 binding agent comprises an amino acid sequence selected from the following sequences:

2LIG2

QVQLQESGGGLVQAGGSLRLSCAASGFTLDYYAIGWFRQAPGKEREEVSCISSSDGSTYYADSVKGRFTISRDN
AKNTVNLQMNSLKPEDTAVYYCATDGWSSCRHGIN-EYLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 286);

or

2LIG3

QVQLQESGGGLVQAGGSLRLSCTASGTIFSINHMDWFRQAPGKQRELVALITSDGFPTYADSAKGRFTISRDN
KKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 287);

or

2LIG16

QVQLQESGGGLVQPGGSLRLSCAASGFTFDDYGMSWVRQAPGKGLEWVSAIRWNGGSTNYADSVKGRFTISR
DNAKNTLYLQMNSLKSEDTAVYYCA-QGY-CYSGYGCPRGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 288);

or

2LIG22

QVQLQESGGGLVQPGGSLRLSCAASGFTLDYYAINWFRQAPGKEREEVSCISSSDGSTYYADSVKGRFTISRDN
AKNTVYLQMNSLKPEDTAVYYCATSGWRLCRPTDEYDYSYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 289);

or

2LIG27

QVQLQESGGGVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPTYADSAKGRFTISRDN
KKTVSLQMNSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 290);

or

WO 2019/032663

PCT/US2018/045743

2LIG29

QVQLQESGGGLVQTGGSLRLSCAASGFTFSSYGMSWVRQTPGKGPEWWSAIDSGGGSTSYADSVKGRFTISR
DNAKNTLYLQMNSLKPEDTAVYYCA-QGYG-CSGYGCSDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 291);

or

2LIG30

QVQLQESGGGLVQPGGSLRLSCAASGKIFSGNDMGWYRQAPGKQRELVGIITSGGITDYADAVKGRFTISR
DNAKNMMLYQMNSLKPEDTAVYYCNMRDRTIWWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 292);

or

2LIG34

QVQLQESGGGSVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPTYADSAKGRFTISR
DNKKTVSLQMNSLKPEDTAVYYCHVSSGVVNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 293);

or

2LIG35

QVQLQESGGGLVQPGGSLRLSCAASGFTFSSYGMSWVRQTPGKGPEWWSAIDSGGGSTSYADSVKGRFTTSR
DNAKNTLYLQMNSLKPEDTAVYYCA-QGYG-CSGYGCSDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 294);

or

2LIG48

QVQLQESGGGLVQPGGSLRLSCAASGFTFNDYAMSWVRQAPGKGLEWVSGIRSNGGYTNYADSVKGRFTISR
DNAKNTLYLQMNSLKSEDTAVYYCA-QGYG-CSGYGCYPGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 295);

or

2LIG65

QVQLQESGGGLVQAGGSLRLSCAASGFNLDPYAIAWFRQAPGKEREEVSCISSSDVGTYADSVKGRFTISR
DNAKNTLYLQMNSLKPEDTAVYYCATDGYYCYSDYPHPLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 296);

or

2LIG85

QVQLQESGGGLVQPGGSLRLSCAASGFTFTAYAMSWFRQAPGKEREEVSCINSSDGSTYYADSVKGRFTISRDN
NAKNTVYLMNSLKPEDTAVYHCAATDGWRDCTWSNEYAYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 297);

or

2LIG86

QVQLQESGGGLVQPGGSLRLSCAASGFTFDYYAIGWFRQAPGKEREEVSCISGSDSSTYYADSVKGRFTIVRDN
AQNTVYLMNSLKPEDTAIYYCAVTGWRTCRGLNEYDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 298);

or

2LIG89

QVQLQESGGGLVQPGGSLRLSCAASGFNLDPYAIAWFRQAPGKEREEVSCISSSDVGTYADSVKGRFTISRDN
TKKTVYLMNSLKPEDTAVYYCATDGYYYCSDYPHPLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 299);

or

2LIG97

QVQLQESGGGLVQAGESLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPTYADSAKGRFTISRDN
KTVSLQMNLSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 300);

or

2LIG99

QVQLQESGGGLVQAGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPAYADSAKGRFTISRDN
KKTVSLQMNLSLKPEDTAVYYCHVSSGVYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 301);

or

2LIG109

QVQLQESGGGLVQSGGSLRLSCKTSGFTFSSYGMSWVRQTPGKGPEWWSAIDSGGGSTSYADSVKGRFTISR
DNAKNTLYLMNSLKPEDTAVYYCAQGYG-CSGYGCSDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 302);

or

2LIG127

QVQLQESGGGLVQPGGSLRLSCAASGFNLDPYAIGWFRQAPGKEREEVSCISSGDGSKYYADSVKGRFTMSRD
NAKKTVYLMNSLKPEDTAVYYCATDGYYYCSDYPHPLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 303);

or

2LIG139

QVQLQESGGGLVQPGGSLRLSCAVSGFNLDPYAIAWFRQAPGKEREEVSCISSSDVGTTYADSVKGRFTISRDN
AKKTVYLMNSLKPEDTAVYYCATDGYYCSDYPHPLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 304);

or

2LIG176

QVQLQESGGGLVQAGGSLRLSCAASESIFSIEAMGWYRQAPGKQRELVAIFGGGFTNYADSVKGRFTISRDN
NRTVYLMNSLKPEDTAVYYCNADLVSGSSRLYDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 305);

or

2LIG189

QVQLQESGGGLVQAGGSLRLSCAASGKIFSGNDMGWYRQAPGKQRELVGIITSGGITDYADAVKGRFTISRDN
KNMMYLMNSLKPEDTAVYYCNMRDRTIWWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 306);

or

3LIG3

QVQLQESGGGLVQPGGSLRLSCAASGFTLDYYAIGWFRQAPGKEREEVSCISSSDGSTYYADSVKGRFTISRDN
AKNTVNLQMNLSLKPEDTAVYYCATDGWSSCRHGINEYLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 307);

or

3LIG7

QVQLQESGGGLVQAGGSLRLSCAASGFTFSSYGMSWVRQTPGKGPEWWSAIDSGGGSTSYADSVKGRFTISR
DNAKNTLYLMNSLKPEDTAVYYCAQGY-CSGYGCSDYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 308);

or

3LIG8

QVQLQESGGGLVQPGGSLRLSCTASGTIFSINRMDWFRQAPGKQRELVALITSDGTPTYADSAKGRFTISRDN
KKTVSLQMNLSLKPEDTAVYYCHVSSGVVNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID NO: 309);

or

3LIG9

QVQLQESGGGLVQPGGSLRLSCAASGFTFSSYGMSWVRQTPGKGPEWWSAIDSGGGSTSYADSVKGRFTISR
DNAKNTLYLQMNSLKPEDTAVYYCAQGYYCSGYGCSYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 310);

or

3LIG18

QVQLQESGGGLVQPGGSLRLSCAASGFNLDPYAIAWFRQAPGKEREEVSCISSSDVGTYADSVKGRFTISRDN
AKKTVYLMNSLKPEDTAVYYCATDGYYYCSDYPHPLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 311);

or

3LIG20

QVQLQESGGGLVXAGGSLRLSCAASGRTFSISAMGWFRQAPGKEREFVAAITWSGGSTSYTDSVKGRFTISR
NAKNTLYLQMNSLKPEDTAIYYCAAMGRTNYGVIYDPNMYNYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 312);

or

3LIG28

QVQLQESGGGLVQPGGSLRLSCAASGFTLDYYAINWFRQAPGKEREEVSCISSSDGSTYYADSVKGRFTISRDN
AKNTVYLMNSLKPEDTAVYYCATSGWRLCRPTDEYDLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 313);

or

3LIG29

QVQLQESGGGLVQAGGSMRLSCAASGFTFSSYGMSWVRQTPGKGPEWWSAIDSGGGSTSYADSVKGRFTISR
DNAKNTLYLQMNSLKPEDTAVYYCAQGYYCSGYGCSYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ
ID NO: 314);

or

3LIG30

QVQLQESGGGTQAGGSLRLSCAASGFTFNDYAMSWVRQAPGKGLEWWSGIRSNGGYTNYADSVKGRFTISR
DNAKNTLYLQMNSLKSEDTAVYYCAQGYYCSGYGCYPGQGTQVTVSSAAAYPYDVPDYGSHHHHHH (SEQ ID
NO: 315);

or

3LIG33

QVQLQESGGGLVQPGTSLRLSCAASGFTLDYYAIGWFRQAPGKEREEVSCISSSDGSTYYADSVKGRFTISRDN
AKNTVNLQMNSLKPEDTAVYYCATDGWSSCRHGINEYLYWGQGTQVTVSSAAAYPYDVPDYGSHHHHHH
(SEQ ID NO: 316).

In various exemplary embodiments, the PD-L1 binding agent comprises an amino acid sequence selected from any one of the above sequences without the terminal histidine tag sequence (*i.e.*, HHHHHH; SEQ ID NO: 84).

In some embodiments, the PD-L1 binding agent comprises an amino acid sequence selected from SEQ ID Nos: 286-316 (provided above) without the HA tag (*i.e.*, YPYDVPDYGS; SEQ ID NO: 85).

In some embodiments, the PD-L1 binding agent comprises an amino acid sequence selected from SEQ ID Nos: 286-316 (provided above) without the AAA linker.

In some embodiments, the PD-L1 binding agent comprises an amino acid sequence selected from SEQ ID Nos: 286-316 (provided above) without the AAA linker, HA tag, and terminal histidine tag sequence (*i.e.*, AAAYPYDVPDYGSHHHHHH; SEQ ID NO: 86).

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as “analog”) of the PD-L1 binding agent of the invention as described herein. In various embodiments, the amino acid sequence of the PD-L1 binding agent further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety comprising a sequence that is at least 60% identical to any one of the sequences disclosed herein. For example, the PD-1 or PD-L1 binding agent may comprise a targeting moiety comprising a sequence that is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any of the sequences disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, about 99% or about 100% sequence identity to any one of the sequences disclosed herein).

In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety comprising an amino acid sequence having one or more amino acid mutations with respect to any one of the sequences disclosed herein. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety comprising an amino acid sequence having one, or two, or three, or four, or five, or six, or seven, or eight, or nine, or ten, or fifteen, or twenty amino acid mutations with respect to any one of the sequences disclosed herein. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

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

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

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

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

In various embodiments, the substitutions may also include non-classical amino acids. Exemplary non-classical amino acids include, but are not limited to, selenocysteine, pyrrolysine, *N*-formylmethionine β -alanine, GABA and δ -Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α -methyl amino acids, N α -methyl amino acids, and amino acid analogs in general.

In various embodiments, the amino acid mutation may be in the CDRs of the targeting moiety (e.g., the CDR1, CDR2 or CDR3 regions). In another embodiment, amino acid alteration may be in the framework regions (FRs) of the targeting moiety (e.g., the FR1, FR2, FR3, or FR4 regions).

Modification of the amino acid sequences may be achieved using any known technique in the art e.g., site-directed mutagenesis or PCR based mutagenesis. Such techniques are described, for example, in Sambrook *et al.*,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., 1989 and Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1989.

In various embodiments, the mutations do not substantially reduce the present PD-1 or PD-L1 binding agent's capability to specifically bind to PD-1 or PD-L1. In various embodiments, the mutations do not substantially reduce the present PD-1 or PD-L1 binding agent's capability to specifically bind to PD-1 or PD-L1 and without functionally modulating (*e.g.*, partially or fully neutralizing) PD-1 or PD-L1.

In various embodiments, the binding affinity of the PD-1 or PD-L1 binding agent of the invention for the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or monomeric and/or dimeric forms and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric and/or dimeric forms) of human PD-1 or PD-L1 may be described by the equilibrium dissociation constant (K_D). In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric and/or dimeric forms) of human PD-1 or PD-L1 with a K_D of less than about 1 μ M, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, or about 5 nM, or about 1 nM.

In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds but does not functionally modulate (*e.g.*, partially or fully neutralize) the antigen of interest, *i.e.*, PD-1 or PD-L1. For instance, in various embodiments, the targeting moiety of the PD-1 or PD-L1 binding agent simply targets the antigen but does not substantially functionally modulate (*e.g.* partially or fully inhibit, reduce or neutralize) a biological effect that the antigen has. In various embodiments, the targeting moiety of the PD-1 or PD-L1 binding agent binds an epitope that is physically separate from an antigen site that is important for its biological activity (*e.g.* an antigen's active site).

In various embodiments, these binding agents bind to, and functionally modulate (*e.g.* partially or fully neutralize) PD-1 or PD-L1.

Therapeutic Agents Comprising the Present PD-1 or PD-L1 Binding Agents

Chimeras and Fusions with Signaling Agents

In various embodiments, the PD-1 or PD-L1 binding agent of the invention is part of a chimera or fusion with one or more signaling agents. Accordingly, the present invention provides for chimeric or fusion proteins that include, for example, a targeting moiety against PD-1 or PD-L1 and one or more signaling agents.

In various embodiments, the signaling agent is modified to have reduced affinity or activity for one or more of its receptors, which allows for attenuation of activity (inclusive of agonism or antagonism) and/or prevents non-specific signaling or undesirable sequestration of the chimeric or fusion protein. In various embodiments, the signaling agent is antagonistic in its wild type form and bears one or more mutations that attenuate its antagonistic activity.

In various embodiments, the signaling agent is antagonistic due to one or more mutations, *e.g.* an agonistic signaling agent is converted to an antagonistic signaling agent and, such a converted signaling agent, optionally, also bears one or more mutations that attenuate its antagonistic activity (*e.g.* as described in WO 2015/007520, the entire contents of which are hereby incorporated by reference).

Accordingly, in various embodiments, the signaling agent is a modified (*e.g.* mutant) form of the signaling agent having one or more modifications (*e.g.* mutations). In various embodiments, the mutations allow for the modified signaling agent to have one or more of attenuated activity such as one or more of reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmutated, *i.e.* the wild type form of the signaling agent (*e.g.* comparing the same signaling agent in a wild type form versus a modified (*e.g.* mutant) form). In some embodiments, the mutations which attenuate or reduce binding or affinity include those mutations which substantially reduce or ablate binding or activity. In some embodiments, the mutations which attenuate or reduce binding or affinity are different than those mutations which substantially reduce or ablate binding or activity. Consequentially, in various embodiments, the mutations allow for the signaling agent to have improved safety, *e.g.* reduced systemic toxicity, reduced side effects, and reduced off-target effects relative to unmutated, *i.e.* wild type, signaling agent (*e.g.* comparing the same signaling agent in a wild type form versus a modified (*e.g.* mutant) form).

As described herein, the agent may have improved safety due to one of more modifications, *e.g.* mutations. In various embodiments, improved safety means that the present chimeric protein provides lower toxicity (*e.g.* systemic toxicity and/or tissue/organ-associated toxicities); and/or lessened or substantially eliminated side effects; and/or increased tolerability, lessened or substantially eliminated adverse events; and/or reduced or substantially eliminated off-target effects; and/or an increased therapeutic window.

In various embodiments, the signaling agent is modified to have one or more mutations that reduce its binding affinity or activity for one or more of its receptors. In some embodiments, the signaling agent is modified to have one or more mutations that substantially reduce or ablate binding affinity or activity for the receptors. In some embodiments, the activity provided by the wild type signaling agent is agonism at the receptor (*e.g.* activation of a cellular effect at a site of therapy). For example, the wild type signaling agent may activate its receptor. In such embodiments, the mutations result in the modified signaling agent to have reduced or ablated activating activity at the receptor. For example, the mutations may result in the modified signaling agent to deliver a reduced activating signal to a target cell or the activating signal could be ablated. In some embodiments, the activity provided by the wild type signaling agent is antagonism at the receptor (*e.g.* blocking or dampening of a cellular effect at a site of therapy). For example, the wild type signaling agent may antagonize or inhibit the receptor. In these embodiments, the mutations result in the modified signaling agent to have a reduced or ablated antagonizing activity at the receptor. For example, the mutations may result in the modified signaling agent to deliver a reduced inhibitory signal to a target cell or the inhibitory signal could be ablated. In various embodiments, the signaling agent is antagonistic due to one or more mutations, *e.g.* an agonistic signaling agent is converted to an antagonistic signaling agent (*e.g.* as described in WO 2015/007520, the entire contents of which are hereby incorporated by reference) and, such a converted signaling agent, optionally, also bears one or mutations that reduce its binding

affinity or activity for one or more of its receptors or that substantially reduce or ablate binding affinity or activity for one or more of its receptors.

In some embodiments, the reduced affinity or activity at the receptor is restorable by attachment with one or more of the targeting moieties as described herein (e.g., targeting moiety against PD-1 or PD-L1). In other embodiments, the reduced affinity or activity at the receptor is not substantially restorable by the activity of one or more of the targeting moieties.

In various embodiments, the chimeric proteins of the present invention reduce off-target effects because their signaling agents have mutations that weaken or ablate binding affinity or activity at a receptor. In various embodiments, this reduction in side effects is observed relative with, for example, the wild type signaling agents. In various embodiments, the signaling agent is active on target cells because the targeting moiety(ies) compensates for the missing/insufficient binding (e.g., without limitation and/or avidity) required for substantial activation. In various embodiments, the modified signaling agent is substantially inactive *en route* to the site of therapeutic activity and has its effect substantially on specifically targeted cell types which greatly reduces undesired side effects.

In some embodiments, the signaling agent may include one or more mutations that attenuate or reduce binding or affinity for one receptor (*i.e.*, a therapeutic receptor) and one or more mutations that substantially reduce or ablate binding or activity at a second receptor. In such embodiments, these mutations may be at the same or at different positions (*i.e.*, the same mutation or multiple mutations). In some embodiments, the mutation(s) that reduce binding and/or activity at one receptor is different than the mutation(s) that substantially reduce or ablate at another receptor. In some embodiments, the mutation(s) that reduce binding and/or activity at one receptor is the same as the mutation(s) that substantially reduce or ablate at another receptor. In some embodiments, the present chimeric proteins have a modified signaling agent that has both mutations that attenuate binding and/or activity at a therapeutic receptor and therefore allow for a more controlled, on-target therapeutic effect (e.g. relative wild type signaling agent) and mutations that substantially reduce or ablate binding and/or activity at another receptor and therefore reduce side effects (e.g. relative to wild type signaling agent).

In some embodiments, the substantial reduction or ablation of binding or activity is not substantially restorable with a targeting moiety (e.g., a targeting moiety against PD-1 or PD-L1 or any other targeting moiety described herein). In some embodiments, the substantial reduction or ablation of binding or activity is restorable with a targeting moiety. In various embodiments, substantially reducing or ablating binding or activity at a second receptor also may prevent deleterious effects that are mediated by the other receptor. Alternatively, or in addition, substantially reducing or ablating binding or activity at the other receptor causes the therapeutic effect to improve as there is a reduced or eliminated sequestration of the therapeutic chimeric proteins away from the site of therapeutic action. For instance, in some embodiments, this obviates the need of high doses of the present chimeric proteins that compensate for loss at the other receptor. Such ability to reduce dose further provides a lower likelihood of side effects.

In various embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced, substantially reduced, or ablated affinity, *e.g.* binding (*e.g.* K_D) and/or activation (for instance, when the modified signaling agent is an agonist of its receptor, measurable as, for example, K_A and/or EC_{50}) and/or inhibition (for instance, when the modified signaling agent is an antagonist of its receptor, measurable as, for example, K_I and/or IC_{50}), for one or more of its receptors. In various embodiments, the reduced affinity at the immunomodulating agent's receptor allows for attenuation of activity (inclusive of agonism or antagonism). In such embodiments, the modified signaling agent has about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 10%-20%, about 20%-40%, about 50%, about 40%-60%, about 60%-80%, about 80%-100% of the affinity for the receptor relative to the wild type signaling agent. In some embodiments, the binding affinity is at least about 2-fold lower, about 3-fold lower, about 4-fold lower, about 5-fold lower, about 6-fold lower, about 7-fold lower, about 8-fold lower, about 9-fold lower, at least about 10-fold lower, at least about 15-fold lower, at least about 20-fold lower, at least about 25-fold lower, at least about 30-fold lower, at least about 35-fold lower, at least about 40-fold lower, at least about 45-fold lower, at least about 50-fold lower, at least about 100-fold lower, at least about 150-fold lower, or about 10-50-fold lower, about 50-100-fold lower, about 100-150-fold lower, about 150-200-fold lower, or more than 200-fold lower relative to the wild type signaling agent.

In embodiments wherein the modified signaling agent has mutations that reduce binding at one receptor and substantially reduce or ablate binding at a second receptor, the attenuation or reduction in binding affinity of a modified signaling agent for one receptor is less than the substantial reduction or ablation in affinity for the other receptor. In some embodiments, the attenuation or reduction in binding affinity of a modified signaling agent for one receptor is less than the substantial reduction or ablation in affinity for the other receptor by about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In various embodiments, substantial reduction or ablation refers to a greater reduction in binding affinity and/or activity than attenuation or reduction.

In various embodiments, the modified signaling agent comprises one or more mutations that reduce the endogenous activity of the signaling agent to about 75%, or about 70%, or about 60%, or about 50%, or about 40%, or about 30%, or about 25%, or about 20%, or about 10%, or about 5%, or about 3%, or about 1%, *e.g.*, relative to the wild type signaling agent.

In some embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced affinity for its receptor that is lower than the binding affinity of the targeting moiety(ies) for its(their) receptor(s). In some embodiments, this binding affinity differential is between signaling agent/receptor and targeting moiety/receptor on the same cell. In some embodiments, this binding affinity differential allows for the signaling agent, *e.g.* mutated signaling agent, to have localized, on-target effects and to minimize off-target effects that underlie side effects that are observed with wild type signaling agent. In some embodiments, this

binding affinity is at least about 2-fold, or at least about 5-fold, or at least about 10-fold, or at least about 15-fold lower, or at least about 25-fold, or at least about 50-fold lower, or at least about 100-fold, or at least about 150-fold.

Receptor binding activity may be measured using methods known in the art. For example, affinity and/or binding activity may be assessed by Scatchard plot analysis and computer-fitting of binding data (e.g. Scatchard, 1949) or by reflectometric interference spectroscopy under flow through conditions, as described by Brecht *et al.* (1993), the entire contents of all of which are hereby incorporated by reference.

In various embodiments, the signaling agent is an immune-modulating agent, e.g. one or more of an interleukin, interferon, and tumor necrosis factor.

In some embodiments, the signaling agent is an interleukin or a modified interleukin, including for example IL-1; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-8; IL-9; IL-10; IL-11; IL-12; IL-13; IL-14; IL-15; IL-16; IL-17; IL-18; IL-19; IL-20; IL-21; IL-22; IL-23; IL-24; IL-25; IL-26; IL-27; IL-28; IL-29; IL-30; IL-31; IL-32; IL-33; IL-35; IL-36 or a fragment, variant, analogue, or family-member thereof. Interleukins are a group of multi-functional cytokines synthesized by lymphocytes, monocytes, and macrophages. Known functions include stimulating proliferation of immune cells (e.g., T helper cells, B cells, eosinophils, and lymphocytes), chemotaxis of neutrophils and T lymphocytes, and/or inhibition of interferons. Interleukin activity can be determined using assays known in the art: Matthews *et al.*, in *Lymphokines and Interferens: A Practical Approach*, Clemens *et al.*, eds, IRL Press, Washington, D.C. 1987, pp. 221-225; and Orencole & Dinarello (1989) Cytokine 1, 14-20.

In some embodiments, the signaling agent is an interferon or a modified version of an interferon such as interferon types I, II, and III. Illustrative interferons, including for example, interferon- α -1, 2, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, and 21, interferon- β and interferon- γ , interferon κ , interferon ϵ , interferon τ , and interferon ω .

In some embodiments, the signaling agent is a tumor necrosis factor (TNF) or a modified version of a tumor necrosis factor (TNF) or a protein in the TNF family, including but not limited to, TNF- α , TNF- β , LT- β , CD40L, CD27L, CD30L, FASL, 4-1BBL, OX40L, and TRAIL.

The amino acid sequences of the wild type signaling agents described herein are well known in the art. Accordingly, in various embodiments the modified signaling agent comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with the known wild type amino acid sequences of the signaling agents described herein (e.g. about 60%, or about

61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In various embodiments the modified signaling agent comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with any amino acid sequences of the signaling agents described herein (e.g. about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In various embodiments, the modified signaling agent comprises an amino acid sequence having one or more amino acid mutations. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations. In some embodiments, the amino acid mutations are amino acid substitutions, and may include conservative and/or non-conservative substitutions, as described elsewhere herein. In various embodiments, the substitutions may also include non-classical amino acids as described elsewhere herein.

As described herein, the modified signaling agents bear mutations that affect affinity and/or activity at one or more receptors. In various embodiments, there is reduced affinity and/or activity at a therapeutic receptor, e.g. a receptor through which a desired therapeutic effect is mediated (e.g. agonism or antagonism). In various embodiments, the modified signaling agents bear mutations that substantially reduce or ablate affinity and/or activity at a receptor, e.g. a receptor through which a desired therapeutic effect is not mediated (e.g. as the result of promiscuity of binding). The receptors of any modified signaling agents, e.g. one of the cytokines, growth factors, and hormones as described herein, are known in the art.

Illustrative mutations which provide reduced affinity and/or activity (*e.g.* agonistic) at a receptor are found in WO 2013/107791 and PCT/EP2017/061544 (*e.g.* with regard to interferons), WO 2015/007542 (*e.g.* with regard to interleukins), and WO 2015/007903 (*e.g.* with regard to TNF), the entire contents of each of which are hereby incorporated by reference. Illustrative mutations which provide reduced affinity and/or activity (*e.g.* antagonistic) at a therapeutic receptor are found in WO 2015/007520, the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced affinity and/or activity for a type I cytokine receptor, a type II cytokine receptor, a chemokine receptor, a receptor in the Tumor Necrosis Factor Receptor (TNFR) superfamily, TGF-beta Receptors, a receptor in the immunoglobulin (Ig) superfamily, and/or a receptor in the tyrosine kinase superfamily.

In various embodiments, the receptor for the signaling agent is a Type I cytokine receptor. Type I cytokine receptors are known in the art and include, but are not limited to receptors for IL2 (beta-subunit), IL3, IL4, IL5, IL6, IL7, IL9, IL11, IL12, GM-CSF, G-CSF, LIF, CNTF, and also the receptors for Thrombopoietin (TPO), Prolactin, and Growth hormone. Illustrative type I cytokine receptors include, but are not limited to, GM-CSF receptor, G-CSF receptor, LIF receptor, CNTF receptor, TPO receptor, and type I IL receptors.

In various embodiments, the receptor for the signaling agent is a Type II cytokine receptor. Type II cytokine receptors are multimeric receptors composed of heterologous subunits, and are receptors mainly for interferons. This family of receptors includes, but is not limited to, receptors for interferon- α , interferon- β and interferon- γ , IL10, IL22, and tissue factor. Illustrative type II cytokine receptors include, but are not limited to, IFN- α receptor (*e.g.* IFNAR1 and IFNAR2), IFN- β receptor, IFN- γ receptor (*e.g.* IFNGR1 and IFNGR2), and type II IL receptors.

In various embodiments, the receptor for the signaling agent is a G protein-coupled receptor. Chemokine receptors are G protein-coupled receptors with seven transmembrane structure and coupled to G-protein for signal transduction. Chemokine receptors include, but are not limited to, CC chemokine receptors, CXC chemokine receptors, CX3C chemokine receptors, and XC chemokine receptor (XCR1). Exemplary chemokine receptors include, but are not limited to, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR3B, CXCR4, CXCR5, CSCR6, CXCR7, XCR1, and CX3CR1.

In various embodiments, the receptor for the signaling agent is a TNFR family member. Tumor necrosis factor receptor (TNFR) family members share a cysteine-rich domain (CRD) formed of three disulfide bonds surrounding a core motif of CXXCXXC creating an elongated molecule. Exemplary tumor necrosis factor receptor family members include: CDI 20a (TNFRSF1A), CD 120b (TNFRSF1B), Lymphotoxin beta receptor (LTBR, TNFRSF3), CD 134 (TNFRSF4), CD40 (CD40, TNFRSF5), FAS (FAS, TNFRSF6), TNFRSF6B (TNFRSF6B), CD27 (CD27, TNFRSF7), CD30 (TNFRSF8), CD137 (TNFRSF9), TNFRSF10A (TNFRSF10A), TNFRSF10B (TNFRSF10B), TNFRSF10C (TNFRSF10C), TNFRSF10D (TNFRSF10D), RANK (TNFRSF1A), Osteoprotegerin (TNFRSF1B), TNFRSF12A (TNFRSF12A), TNFRSF13B (TNFRSF13B), TNFRSF13C (TNFRSF13C), TNFRSF14 (TNFRSF14), Nerve growth factor receptor (NGFR, TNFRSF16), TNFRSF17 (TNFRSF17), TNFRSF18 (TNFRSF18),

TNFRSF19 (TNFRSF19), TNFRSF21 (TNFRSF21), and TNFRSF25 (TNFRSF25). In an embodiment, the TNFR family member is CD120a (TNFRSF1A) or TNF-R1. In another embodiment, the TNFR family member is CD 120b (TNFRSF1B) or TNF-R2.

In various embodiments, the receptor for the signaling agent is a TGF-beta receptor. TGF-beta receptors are single pass serine/threonine kinase receptors. TGF-beta receptors include, but are not limited to, TGFBR1, TGFBR2, and TGFBR3.

In various embodiments, the receptor for the signaling agent is an Ig superfamily receptor. Receptors in the immunoglobulin (Ig) superfamily share structural homology with immunoglobulins. Receptors in the Ig superfamily include, but are not limited to, interleukin-1 receptors, CSF-1R, PDGFR (e.g. PDGFRA and PDGFRB), and SCFR.

In various embodiments, the receptor for the signaling agent is a tyrosine kinase superfamily receptor. Receptors in the tyrosine kinase superfamily are well known in the art. There are about 58 known receptor tyrosine kinases (RTKs), grouped into 20 subfamilies. Receptors in the tyrosine kinase superfamily include, but are not limited to, FGF receptors and their various isoforms such as FGFR1, FGFR2, FGFR3, FGFR4, and FGFR5.

In an embodiment, the modified signaling agent is interferon α . In such embodiments, the modified IFN- α agent has reduced affinity and/or activity for the IFN- α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains. In some embodiments, the modified IFN- α agent has substantially reduced or ablated affinity and/or activity for the IFN- α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains.

Mutant forms of interferon α are known to the person skilled in the art. In an illustrative embodiment, the modified signaling agent is the allelic form IFN- α 2a having the amino acid sequence of SEQ ID NO: 317.

In an illustrative embodiment, the modified signaling agent is the allelic form IFN- α 2b having the amino acid sequence of (which differs from IFN- α 2a at amino acid position 23) SEQ ID NO: 318.

In some embodiments, said IFN- α 2 mutant (IFN- α 2a or IFN- α 2b) is mutated at one or more amino acids at positions 144-154, such as amino acid positions 148, 149 and/or 153. In some embodiments, the IFN- α 2 mutant comprises one or more mutations selected from L153A, R149A, and M148A. Such mutants are described, for example, in WO2013/107791 and Piehler *et al.*, (2000) J. Biol. Chem, 275:40425-33, the entire contents of all of which are hereby incorporated by reference.

In some embodiments, the IFN- α 2 mutants have reduced affinity and/or activity for IFNAR1. In some embodiments, the IFN- α 2 mutant comprises one or more mutations selected from F64A, N65A, T69A, L80A, Y85A, and Y89A, as described in WO2010/030671, the entire contents of which is hereby incorporated by reference.

In some embodiments, the IFN- α 2 mutant comprises one or more mutations selected from K133A, R144A, R149A, and L153A as described in WO2008/124086, the entire contents of which is hereby incorporated by reference.

In some embodiments, the IFN- α 2 mutant comprises one or more mutations selected from R120E and R120E/K121E, as described in WO2015/007520 and WO2010/030671, the entire contents of which are hereby

incorporated by reference. In such embodiments, said IFN- α 2 mutant antagonizes wild type IFN- α 2 activity. In such embodiments, said mutant IFN- α 2 has reduced affinity and/or activity for IFNAR1 while affinity and/or activity of IFNAR2 is retained.

In some embodiments, the human IFN- α 2 mutant comprises (1) one or more mutations selected from R120E and R120E/K121E, which, without wishing to be bound by theory, create an antagonistic effect and (2) one or more mutations selected from K133A, R144A, R149A, and L153A, which, without wishing to be bound by theory, allow for an attenuated effect at, for example, IFNAR2. In an embodiment, the human IFN- α 2 mutant comprises R120E and L153A.

In some embodiments, the human IFN- α 2 mutant comprises one or more mutations selected from, L15A, A19W, R22A, R23A, L26A, F27A, L30A, L30V, K31A, D32A, R33K, R33A, R33Q, H34A, D35A, Q40A, D114R, L117A, R120A, R125A, K134A, R144A, A145G, A145M, M148A, R149A, S152A, L153A, and N156A as disclosed in WO 2013/059885, the entire disclosures of which are hereby incorporated by reference. In some embodiments, the human IFN- α 2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L30A as disclosed in WO 2013/059885. In some embodiments, the human IFN- α 2 mutant comprises the mutations H57Y, E58N, Q61S, and/or R33A as disclosed in WO 2013/059885. In some embodiments, the human IFN- α 2 mutant comprises the mutations H57Y, E58N, Q61S, and/or M148A as disclosed in WO 2013/059885. In some embodiments, the human IFN- α 2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L153A as disclosed in WO 2013/059885. In some embodiments, the human IFN- α 2 mutant comprises the mutations N65A, L80A, Y85A, and/or Y89A as disclosed in WO 2013/059885. In some embodiments, the human IFN- α 2 mutant comprises the mutations N65A, L80A, Y85A, Y89A, and/or D114A as disclosed in WO 2013/059885.

In an embodiment, the modified signaling agent is interferon β . In such embodiments, the modified interferon β agent has reduced affinity and/or activity for the IFN- α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains. In some embodiments, the modified interferon β agent has substantially reduced or ablated affinity and/or activity for the IFN- α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains.

In an illustrative embodiment, the modified signaling agent is IFN- β . In various embodiments, the IFN- β encompasses functional derivatives, analogs, precursors, isoforms, splice variants, or fragments of IFN- β . In various embodiments, the IFN- β encompasses IFN- β derived from any species. In an embodiment, the chimeric protein comprises a modified version of mouse IFN- β . In another embodiment, the chimeric protein comprises a modified version of human IFN- β . Human IFN- β is a polypeptide with a molecular weight of about 22 kDa comprising 166 amino acid residues. The amino acid sequence of human IFN- β is SEQ ID NO: 319.

In some embodiments, the human IFN- β is IFN- β -1a which is a glycosylated form of human IFN- β . In some embodiments, the human IFN- β is IFN- β -1b which is a non-glycosylated form of human IFN- β that has a Met-1 deletion and a Cys-17 to Ser mutation.

In various embodiments, the modified IFN- β has one or more mutations that reduce its binding to or its affinity for the IFNAR1 subunit of IFNAR. In one embodiment, the modified IFN- β has reduced affinity and/or activity at

IFNAR1. In various embodiments, the modified IFN- β is human IFN- β and has one or more mutations at positions F67, R71, L88, Y92, I95, N96, K123, and R124. In some embodiments, the one or more mutations are substitutions selected from F67G, F67S, R71A, L88G, L88S, Y92G, Y92S, I95A, N96G, K123G, and R124G. In an embodiment, the modified IFN- β comprises the F67G mutation. In an embodiment, the modified IFN- β comprises the K123G mutation. In an embodiment, the modified IFN- β comprises the F67G and R71A mutations. In an embodiment, the modified IFN- β comprises the L88G and Y92G mutations. In an embodiment, the modified IFN- β comprises the Y92G, I95A, and N96G mutations. In an embodiment, the modified IFN- β comprises the K123G and R124G mutations. In an embodiment, the modified IFN- β comprises the F67G, L88G, and Y92G mutations. In an embodiment, the modified IFN- β comprises the F67S, L88S, and Y92S mutations.

In some embodiments, the modified IFN- β has one or more mutations that reduce its binding to or its affinity for the IFNAR2 subunit of IFNAR. In one embodiment, the modified IFN- β has reduced affinity and/or activity at IFNAR2. In various embodiments, the modified IFN- β is human IFN- β and has one or more mutations at positions W22, R27, L32, R35, V148, L151, R152, and Y155. In some embodiments, the one or more mutations are substitutions selected from W22G, R27G, L32A, L32G, R35A, R35G, V148G, L151G, R152A, R152G, and Y155G. In an embodiment, the modified IFN- β comprises the W22G mutation. In an embodiment, the modified IFN- β comprises the L32A mutation. In an embodiment, the modified IFN- β comprises the L32G mutation. In an embodiment, the modified IFN- β comprises the R35A mutation. In an embodiment, the modified IFN- β comprises the R35G mutation. In an embodiment, the modified IFN- β comprises the V148G mutation. In an embodiment, the modified IFN- β comprises the R152A mutation. In an embodiment, the modified IFN- β comprises the R152G mutation. In an embodiment, the modified IFN- β comprises the Y155G mutation. In an embodiment, the modified IFN- β comprises the W22G and R27G mutations. In an embodiment, the modified IFN- β comprises the L32A and R35A mutation. In an embodiment, the modified IFN- β comprises the L151G and R152A mutations. In an embodiment, the modified IFN- β comprises the V148G and R152A mutations.

In some embodiments, the modified IFN- β has one or more of the following mutations: R35A, R35T, E42K, M62I, G78S, A141Y, A142T, E149K, and R152H. In some embodiments, the modified IFN- β has one or more of the following mutations: R35A, R35T, E42K, M62I, G78S, A141Y, A142T, E149K, and R152H in combination with C17S or C17A.

In some embodiments, the modified IFN- β has one or more of the following mutations: R35A, R35T, E42K, M62I, G78S, A141Y, A142T, E149K, and R152H in combination with any of the other IFN- β mutations described herein.

The crystal structure of human IFN- β is known and is described in Karpusas et al., (1998) PNAS, 94(22): 11813–11818. Specifically, the structure of human IFN- β has been shown to include five α -helices (*i.e.*, A, B, C, D, and E) and four loop regions that connect these helices (*i.e.*, AB, BC, CD, and DE loops). In various embodiments, the modified IFN- β has one or more mutations in the A, B, C, D, E helices and/or the AB, BC, CD, and DE loops which reduce its binding affinity or activity at a therapeutic receptor such as IFNAR. Exemplary mutations are described in WO2000/023114 and US20150011732, the entire contents of which are hereby incorporated by reference. In

an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 15, 16, 18, 19, 22, and/or 23. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 28-30, 32, and 33. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 36, 37, 39, and 42. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 64 and 67 and a serine substitution at position 68. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 71-73. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 92, 96, 99, and 100. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 128, 130, 131, and 134. In an exemplary embodiment, the modified IFN- β is human IFN- β comprising alanine substitutions at amino acid positions 149, 153, 156, and 159. In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at W22, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at R27, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at W22, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at R27, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at L32, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at R35, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at L32, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V) and a mutation at R35, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at F67, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at R71, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at F67, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at R71, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at L88, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at Y92, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at F67, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at L88, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V) and a mutation at Y92, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at L88, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V) and a mutation at Y92, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at I95, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), methionine (M), and valine (V) and a mutation at Y92, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at N96, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at Y92, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at Y92, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at I95, the mutation being an aliphatic hydrophobic residue selected from glycine

(G), alanine (A), leucine (L), methionine (M), and valine (V) and a mutation at N96, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at K123, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at R124, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 188 and a mutation at K123, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at R124, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at L151, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at R152, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at L151, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), isoleucine (I), methionine (M), and valine (V) and a mutation at R152, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at V148, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), and methionine (M).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at V148, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V) and a mutation at R152, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the mutant IFN- β comprises SEQ ID NO: 319 and a mutation at Y155, the mutation being an aliphatic hydrophobic residue selected from glycine (G), alanine (A), leucine (L), isoleucine (I), methionine (M), and valine (V).

In some embodiments, the present invention relates to a chimeric protein comprising: (a) a modified IFN- β , having the amino acid sequence of SEQ ID NO: 319 and a mutation at position W22, wherein the mutation is an aliphatic hydrophobic residue; and (b) one or more targeting moieties, said targeting moieties comprising recognition domains which specifically bind to antigens or receptors of interest (e.g., PD-1 or PD-L1), the modified IFN- β and the one or more targeting moieties are optionally connected with one or more linkers. In various embodiments the mutation at position W22 is aliphatic hydrophobic residue is selected from G, A, L, I, M, and V. In various embodiments the mutation at position W22 is G.

Additional exemplary IFN- β mutants are provided in PCT/EP2017/061544, the entire disclosure of which is incorporated by reference herein.

In an embodiment, the modified signaling agent is interferon γ . In such embodiments, the modified interferon γ agent has reduced affinity and/or activity for the interferon-gamma receptor (IFNGR), *i.e.*, IFNGR1 and IFNGR2 chains. In some embodiments, the modified interferon γ agent has substantially reduced or ablated affinity and/or activity for the interferon-gamma receptor (IFNGR), *i.e.*, IFNGR1 and/or IFNGR2 chains.

IFN- γ is the only member of the type II class of interferons. IFN- γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response. IFN- γ is also produced by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells, macrophages, dendritic cells, and B cells. Activated IFN- γ forms a dimer which acts through a heterodimeric receptor (*i.e.*, IFN- γ receptor or IFN- γ R) composed of IFN- γ receptor 1 and IFN- γ receptor 2 subunits. IFN- γ receptor 1 is the major ligand-binding subunit, while IFN- γ receptor 2 is necessary for signal transduction and also increases the affinity of IFN- γ receptor 1 for its ligand. Binding of the IFN- γ dimer to the receptor activates the JAK-STAT signaling pathway to elicit various biological effects.

In various embodiments, the the modified signaling agent comprises a modified version of IFN- γ as a signaling agent. In various embodiments, the IFN- γ encompasses functional derivatives, analogs, precursors, isoforms, splice variants, or fragments of IFN- γ . In various embodiments, the IFN- γ encompasses IFN- γ derived from any species. In an embodiment, the modified signaling agent comprises a modified version of mouse IFN- γ . In another embodiment, the modified signaling agent comprises a modified version of human IFN- γ .

Human IFN- γ is a polypeptide comprising 166 amino acid residues. In an embodiment, the human IFN- γ has the amino acid sequence of SEQ ID NO: 320.

As used herein, human IFN- γ may also refer to mature human IFN- γ without the N-terminal signal peptide. In this embodiment, the mature human IFN- γ comprises 143 amino acids and has the amino acid sequence of SEQ ID NO: 321.

In some embodiments, the human IFN- γ is a glycosylated form of human IFN- γ . In some embodiments, the human IFN- γ is a non-glycosylated form of human IFN- γ .

The sequences of IFN- γ are known in the art. In various embodiments the modified IFN- γ comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at

least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with the known wild type amino acid sequences of IFN- γ (e.g., about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In some embodiments the modified IFN- γ comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with human IFN- γ having an amino acid sequence of SEQ ID NO: 320 (e.g., about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In some embodiments the modified IFN- γ comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at

least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with human IFN- γ having an amino acid sequence of SEQ ID NO: 321 (e.g., about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In various embodiments, the modified IFN- γ comprises an amino acid sequence having one or more amino acid mutations. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

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

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

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

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

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

In various embodiments, the IFN- γ is modified to have one or more mutations. In some embodiments, the mutations allow for the modified IFN- γ to have one or more of attenuated activity such as one or more of reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmutated, e.g., the wild type form

of IFN- γ . For instance, the one or more of attenuated activity such as reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmutated, e.g., the wild type form of IFN- γ may be at a therapeutic receptor such as the IFN- γ receptor. Consequentially, in various embodiments, the mutations allow for the modified soluble agent to have reduced systemic toxicity, reduced side effects, and reduced off-target effects relative to unmutated, e.g., the wild type form of IFN- γ .

In various embodiments, the IFN- γ is modified to have a mutation that reduces its binding affinity and/or activity at a therapeutic receptor such as the IFN- γ receptor comprising the IFN- γ receptor 1 and IFN- γ receptor 2 subunits. In some embodiments, the activity provided by the wild type IFN- γ is agonism at the therapeutic receptor (e.g., activation of a cellular effect at a site of therapy). For example, the wild type IFN- γ may activate the therapeutic receptor. In such embodiments, the mutation results in the modified IFN- γ to have reduced activating activity at the therapeutic receptor.

In some embodiments, the reduced affinity and/or activity at the therapeutic receptor (e.g., IFN- γ receptor) is restorable by attachment with a targeting moiety. In other embodiments, the reduced affinity and/or activity at the therapeutic receptor is not substantially restorable by attachment with the targeting moiety. In various embodiments, the therapeutic chimeric proteins of the present invention reduce off-target effects because the IFN- γ has mutations that weaken binding affinity and/or activity at a therapeutic receptor. In various embodiments, this reduces side effects observed with, for example, the wild type IFN- γ . In various embodiments, the modified IFN- γ is substantially inactive en route to the site of therapeutic activity and has its effect substantially on specifically targeted cell types which greatly reduces undesired side effects.

In various embodiments, the modified IFN- γ has one or more mutations that cause the IFN- γ to have attenuated or reduced affinity and/or activity, e.g., binding (e.g., KD) and/or activation (measurable as, for example, KA and/or EC50) for one or more therapeutic receptors (e.g., IFN- γ receptor). In various embodiments, the reduced affinity and/or activity at the therapeutic receptor allows for attenuation of activity and/or signaling from the therapeutic receptor.

In various embodiments, the modified IFN- γ has one or more mutations that reduce its binding to or its affinity for and/or biological activity for the IFN- γ receptor 1 subunit. In one embodiment, the modified IFN- γ has reduced affinity and/or activity at the IFN- γ receptor 1 subunit. In various embodiments, the modified IFN- γ is human IFN- γ that has one or more mutations at amino acid residues involved with binding to the IFN- γ receptor 1 subunit. In some embodiments, the modified IFN- γ is human IFN- γ that has one or more mutations at amino acids located at the interface with the IFN- γ receptor 1 subunit. In various embodiments, the one or more mutations are at amino acids selected from, but not limited to Q1, V5, E9, K12, H19, S20, V22, A23, D24, N25, G26, T27, L30, K108, H111, E112, I114, Q115, A118, E119, and K125 (each with respect SEQ ID NO: 321, which is a wild type human IFN- γ and which lacks its N-terminal signal sequence). In some embodiments, the one or more mutations are substitutions selected from V5E, S20E, V22A, A23G, A23F, D24G, G26Q, H111A, H111D, I114A, Q115A, and

A118G (each with respect SEQ ID NO: 321). In embodiments, the one or more mutations are substitutions selected from V22A, A23G, D24G, H111A, H111D, I114A, Q115A, and A118G.

In an embodiment, the modified IFN- γ comprises the mutations A23G and D24G. In another embodiment, the modified IFN- γ comprises the mutations I114A and A118G. In a further embodiment, the modified IFN- γ comprises the mutations V5E, S20E, A23F, and G26Q.

In various embodiments, the modified IFN- γ has one or more of the following mutations: deletion of residue A23, deletion of residue D24, an S20I substitution, an A23V substitution, a D21K substitution and a D24A substitution.

In some embodiments, the modified IFN- γ has one or more mutations that reduce its binding to or its affinity and/or biological activity for the IFN- γ receptor 2 subunit.

In some embodiments, the modified IFN- γ has one or more mutations that reduce its binding to or its affinity and/or biological activity for both IFN- γ receptor 1 and IFN- γ receptor 2 subunits.

In some embodiments, the modified IFN- γ has one or more mutations that reduce its binding to or its affinity and/or biological activity for IFN- γ receptor 1 and one or more mutations that substantially reduce or ablate binding to or its affinity and/or biological activity for IFN- γ receptor 2. In some embodiments, chimeric proteins with such modified IFN- γ can provide target-selective IFN- γ receptor 1 activity (e.g., IFN- γ receptor 1 activity is restorable via targeting through the targeting moiety).

In some embodiments, the modified IFN- γ has one or more mutations that reduce its binding to or its affinity and/or biological activity for IFN- γ receptor 1 and one or more mutations that reduce its binding to or its affinity and/or biological activity for IFN- γ receptor 1. In some embodiments, chimeric proteins with such modified IFN- γ can provide target-selective IFN- γ receptor 1 and/or IFN- γ receptor 1 activity (e.g., IFN- γ receptor 1 and IFN- γ receptor 2 activities are restorable via targeting through the targeting moiety).

In various embodiments, the modified IFN- γ is truncated at the C-terminus. In some embodiments, the modified IFN- γ is mature IFN- γ comprising the amino acid sequence of SEQ ID NO: 321 with deletions of the C-terminal terminus. In such embodiments, the mature IFN- γ may comprise a C-terminal truncation of at least about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, or about 25 amino acid residues. In an embodiment, the modified IFN- γ is mature IFN- γ comprising the amino acid sequence of SEQ ID NO: 321 with C-terminal deletions of 5 amino acids. In an embodiment, the modified IFN- γ is mature IFN- γ comprising the amino acid sequence of SEQ ID NO: 321 with C-terminal deletions of 7 amino acids. In an embodiment, the modified IFN- γ is mature IFN- γ comprising the amino acid sequence of SEQ ID NO: 321 with C-terminal deletions of 14 amino acids. In an embodiment, the modified IFN- γ is mature IFN- γ comprising the amino acid sequence of SEQ ID NO: 321 with C-terminal deletions of 15 amino acids. In an embodiment, the modified IFN- γ is mature IFN- γ comprising the amino acid sequence of SEQ ID NO: 321 with C-terminal deletions of 16 amino acids. Additional modified IFN- γ with C-terminal truncations that may be utilized in the present invention is

described in Haelewyn et al., *Biochem. J.* (1997), 324:591-595 and Lundell et al., *Protein Eng.* (1991) 4:335-341, the entire contents are hereby incorporated by reference

In various embodiments, the modified IFN- γ is a single chain IFN- γ as described, for example, in Randal et al. (2001) *Structure* 9:155-163 and Randal et al. (1998) *Protein Sci.* 7:1057-1060, the entire contents are hereby incorporated by reference. In some embodiments, the single chain IFN- γ comprises a first IFN- γ chain linked at its C-terminus to the N-terminus of a second IFN- γ chain. In various embodiments, the first and second IFN- γ chains are linked by a linker, as described elsewhere herein.

In some embodiments, the first IFN- γ chain comprises a C-terminal truncation of at least about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, or about 25 amino acid residues. In an embodiment, the first IFN- γ chain comprises a C-terminal truncation of about 24 amino acid residues. In some embodiments, the second IFN- γ chain comprises an N-terminal truncation of at least about 1, about 2, about 3, about 4, or about 5 amino acid residues. In an embodiment, the second IFN- γ chain comprises an N-terminal truncation of about 3 amino acid residues. In some embodiments, the second IFN- γ chain comprises a C-terminal truncation of at least about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, or about 25 amino acid residues. In various embodiments, the first and/or second IFN- γ chains comprise one or more amino acid mutations at Q1, V5, E9, K12, H19, S20, V22, A23, D24, N25, G26, T27, L30, K108, H111, E112, I114, Q115, A118, E119, and K125, as described elsewhere herein. In various embodiments, the first and/or second IFN- γ chains comprise one or more substitutions selected from V5E, S20E, V22A, A23G, A23F, D24G, G26Q, H111A, H111D, I114A, Q115A, and A118G. In various embodiments, the first and/or second IFN- γ chains comprise one or more substitutions selected from V22A, A23G, D24G, H111A, H111D, I114A, Q115A, and A118G. In various embodiments, the first and/or second IFN- γ chains comprise the A23G and the D24G substitution. In various embodiments, the first and/or second IFN- γ chains comprise the I114A and the A118G substitution. In another embodiment, the mutations are V5E, S20E, A23F, and G26Q.

In various embodiments, a first and/or second IFN- γ chain comprises one or more substitutions as disclosed herein and the first and/or second IFN- γ chain comprises a C-terminal truncation as disclosed herein.

In various embodiments, a first and/or second IFN- γ chain comprises one or more substitutions as disclosed herein and a C-terminal truncation as disclosed herein.

The crystal structure of human IFN- γ is known and is described in, for example, Ealick et al., (1991) *Science*, 252: 698-702. Specifically, the structure of human IFN- γ has been shown to include a core of six α -helices and an extended unfolded sequence in the C-terminal region. In various embodiments, the modified IFN- γ has one or more mutations in the one or more helices which reduce its binding affinity and/or biological activity at a therapeutic receptor (e.g., IFN- γ receptor).

In various embodiments, the modified IFN- γ has about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 10%-20%, about 20%-40%, about 50%, about 40%-60%, about 60%-80%, about 80%-100% of the affinity and/or biological activity for the therapeutic receptor (e.g., IFN- γ receptor or any one of its IFN- γ receptor 1 and IFN- γ receptor 2 subunits) relative to the wild type IFN- γ . In some embodiments, the binding affinity and/or biological activity is at least about 2-fold lower, about 3-fold lower, about 4-fold lower, about 5-fold lower, about 6-fold lower, about 7-fold lower, about 8-fold lower, about 9-fold lower, at least about 10-fold lower, at least about 15-fold lower, at least about 20-fold lower, at least about 25-fold lower, at least about 30-fold lower, at least about 35-fold lower, at least about 40-fold lower, at least about 45-fold lower, at least about 50-fold lower, at least about 100-fold lower, at least about 150-fold lower, or about 10-50-fold lower, about 50-100-fold lower, about 100-150-fold lower, about 150-200-fold lower, or more than 200-fold lower relative to the wild type IFN- γ .

In various embodiments, the modified IFN- γ comprises one or more mutations that reduce the endogenous activity of the IFN- γ to about 75%, or about 70%, or about 60%, or about 50%, or about 40%, or about 30%, or about 25%, or about 20%, or about 10%, or about 5%, or about 3%, or about 1%, e.g., relative to the wild type IFN- γ .

In some embodiments, the modified IFN- γ comprises one or more mutations that cause the modified IFN- γ to have reduced affinity and/or biological activity for a receptor. In some embodiments, the modified IFN- γ 's binding affinity and/or biological activity for a receptor is lower than the binding affinity and/or biological activity of the targeting moiety for its receptor. In some embodiments, this binding affinity and/or biological activity differential is between the modified IFN- γ /receptor and targeting moiety/receptor on the same cell. In some embodiments, this binding affinity and/or biological activity, differential allows for the modified IFN- γ to have localized, on-target effects and to minimize off-target effects that underlie side effects that are observed with wild type IFN- γ . In some embodiments, this binding affinity and/or biological activity is at least about 2-fold, or at least about 5-fold, or at least about 10-fold, or at least about 15-fold lower, or at least about 25-fold, or at least about 50-fold lower, or at least about 100-fold, or at least about 150-fold less.

Receptor binding activity may be measured using methods known in the art. For example, affinity and/or binding activity may be assessed by Scatchard plot analysis and computer-fitting of binding data (e.g., Scatchard, 1949) or by reflectometric interference spectroscopy under flow through conditions, as described by Brecht et al. (1993), the entire contents of all of which are hereby incorporated by reference.

In some embodiments, the modified signaling agent is a consensus interferon. The consensus interferon is generated by scanning the sequences of several human non-allelic IFN- α subtypes and assigning the most frequently observed amino acid in each corresponding position. The consensus interferon differs from IFN- α 2b at 20 out of 166 amino acids (88% homology), and comparison with IFN- β shows identity at over 30% of the amino acid positions. In various embodiments, the consensus interferon comprises the amino acid sequence of SEQ ID NO: 322.

In some embodiments, the consensus interferon comprises the amino acid sequence of SEQ ID NO: 323, which differs from the amino acid sequence of SEQ ID NO: 322 by one amino acid, i.e., SEQ ID NO: 323 lacks the initial methionine residue of SEQ ID NO: 322.

In various embodiments, the consensus interferon comprises a modified version of the consensus interferon, i.e., a consensus interferon variant, as a signaling agent. In various embodiments, the consensus interferon variant encompasses functional derivatives, analogs, precursors, isoforms, splice variants, or fragments of the consensus interferon.

In an embodiment, the consensus interferon variants are selected from the consensus interferon variants disclosed in U.S. Patent Nos. 4,695,623, 4,897,471, 5,541,293, and 8,496,921, the entire contents of all of which are hereby incorporated by reference. For example, the consensus interferon variant may comprise the amino acid sequence of IFN-CON2 or IFN-CON3 as disclosed in U.S. Patent Nos. 4,695,623, 4,897,471, and 5,541,293. In an embodiment, the consensus interferon variant comprises the amino acid sequence of IFN-CON₂, SEQ ID NO: 324.

In an embodiment, the consensus interferon variant comprises the amino acid sequence of IFN-CON₃, SEQ ID NO: 325.

In an embodiment, the consensus interferon variant comprises the amino acid sequence of any one of the variants disclosed in U.S. Patent No. 8,496,921. For example, the consensus variant may comprise the amino acid sequence of SEQ ID NO: 326.

In another embodiment, the consensus interferon variant may comprise the amino acid sequence of SEQ ID NO: 327.

In some embodiments, the consensus interferon variant may be PEGylated, i.e., comprises a PEG moiety. In an embodiment, the consensus interferon variant may comprise a PEG moiety attached at the S156C position of SEQ ID NO: 327.

In some embodiments, the engineered interferon is a variant of human IFN- α 2a, with an insertion of Asp at approximately position 41 in the sequence SEQ ID NO: 328 to yield SEQ ID NO: 329 (which resulted in a renumbering of the sequence relative to IFN- α 2a sequence) and the following mutations of Arg23Lys, Leu26Pro, Glu53Gln, Thr54Ala, Pro56Ser, Asp86Glu, Ile104Thr, Gly106Glu, Thr110Glu, Lys117Asn, Arg125Lys, and Lys136Thr. All embodiments herein that describe consensus interferons apply equally to this engineered interferon.

In various embodiments, the consensus interferon variant comprises an amino acid sequence having one or more amino acid mutations. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

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

In various embodiments, the substitutions may also include non-classical amino acids (e.g. selenocysteine, pyrrolysine, N-formylmethionine β -alanine, GABA and δ -Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-

isomers of the common amino acids, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α -methyl amino acids, N α -methyl amino acids, and amino acid analogs in general).

In various embodiments, the consensus interferon is modified to have one or more mutations. In some embodiments, the mutations allow for the consensus interferon variant to have one or more of attenuated activity such as one or more of reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmutated, e.g., the wild type form of the consensus interferon (e.g., the consensus interferon having an amino acid sequence of SEQ ID NO: 325 or 326). For instance, the one or more of attenuated activity such as reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmutated, e.g., the wild type form of the consensus interferon, may be at a therapeutic receptor such as IFNAR. Consequentially, in various embodiments, the mutations allow for the consensus interferon variant to have reduced systemic toxicity, reduced side effects, and reduced off-target effects relative to unmutated, e.g. the wild type form of the consensus interferon.

In various embodiments, the consensus interferon is modified to have a mutation that reduces its binding affinity or activity at a therapeutic receptor such as IFNAR. In some embodiments, the activity provided by the consensus interferon is agonism at the therapeutic receptor (e.g. activation of a cellular effect at a site of therapy). For example, the consensus interferon may activate the therapeutic receptor. In such embodiments, the mutation results in the consensus interferon variant to have reduced activating activity at the therapeutic receptor.

In some embodiments, the reduced affinity or activity at the therapeutic receptor is restorable by attachment with a targeting moiety. In other embodiments, the reduced affinity or activity at the therapeutic receptor is not substantially restorable by attachment with the targeting moiety. In various embodiments, the therapeutic Fc-based chimeric proteins of the present invention reduce off-target effects because the consensus interferon variant has mutations that weaken binding affinity or activity at a therapeutic receptor. In various embodiments, this reduces side effects observed with, for example, the wild type consensus interferon. In various embodiments, the consensus interferon variant is substantially inactive en route to the site of therapeutic activity and has its effect substantially on specifically targeted cell types which greatly reduces undesired side effects.

In various embodiments, the consensus interferon variant has one or more mutations that cause the consensus interferon variant to have attenuated or reduced affinity, e.g. binding (e.g. KD) and/or activation (measurable as, for example, KA and/or EC50) for one or more therapeutic receptors. In various embodiments, the reduced affinity at the therapeutic receptor allows for attenuation of activity and/or signaling from the therapeutic receptor.

In various embodiments, the consensus interferon variant has one or more mutations that reduce its binding to or its affinity for the IFNAR1 subunit of IFNAR. In one embodiment, the consensus interferon variant has reduced affinity and/or activity at IFNAR1. In some embodiments, the consensus interferon variant has one or more

mutations that reduce its binding to or its affinity for the IFNAR2 subunit of IFNAR. In some embodiments, the consensus interferon variant has one or more mutations that reduce its binding to or its affinity for both IFNAR1 and IFNAR2 subunits.

In some embodiments, the consensus interferon variant has one or more mutations that reduce its binding to or its affinity for IFNAR1 and one or more mutations that substantially reduce or ablate binding to or its affinity for IFNAR2. In some embodiments, Fc-based chimeric proteins with such consensus interferon variant can provide target-selective IFNAR1 activity (e.g. IFNAR1 activity is restorable via targeting through the targeting moiety, e.g., SIRP α).

In some embodiments, the consensus interferon variant has one or more mutations that reduce its binding to or its affinity for IFNAR2 and one or more mutations that substantially reduce or ablate binding to or its affinity for IFNAR1. In some embodiments, Fc-based chimeric proteins with such consensus interferon variant can provide target-selective IFNAR2 activity (e.g. IFNAR2 activity is restorable via targeting through the targeting moiety, e.g., SIRP α).

In some embodiments, the consensus interferon variant has one or more mutations that reduce its binding to or its affinity for IFNAR1 and one or more mutations that reduce its binding to or its affinity for IFNAR2. In some embodiments, Fc-based chimeric proteins with such consensus interferon variant can provide target-selective IFNAR1 and/or IFNAR2 activity (e.g. IFNAR1 and/or IFNAR2 activity is restorable via targeting through the targeting moiety, e.g., SIRP α).

In some embodiments, the consensus interferon is modified to have a mutation at one or more amino acids at positions 145-155, such as amino acid positions 149, 150 and/or 154, with reference to SEQ ID NO: 326. In some embodiments, the consensus interferon is modified to have a mutation at one or more amino acids at positions 145-155, such as amino acid positions 149, 150 and/or 154, with reference to SEQ ID NO: 326, the substitutions optionally being hydrophobic and selected from alanine, valine, leucine, and isoleucine. In some embodiments, the consensus interferon mutant comprises one or more mutations selected from M149A, R150A, and L154A, and, with reference to SEQ ID NO: 323.

In an embodiment, the consensus interferon is modified to have a mutation at amino acid position 121 (i.e., K121), with reference to SEQ ID NO: 323. In an embodiment, the consensus interferon comprises a K121E mutation, with reference to SEQ ID NO: 323.

In some embodiments, the modified signaling agent is vascular endothelial growth factor (VEGF). VEGF is a potent growth factor that plays major roles in physiological but also pathological angiogenesis, regulates vascular permeability and can act as a growth factor on cells expressing VEGF receptors. Additional functions include, among others, stimulation of cell migration in macrophage lineage and endothelial cells. Several members of the VEGF family of growth factors exist, as well as at least three receptors (VEGFR-1, VEGFR -2, and VEGFR -3). Members of the VEGF family can bind and activate more than one VEGFR type. For example, VEGF-A binds VEGFR-1 and -2, while VEGF-C can bind VEGFR-2 and -3. VEGFR-1 and -2 activation regulates angiogenesis

while VEGFR-3 activation is associated with lymphangiogenesis. The major pro-angiogenic signal is generated from activation of VEGFR-2. VEGFR-1 activation has been reported to be possibly associated with negative role in angiogenesis. It has also been reported that VEGFR-1 signaling is important for progression of tumors *in vivo* via bone marrow-derived VEGFR-1 positive cells (contributing to formation of premetastatic niche in the bone). Several therapies based on VEGF-A directed/neutralizing therapeutic antibodies have been developed, primarily for use in treatment of various human tumors relying on angiogenesis. These are not without side effects though. This may not be surprising considering that these operate as general, non-cell/tissue specific VEGF/VEGFR interaction inhibitors. Hence, it would be desirable to restrict VEGF (*e.g.* VEGF-A)/VEGFR-2 inhibition to specific target cells (*e.g.* tumor vasculature endothelial cells).

In some embodiments, the VEGF is VEGF-A, VEGF-B, VEGF-C, VEGF-D, or VEGF-E and isoforms thereof including the various isoforms of VEGF-A such as VEGF₁₂₁, VEGF_{121b}, VEGF₁₄₅, VEGF₁₆₅, VEGF_{165b}, VEGF₁₈₉, and VEGF₂₀₆. In some embodiments, the modified signaling agent has reduced affinity and/or activity for VEGFR-1 (Flt-1) and/or VEGFR-2 (KDR/Flk-1). In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for VEGFR-1 (Flt-1) and/or VEGFR-2 (KDR/Flk-1). In an embodiment, the modified signaling agent has reduced affinity and/or activity for VEGFR-2 (KDR/Flk-1) and/or substantially reduced or ablated affinity and/or activity for VEGFR-1 (Flt-1). Such an embodiment finds use, for example, in wound healing methods or treatment of ischemia-related diseases (without wishing to be bound by theory, mediated by VEGFR-2's effects on endothelial cell function and angiogenesis). In various embodiments, binding to VEGFR-1 (Flt-1), which is linked to cancers and pro-inflammatory activities, is avoided. In various embodiments, VEGFR-1 (Flt-1) acts a decoy receptor and therefore substantially reduces or ablates affinity at this receptor avoids sequestration of the therapeutic agent. In an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for VEGFR-1 (Flt-1) and/or substantially reduced or ablated affinity and/or activity for VEGFR-2 (KDR/Flk-1). In some embodiments, the VEGF is VEGF-C or VEGF-D. In such embodiments, the modified signaling agent has reduced affinity and/or activity for VEGFR-3. Alternatively, the modified signaling agent has substantially reduced or ablated affinity and/or activity for VEGFR-3.

Proangiogenic therapies are also important in various diseases (*e.g.* ischemic heart disease, bleeding *etc.*), and include VEGF-based therapeutics. Activation of VEGFR-2 is proangiogenic (acting on endothelial cells). Activation of VEGFR-1 can cause stimulation of migration of inflammatory cells (including, for example, macrophages) and lead to inflammation associated hypervascular permeability. Activation of VEGFR-1 can also promote bone marrow associated tumor niche formation. Thus, VEGF based therapeutic selective for VEGFR-2 activation would be desirable in this case. In addition, cell specific targeting, *e.g.* to endothelial cells, would be desirable.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (*e.g.* antagonistic) for VEGFR-2 and/or has substantially reduced or ablated affinity and/or activity for VEGFR-1. When targeted to tumor vasculature endothelial cells via a targeting moiety that binds to a tumor endothelial cell marker (*e.g.* PSMA and others), such construct inhibits VEGFR-2 activation specifically on such marker-positive cells, while not activating VEGFR-1 *en route* and on target cells (if activity ablated), thus eliminating induction of inflammatory responses,

for example. This would provide a more selective and safe anti-angiogenic therapy for many tumor types as compared to VEGF-A neutralizing therapies.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (e.g. agonistic) for VEGFR-2 and/or has substantially reduced or ablated affinity and/or activity for VEGFR-1. Through targeting to vascular endothelial cells, such construct, in some embodiments, promotes angiogenesis without causing VEGFR-1 associated induction of inflammatory responses. Hence, such a construct would have targeted proangiogenic effects with substantially reduced risk of side effects caused by systemic activation of VEGFR-2 as well as VEGFR-1.

In an illustrative embodiment, the modified signaling agent is VEGF₁₆₅, which has the amino acid sequence of SEQ ID NO: 330.

In another illustrative embodiment, the modified signaling agent is VEGF_{165b}, which has the amino acid sequence of SEQ ID NO: 331.

In these embodiments, the modified signaling agent has a mutation at amino acid I83 (e.g., a substitution mutation at I83, e.g., I83K, I83R, or I83H). Without wishing to be bound by theory, it is believed that such mutations may result in reduced receptor binding affinity. See, for example, U.S. Patent No. 9,078,860, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is TNF- α . TNF is a pleiotropic cytokine with many diverse functions, including regulation of cell growth, differentiation, apoptosis, tumorigenesis, viral replication, autoimmunity, immune cell functions and trafficking, inflammation, and septic shock. It binds to two distinct membrane receptors on target cells: TNFR1 (p55) and TNFR2 (p75). TNFR1 exhibits a very broad expression pattern whereas TNFR2 is expressed preferentially on certain populations of lymphocytes, Tregs, endothelial cells, certain neurons, microglia, cardiac myocytes and mesenchymal stem cells. Very distinct biological pathways are activated in response to receptor activation, although there is also some overlap. As a general rule, without wishing to be bound by theory, TNFR1 signaling is associated with induction of apoptosis (cell death) and TNFR2 signaling is associated with activation of cell survival signals (e.g. activation of NF κ B pathway). Administration of TNF is systemically toxic, and this is largely due to TNFR1 engagement. However, it should be noted that activation of TNFR2 is also associated with a broad range of activities and, as with TNFR1, in the context of developing TNF based therapeutics, control over TNF targeting and activity is important.

In some embodiments, the modified signaling agent has reduced affinity and/or activity for TNFR1 and/or TNFR2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TNFR1 and/or TNFR2. TNFR1 is expressed in most tissues, and is involved in cell death signaling while, by contrast, TNFR2 is involved in cell survival signaling. Accordingly, in embodiments directed to methods of treating cancer, the modified signaling agent has reduced affinity and/or activity for TNFR1 and/or substantially reduced or ablated affinity and/or activity for TNFR2. In these embodiments, the chimeric proteins may be targeted to a cell for which apoptosis is desired, e.g. a tumor cell or a tumor vasculature endothelial cell. In embodiments directed

to methods of promoting cell survival, for example, in neurogenesis for the treatment of neurodegenerative disorders, the modified signaling agent has reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1. Stated another way, the present chimeric proteins, in some embodiments, comprise modified TNF- α agent that allows of favoring either death or survival signals.

In some embodiments, the chimeric protein has a modified TNF having reduced affinity and/or activity for TNFR1 and/or substantially reduced or ablated affinity and/or activity for TNFR2. Such a chimera, in some embodiments, is a more potent inducer of apoptosis as compared to a wild type TNF and/or a chimera bearing only mutation(s) causing reduced affinity and/or activity for TNFR1. Such a chimera, in some embodiments, finds use in inducing tumor cell death or a tumor vasculature endothelial cell death (e.g. in the treatment of cancers). Also, in some embodiments, these chimeras avoid or reduce activation of T_{reg} cells via TNFR2, for example, thus further supporting TNFR1-mediated antitumor activity *in vivo*.

In some embodiments, the chimeric protein has a modified TNF having reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1. Such a chimera, in some embodiments, is a more potent activator of cell survival in some cell types, which may be a specific therapeutic objective in various disease settings, including without limitation, stimulation of neurogenesis. In addition, such a TNFR2-favoring chimeras also are useful in the treatment of autoimmune diseases (e.g. Crohn's, diabetes, MS, colitis *etc.* and many others described herein). In some embodiments, the chimera is targeted to auto-reactive T cells. In some embodiments, the chimera promotes T_{reg} cell activation and indirect suppression of cytotoxic T cells.

In some embodiments, the chimera causes the death of auto-reactive T cells, e.g. by activation of TNFR2 and/or avoidance of TNFR1 (e.g. a modified TNF having reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1). Without wishing to be bound by theory these auto-reactive T cells, have their apoptosis/survival signals altered e.g. by NF κ B pathway activity/signaling alterations. In some embodiments, the chimera causes the death of autoreactive T cells having lesions or modifications in the NF κ B pathway, which underlie an imbalance of their cell death (apoptosis)/survival signaling properties and, optionally, altered susceptibility to certain death-inducing signals (e.g., TNFR2 activation).

In some embodiments, a TNFR2 based chimera has additional therapeutic applications in diseases, including various autoimmune diseases, heart disease, de-myelinating and neurodegenerative disorders, and infectious disease, among others.

In an embodiment, the wild type TNF- α has the amino acid sequence of SEQ ID NO: 332.

In such embodiments, the modified TNF- α agent has mutations at one or more amino acid positions 29, 31, 32, 84, 85, 86, 87, 88, 89, 145, 146 and 147 which produces a modified TNF- α with reduced receptor binding affinity. See, for example, U.S. Patent No. 7,993,636, the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified human TNF- α moiety has mutations at one or more amino acid positions R32, N34, Q67, H73, L75, T77, S86, Y87, V91, I97, T105, P106, A109, P113, Y115, E127, N137, D143, A145, and

E146 as described, for example, in WO/2015/007903, the entire contents of which is hereby incorporated by reference (numbering according to the human TNF sequence, Genbank accession number BAG70306, version BAG70306.1 GI: 197692685). In some embodiments, the modified human TNF- α moiety has substitution mutations selected from L29S, R32G, R32W, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, S86T, Y87Q, Y87L, Y87A, Y87F, Y87H, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G, A145R, A145T, E146D, E146K, and S147D. In an embodiment, the human TNF- α moiety has a mutation selected from Y87Q, Y87L, Y87A, Y87F, and Y87H. In another embodiment, the human TNF- α moiety has a mutation selected from I97A, I97Q, and I97S. In a further embodiment, the human TNF- α moiety has a mutation selected from Y115A and Y115G. In an embodiment, the human TNF- α moiety has an E146K mutation. In an embodiment, the human TNF- α moiety has an Y87H and an E146K mutation. In an embodiment, the human TNF- α moiety has an Y87H and an A145R mutation. In an embodiment, the human TNF- α moiety has a R32W and a S86T mutation. In an embodiment, the human TNF- α moiety has a R32W and an E146K mutation. In an embodiment, the human TNF- α moiety has a L29S and a R32W mutation. In an embodiment, the human TNF- α moiety has a D143N and an A145R mutation. In an embodiment, the human TNF- α moiety has a D143N and an A145R mutation. In an embodiment, the human TNF- α moiety has an A145T, an E146D, and a S147D mutation. In an embodiment, the human TNF- α moiety has an A145T and a S147D mutation.

In some embodiments, the modified TNF- α agent has one or more mutations selected from N39Y, S147Y, and Y87H, as described in WO2008/124086, the entire contents of which is hereby incorporated by reference.

In some embodiments, the modified human TNF- α moiety has mutations that provide receptor selectivity as described in PCT/IB2016/001668, the entire contents of which are hereby incorporated by reference. In some embodiments, the mutations to TNF are TNF-R1 selective. In some embodiments, the mutations to TNF which are TNF-R1 selective are at one or more of positions R32, S86, and E146. In some embodiments, the mutations to TNF which are TNF-R1 selective are one or more of R32W, S86T, and E146K. In some embodiments, the mutations to TNF which are TNF-R1 selective are one or more of R32W, R32W/S86T, R32W/E146K and E146K. In some embodiments, the mutations to TNF are TNF-R2 selective. In some embodiments, the mutations to TNF which are TNF-R2 selective are at one or more of positions A145, E146, and S147. In some embodiments, the mutations to TNF which are TNF-R2 selective are one or more of A145T, A145R, E146D, and S147D. In some embodiments, the mutations to TNF which are TNF-R2 selective are one or more of A145R, A145T/S147D, and A145T/E146D/S147D.

In an embodiment, the modified signaling agent is TNF- β . TNF- β can form a homotrimer or a heterotrimer with LT- β (LT- α 1 β 2). In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TNFR1 and/or TNFR2 and/or herpes virus entry mediator (HEVM) and/or LT- β R.

In an embodiment, the wild type TNF- β has the amino acid sequence of SEQ ID NO: 333.

In such embodiments, the modified soluble agent may comprise mutations at one or more amino acids at positions 106-113, which produce a modified TNF- β with reduced receptor binding affinity to TNFR2. In an embodiment, the

modified soluble agent has one or more substitution mutations at amino acid positions 106-113. In illustrative embodiments, the substitution mutations are selected from Q107E, Q107D, S106E, S106D, Q107R, Q107N, Q107E/S106E, Q107E/S106D, Q107D/S106E, and Q107D/S106D. In another embodiment, the modified soluble agent has an insertion of about 1 to about 3 amino acids at positions 106-113.

In some embodiments, the modified agent is a TNF family member (e.g. TNF-alpha, TNF-beta) which can be a single chain trimeric version as described in WO 2015/007903, the entire contents of which are incorporated by reference.

In some embodiments, the modified agent is a TNF family member (e.g. TNF-alpha, TNF-beta) which has reduced affinity and/or activity, *i.e.* antagonistic activity (e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TNFR1. In these embodiments, the modified agent is a TNF family member (e.g. TNF-alpha, TNF-beta) which also, optionally, has substantially reduced or ablated affinity and/or activity for TNFR2. In some embodiments, the modified agent is a TNF family member (e.g. TNF-alpha, TNF-beta) which has reduced affinity and/or activity, *i.e.* antagonistic activity (e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TNFR2. In these embodiments, the modified agent is a TNF family member (e.g. TNF-alpha, TNF-beta) which also, optionally, has substantially reduced or ablated affinity and/or activity for TNFR1. The constructs of such embodiments find use in, for example, methods of dampening TNF response in a cell specific manner. In some embodiments, the antagonistic TNF family member (e.g. TNF-alpha, TNF-beta) is a single chain trimeric version as described in WO 2015/007903.

In an embodiment, the modified signaling agent is TRAIL. In some embodiments, the modified TRAIL agent has reduced affinity and/or activity for DR4 (TRAIL-RI) and/or DR5 (TRAIL-RII) and/or DcR1 and/or DcR2. In some embodiments, the modified TRAIL agent has substantially reduced or ablated affinity and/or activity for DR4 (TRAIL-RI) and/or DR5 (TRAIL-RII) and/or DcR1 and/or DcR2.

In an embodiment, the wild type TRAIL has the amino acid sequence of SEQ ID NO: 334.

In such embodiments, the modified TRAIL agent may comprise a mutation at amino acid positions T127-R132, E144-R149, E155-H161, Y189-Y209, T214-1220, K224-A226, W231, E236-L239, E249-K251, T261-H264 and H270-E271 (Numbering based on the human sequence, Genbank accession number NP_003801, version 10 NP_003801.1, GI: 4507593; see above).

In some embodiments, the modified TRAIL agent may comprise one or more mutations that substantially reduce its affinity and/or activity for TRAIL-R1. In such embodiments, the modified TRAIL agent may specifically bind to TRIL-R2. Exemplary mutations include mutations at one or more amino acid positions Y189, R191, Q193, H264, I266, and D267. For example, the mutations may be one or more of Y189Q, R191K, Q193R, H264R, I266L and D267Q. In an embodiment, the modified TRAIL agent comprises the mutations Y189Q, R191K, Q193R, H264R, I266L and D267Q.

In some embodiments, the modified TRAIL agent may comprise one or more mutations that substantially reduce its affinity and/or activity for TRAIL-R2. In such embodiments, the modified TRAIL agent may specifically bind to TRIL-R1. Exemplary mutations include mutations at one or more amino acid positions G131, R149, S159, N199, K201, and S215. For example, the mutations may be one or more of G131R, R149I, S159R, N199R, K201H, and S215D. In an embodiment, the modified TRAIL agent comprises the mutations G131R, R149I, S159R, N199R, K201H, and S215D. Additional TRAIL mutations are described in, for example, Trebing et al., (2014) Cell Death and Disease, 5:e1035, the entire disclosure of which is hereby incorporated by reference.

In an embodiment, the modified signaling agent is TGF α . In such embodiments, the modified TGF α agent has reduced affinity and/or activity for the epidermal growth factor receptor (EGFR). In some embodiments, the modified TGF α agent has substantially reduced or ablated affinity and/or activity for the epidermal growth factor receptor (EGFR).

In an embodiment, the modified signaling agent is TGF β . In such embodiments, the modified signaling agent has reduced affinity and/or activity for TGFBR1 and/or TGFBR2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TGFBR1 and/or TGFBR2. In some embodiments, the modified signaling agent optionally has reduced or substantially reduced or ablated affinity and/or activity for TGFBR3 which, without wishing to be bound by theory, may act as a reservoir of ligand for TGF-beta receptors. In some embodiments, the TGF β may favor TGFBR1 over TGFBR2 or TGFBR2 over TGFBR1. Similarly, LAP, without wishing to be bound by theory, may act as a reservoir of ligand for TGF-beta receptors. In some embodiments, the modified signaling agent has reduced affinity and/or activity for TGFBR1 and/or TGFBR2 and/or substantially reduced or ablated affinity and/or activity for Latency Associated Peptide (LAP). In some embodiments, such chimeras find use in Camurati-Engelmann disease, or other diseases associated with inappropriate TGF β signaling.

In some embodiments, the modified agent is a TGF family member (e.g. TGF α , TGF β) which has reduced affinity and/or activity, *i.e.* antagonistic activity (e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) at one or more of TGFBR1, TGFBR2, TGFBR3. In these embodiments, the modified agent is a TGF family member (e.g. TGF α , TGF β) which also, optionally, has substantially reduced or ablated affinity and/or activity at one or more of TGFBR1, TGFBR2, TGFBR3.

In some embodiments, the modified agent is a TGF family member (e.g. TGF α , TGF β) which has reduced affinity and/or activity, *i.e.* antagonistic activity (e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TGFBR1 and/or TGFBR2. In these embodiments, the modified agent is a TGF family member (e.g. TGF α , TGF β) which also, optionally, has substantially reduced or ablated affinity and/or activity at TGFBR3.

In an embodiment, the modified signaling agent is an interleukin. In an embodiment, the modified signaling agent is IL-1. In an embodiment, the modified signaling agent is IL-1 α or IL-1 β . In some embodiments, the modified

signaling agent has reduced affinity and/or activity for IL-1R1 and/or IL-1RAcP. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-1R1 and/or IL-1RAcP. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-1R2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-1R2. For instance, in some embodiments, the present modified IL-1 agents avoid interaction at IL-1R2 and therefore substantially reduce its function as a decoy and/or sink for therapeutic agents.

In an embodiment, the wild type IL-1 β has the amino acid sequence of SEQ ID NO: 335.

IL1 is a proinflammatory cytokine and an important immune system regulator. It is a potent activator of CD4 T cell responses, increases proportion of Th17 cells and expansion of IFN γ and IL-4 producing cells. IL-1 is also a potent regulator of CD8 $^{+}$ T cells, enhancing antigen-specific CD8 $^{+}$ T cell expansion, differentiation, migration to periphery and memory. IL-1 receptors comprise IL-1R1 and IL-1R2. Binding to and signaling through the IL-1R1 constitutes the mechanism whereby IL-1 mediates many of its biological (and pathological) activities. IL1-R2 can function as a decoy receptor, thereby reducing IL-1 availability for interaction and signaling through the IL-1R1.

In some embodiments, the modified IL-1 has reduced affinity and/or activity (*e.g.* agonistic activity) for IL-1R1. In some embodiments, the modified IL-1 has substantially reduced or ablated affinity and/or activity for IL-1R2. In such embodiments, there is restorable IL-1/ IL-1R1 signaling and prevention of loss of therapeutic chimeras at IL-R2 and therefore a reduction in dose of IL-1 that is required (*e.g.* relative to wild type or a chimera bearing only an attenuation mutation for IL-R1). Such constructs find use in, for example, methods of treating cancer, including, for example, stimulating the immune system to mount an anti-cancer response.

In some embodiments, the modified IL-1 has reduced affinity and/or activity (*e.g.* antagonistic activity, *e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, *e.g.*, WO 2015/007520, the entire contents of which are hereby incorporated by reference) for IL-1R1. In some embodiments, the modified IL-1 has substantially reduced or ablated affinity and/or activity for IL-1R2. In such embodiments, there is the IL-1/ IL-1R1 signaling is not restorable and prevention of loss of therapeutic chimeras at IL-R2 and therefore a reduction in dose of IL-1 that is required (*e.g.* relative to wild type or a chimera bearing only an attenuation mutation for IL-R1). Such constructs find use in, for example, methods of treating autoimmune diseases, including, for example, suppressing the immune system.

In such embodiments, the modified signaling agent has a deletion of amino acids 52-54 which produces a modified human IL-1 β with reduced binding affinity for type I IL-1R and reduced biological activity. See, for example, WO 1994/000491, the entire contents of which are hereby incorporated by reference. In some embodiments, the modified human IL-1 β has one or more substitution mutations selected from A117G/P118G, R120X, L122A, T125G/L126G, R127G, Q130X, Q131G, K132A, S137G/Q138Y, L145G, H146X, L145A/L147A, Q148X, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209X, K209A/K210A, K219X, E221X, E221 S/N224A, N224S/K225S, E244K, N245Q (where X can be any change in amino acid, *e.g.*, a non-conservative change), which exhibit reduced binding to IL-1R, as

described, for example, in WO2015/007542 and WO/2015/007536, the entire contents of which is hereby incorporated by reference (numbering base on the human IL-1 β sequence, Genbank accession number NP_000567, version NP-000567.1, GI: 10835145). In some embodiments, the modified human IL-1 β may have one or more mutations selected from R120A, R120G, Q130A, Q130W, H146A, H146G, H146E, H146N, H146R, Q148E, Q148G, Q148L, K209A, K209D, K219S, K219Q, E221S and E221K. In an embodiment, the modified human IL-1 β comprises the mutations Q131G and Q148G. In an embodiment, the modified human IL-1 β comprises the mutations Q148G and K208E. In an embodiment, the modified human IL-1 β comprises the mutations R120G and Q131G. In an embodiment, the modified human IL-1 β comprises the mutations R120G and H146A. In an embodiment, the modified human IL-1 β comprises the mutations R120G and H146N. In an embodiment, the modified human IL-1 β comprises the mutations R120G and H146R. In an embodiment, the modified human IL-1 β comprises the mutations R120G and H146E. In an embodiment, the modified human IL-1 β comprises the mutations R120G and H146G. In an embodiment, the modified human IL-1 β comprises the mutations R120G and K208E. In an embodiment, the modified human IL-1 β comprises the mutations R120G, F162A, and Q164E.

In an embodiment, the modified signaling agent is IL-2. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-2R α and/or IL-2R β and/or IL-2R γ . In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-2R β and/or IL-2R γ . In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-2R α . Such embodiments may be relevant for treatment of cancer, for instance when the modified IL-2 is agonistic at IL-2R β and/or IL-2R γ . For instance, the present constructs may favor attenuated activation of CD8 $^{+}$ T cells (which can provide an anti-tumor effect), which have IL2 receptors β and γ and disfavor T_{regs} (which can provide an immune suppressive, pro-tumor effect), which have IL2 receptors α , β , and γ . Further, in some embodiments, the preferences for IL-2R β and/or IL-2R γ over IL-2R α avoid IL-2 side effects such as pulmonary edema. Also, IL-2-based chimeras are useful for the treatment of autoimmune diseases, for instance when the modified IL-2 is antagonistic (e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) at IL-2R β and/or IL-2R γ . For instance, the present constructs may favor attenuated suppression of CD8 $^{+}$ T cells (and therefore dampen the immune response), which have IL2 receptors β and γ and disfavor T_{regs} which have IL2 receptors α , β , and γ . Alternatively, in some embodiments, the chimeras bearing IL-2 favor the activation of T_{regs}, and therefore immune suppression, and activation of disfavor of CD8 $^{+}$ T cells. For instance, these constructs find use in the treatment of diseases or diseases that would benefit from immune suppression, e.g. autoimmune disorders.

In some embodiments, the chimeric protein has targeting moieties as described herein directed to CD8 $^{+}$ T cells as well as a modified IL-2 agent having reduced affinity and/or activity for IL-2R β and/or IL-2R γ and/or substantially reduced or ablated affinity and/or activity for IL-2R α . In some embodiments, these constructs provide targeted CD8 $^{+}$ T cell activity and are generally inactive (or have substantially reduced activity) towards T_{reg} cells. In some embodiments, such constructs have enhanced immune stimulatory effect compared to wild type IL-2 (e.g., without

wishing to be bound by theory, by not stimulating Tregs), whilst eliminating or reducing the systemic toxicity associated with IL-2.

In an embodiment, the wild type IL-2 has the amino acid sequence of SEQ ID NO: 336.

In such embodiments, the modified IL-2 agent has one or more mutations at amino acids L72 (L72G, L72A, L72S, L72T, L72Q, L72E, L72N, L72D, L72R, or L72K), F42 (F42A, F42G, F42S, F42T, F42Q, F42E, F42N, F42D, F42R, or F42K) and Y45 (Y45A, Y45G, Y45S, Y45T, Y45Q, Y45E, Y45N, Y45D, Y45R or Y45K). Without wishing to be bound by theory, it is believed that these modified IL-2 agents have reduced affinity for the high-affinity IL-2 receptor and preserves affinity to the intermediate-affinity IL-2 receptor, as compared to the wild-type IL-2. See, for example, US Patent Publication No. 2012/0244112, the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified IL-2 agent has one or more mutations at amino acids R38, F42, Y45, and E62. For example, the modified IL-2 agent may comprise one or more of R38A, F42A, Y45A, and E62A. In some embodiments, the modified IL-2 agent may comprise a mutation at C125. For example, the mutation may be C125S. In such embodiments, the modified IL-2 agent may have substantially reduced affinity and/or activity for IL-2R α , as described in, for example, Carmenate et al. (2013) *The Journal of Immunology*, 190:6230-6238, the entire disclosure of which is hereby incorporated by reference. In some embodiments, the modified IL-2 agent with mutations at R38, F42, Y45, and/or E62 is able to induce an expansion of effector cells including CD8⁺ T cells and NK cells but not Treg cells. In some embodiments, the modified IL-2 agent with mutations at R38, F42, Y45, and/or E62 is less toxic than wildtype IL-2 agents. A chimeric protein comprising the modified IL-2 agent with substantially reduced affinity and/or activity for IL-2R α may find application in oncology for example.

In other embodiments, the modified IL-2 agent may have substantially reduced affinity and/or activity for IL-2R β , as described in, for example, WO2016/025385, the entire disclosure of which is hereby incorporated by reference. In such embodiments, the modified IL-2 agent may induce an expansion of Treg cells but not effector cells such as CD8⁺ T cells and NK cells. A chimeric protein comprising the modified IL-2 agent with substantially reduced affinity and/or activity for IL-2R β may find application in the treatment of autoimmune disease for example. In some embodiments, the modified IL-2 agent may comprise one or more mutations at amino acids N88, D20, and/or A126. For example, the modified IL-2 agent may comprise one or more of N88R, N88I, N88G, D20H, Q126L, and Q126F.

In various embodiments, the modified IL-2 agent may comprise a mutation at D109 or C125. For example, the mutation may be D109C or C125S. In some embodiments, the modified IL-2 with a mutation at D109 or C125 may be utilized for attachment to a PEG moiety.

In an embodiment, the modified signaling agent is IL-3. In some embodiments, the modified signaling agent has reduced affinity and/or activity for the IL-3 receptor, which is a heterodimer with a unique alpha chain paired with the common beta (beta c or CD131) subunit. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the IL-3 receptor, which is a heterodimer with a unique alpha chain paired with the common beta (beta c or CD131) subunit.

In an embodiment, the modified signaling agent is IL-4. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for type 1 and/or type 2 IL-4 receptors. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for type 1 and/or type 2 IL-4 receptors. Type 1 IL-4 receptors are composed of the IL-4R α subunit with a common γ chain and specifically bind IL-4. Type 2 IL-4 receptors include an IL-4R α subunit bound to a different subunit known as IL-13R α 1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity the type 2 IL-4 receptors.

In an embodiment, the wild type IL-4 has the amino acid sequence of SEQ ID NO: 337.

In such embodiments, the modified IL-4 agent has one or more mutations at amino acids R121 (R121A, R121D, R121E, R121F, R121H, R121I, R121K, R121N, R121P, R121T, R121W), E122 (E122F), Y124 (Y124A, Y124Q, Y124R, Y124S, Y124T) and S125 (S125A). Without wishing to be bound by theory, it is believed that these modified IL-4 agents maintain the activity mediated by the type I receptor, but significantly reduces the biological activity mediated by the other receptors. See, for example, US Patent No. 6,433,157, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-6. IL-6 signals through a cell-surface type I cytokine receptor complex including the ligand-binding IL-6R chain (CD126), and the signal-transducing component gp130. IL-6 may also bind to a soluble form of IL-6R (sIL-6R), which is the extracellular portion of IL-6R. The sIL-6R/IL-6 complex may be involved in neurites outgrowth and survival of neurons and, hence, may be important in nerve regeneration through remyelination. Accordingly, in some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-6R/gp130 and/or sIL-6R. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-6R/gp130 and/or sIL-6R.

In an embodiment, the wild type IL-6 has the amino acid sequence of IL-6 (mature form, wild type) (SEQ ID NO: 338).

In such embodiments, the modified signaling agent has one or more mutations at amino acids 58, 160, 163, 171 or 177. Without wishing to be bound by theory, it is believed that these modified IL-6 agents exhibit reduced binding affinity to IL-6R α and reduced biological activity. See, for example, WO 97/10338, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-10. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-10 receptor-1 and IL-10 receptor-2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-10 receptor-1 and IL-10 receptor-2

In an embodiment, the modified signaling agent is IL-11. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-11R α and/or IL-11R β and/or gp130. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-11R α and/or IL-11R β and/or gp130.

In an embodiment, the modified signaling agent is IL-12. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-12R β 1 and/or IL-12R β 2. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-12R β 1 and/or IL-12R β 2.

In an embodiment, the modified signaling agent is IL-13. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the IL-4 receptor (IL-4R α) and IL-13R α 1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-4 receptor (IL-4R α) or IL-13R α 1.

In an embodiment, the wild type IL-13 has the amino acid sequence of IL-13 (mature form, wild type) (SEQ ID NO: 339).

In such embodiments, the modified IL-13 agent has one or more mutations at amino acids 13, 16, 17, 66, 69, 99, 102, 104, 105, 106, 107, 108, 109, 112, 113 and 114. Without wishing to be bound by theory, it is believed that these modified IL-13 agents exhibit reduced biological activity. See, for example, WO 2002/018422, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-18. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-18R α and/or IL-18R β . In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-18R α and/or IL-18R β . In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-18R α type II, which is an isoform of IL-18R α that lacks the TIR domain required for signaling.

In an embodiment, the wild type IL-18 has the amino acid sequence of IL-18 (wild type) (SEQ ID NO: 340).

In such embodiments, the modified IL-18 agent may comprise one or more mutations in amino acids or amino acid regions selected from Y37-K44, R49-Q54, D59-R63, E67-C74, R80, M87-A97, N 127-K129, Q139-M149, K165-K171, R183 and Q190-N191, as described in WO/2015/007542, the entire contents of which are hereby incorporated by reference (numbering based on the human IL-18 sequence, Genbank accession number AAV38697, version AAV38697.1, GI: 54696650).

In an embodiment, the modified signaling agent is IL-33. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the ST-2 receptor and IL-1RAcP. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the ST-2 receptor and IL-1RAcP.

In an embodiment, the wild type IL-33 has the amino acid sequence of SEQ ID NO: 341.

In such embodiments, the modified IL-33 agent may comprise one or more mutations in amino acids or amino acid regions selected from I113-Y122, S127-E139, E144-D157, Y163-M183, E200, Q215, L220-C227 and T260-E269, as described in WO/2015/007542, the entire contents of which are hereby incorporated by reference (numbering based on the human sequence, Genbank accession number NP_254274, version NP_254274.1, GI:15559209).

In an embodiment, the modified signaling agent is epidermal growth factor (EGF). EGF is a member of a family of potent growth factors. Members include EGF, HB-EGF, and others such as TGF α , amphiregulin, neuregulins, epiregulin, betacellulin. EGF family receptors include EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. These may

function as homodimeric and /or heterodimeric receptor subtypes. The different EGF family members exhibit differential selectivity for the various receptor subtypes. For example, EGF associates with ErbB1/ErbB1, ErbB1/ErbB2, ErbB4/ErbB2 and some other heterodimeric subtypes. HB-EGF has a similar pattern, although it also associates with ErbB4/4. Modulation of EGF (EGF-like) growth factor signaling, positively or negatively, is of considerable therapeutic interest. For example, inhibition of EGFRs signaling is of interest in the treatment of various cancers where EGFR signaling constitutes a major growth promoting signal. Alternatively, stimulation of EGFRs signaling is of therapeutic interest in, for example, promoting wound healing (acute and chronic), oral mucositis (a major side-effect of various cancer therapies, including, without limitation radiation therapy).

In some embodiments, the modified signaling agent has reduced affinity and/or activity for ErbB1, ErbB2, ErbB3, and/or ErbB4. Such embodiments find use, for example, in methods of treating wounds. In some embodiments, the modified signaling agent binds to one or more ErbB1, ErbB2, ErbB3, and ErbB4 and antagonizes the activity of the receptor. In such embodiments, the modified signaling agent has reduced affinity and/or activity for ErbB1, ErbB2, ErbB3, and/or ErbB4 which allows for the activity of the receptor to be antagonized in an attenuated fashion. Such embodiments find use in, for example, treatments of cancer. In an embodiment, the modified signaling agent has reduced affinity and/or activity for ErbB1. ErbB1 is the therapeutic target of kinase inhibitors - most have side effects because they are not very selective (e.g., gefitinib, erlotinib, afatinib, brigatinib and icotinib). In some embodiments, attenuated antagonistic ErbB1 signaling is more on-target and has less side effects than other agents targeting receptors for EGF.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (e.g. antagonistic e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) for ErbB1 and/or substantially reduced or ablated affinity and/or activity for ErbB4 or other subtypes it may interact with. Through specific targeting via the targeting moiety, cell-selective suppression (antagonism e.g. natural antagonistic activity or antagonistic activity that is the result of one or more mutations, see, e.g., WO 2015/007520, the entire contents of which are hereby incorporated by reference) of ErbB1/ErbB1 receptor activation would be achieved – while not engaging other receptor subtypes potentially associated with inhibition-associated side effects. Hence, in contrast to EGFR kinase inhibitors, which inhibit EGFR activity in all cell types in the body, such a construct would provide a cell-selective (e.g., tumor cell with activated EGFR signaling due to amplification of receptor, overexpression etc.) anti-EGFR (ErbB1) drug effect with reduced side effects.

In some embodiments, the modified signaling agent has reduced affinity and/or activity (e.g. agonistic) for ErbB4 and/or other subtypes it may interact with. Through targeting to specific target cells through the targeting moiety, a selective activation of ErbB1 signaling is achieved (e.g. epithelial cells). Such a construct finds use, in some embodiments, in the treatment of wounds (promoting wound healing) with reduced side effects, especially for treatment of chronic conditions and application other than topical application of a therapeutic (e.g. systemic wound healing).

In an embodiment, the modified signaling agent is insulin or insulin analogs. In some embodiments, the modified insulin or insulin analog has reduced affinity and/or activity for the insulin receptor and/or IGF1 or IGF2 receptor. In some embodiments, the modified insulin or insulin analog has substantially reduced or ablated affinity and/or activity for the insulin receptor and/or IGF1 or IGF2 receptor. Attenuated response at the insulin receptor allows for the control of diabetes, obesity, metabolic disorders and the like while directing away from IGF1 or IGF2 receptor avoids pro-cancer effects.

In an embodiment, the modified signaling agent is insulin-like growth factor-I or insulin-like growth factor-II (IGF-1 or IGF-2). In an embodiment, the modified signaling agent is IGF-1. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the insulin receptor and/or IGF1 receptor. In an embodiment, the modified signaling agent may bind to the IGF1 receptor and antagonize the activity of the receptor. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IGF1 receptor which allows for the activity of the receptor to be antagonized in an attenuated fashion. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the insulin receptor and/or IGF1 receptor. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IGF2 receptor which allows for the activity of the receptor to be antagonized in an attenuated fashion. In an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the insulin receptor and accordingly does not interfere with insulin signaling. In various embodiments, this applies to cancer treatment. In various embodiments, the present agents may prevent IR isoform A from causing resistance to cancer treatments.

In one embodiment, the present chimeric protein has (i) a targeting moiety against PD-1 or PD-L1 and (ii) a targeting moiety which is directed against a tumor cell, along with any of the modified or mutant signaling agents described herein.

In one embodiment, the present chimeric protein has (i) a targeting moiety against PD-1 or PD-L1 and (ii) a targeting moiety which is directed against a checkpoint inhibitor marker, along with any of the modified or mutant interferons described herein.

In various embodiments, the signaling agent is a toxin or toxic enzyme. In some embodiments, the toxin or toxic enzyme is derived from plants and bacteria. Illustrative toxins or toxic enzymes include, but are not limited to, the diphtheria toxin, Pseudomonas toxin, anthrax toxin, ribosome-inactivating proteins (RIPs) such as ricin and saporin, modeccin, abrin, gelonin, and poke weed antiviral protein. Additional toxins include those disclosed in Mathew et al., (2009) Cancer Sci 100(8): 1359-65, the entire disclosures are hereby incorporated by reference. In such embodiments, the chimeric proteins of the invention may be utilized to induce cell death in cell-type specific manner. In such embodiments, the toxin may be modified, e.g. mutated, to reduce affinity and/or activity of the toxin for an attenuated effect, as described with other signaling agents herein.

Multi-Specific Chimeras and Fusions with Signaling Agents

In various embodiments, the PD-1 or PD-L1 binding agent of the invention is part of a chimera or fusion with one or more signaling agents as described herein and/or one or more additional targeting moieties. Accordingly, the

present invention provides for chimeric or fusion proteins that include one or more signaling agents and a targeting moiety against PD-1 or PD-L1 and/or one or more additional targeting moieties.

In various embodiments, the PD-1 or PD-L1 binding agent of the invention is multispecific, *i.e.*, the PD-1 or PD-L1 binding agent comprises two or more targeting moieties having recognition domains that recognize and bind two or more targets, *e.g.* antigens, or receptors, or epitopes. In such embodiments, the PD-1 or PD-L1 binding agent of the invention may comprise two or more targeting moieties having recognition domains that recognize and bind two or more epitopes on the same antigen or on different antigens. In various embodiments, such multi-specific PD-1 or PD-L1 binding agents exhibit advantageous properties such as increased avidity and/or improved selectivity. In an embodiment, the PD-1 or PD-L1 binding agent of the invention comprises two targeting moieties and is bispecific, *i.e.*, binds and recognizes two epitopes on the same antigen or on different antigens.

In various embodiments, the multispecific PD-1 or PD-L1 binding agent of the invention comprises two or more targeting moieties with each targeting moiety being an antibody or an antibody derivative as described herein. In an embodiment, the multispecific PD-1 or PD-L1 binding agent of the invention comprises at least one VHH comprising an antigen recognition domain against PD-1 or PD-L1 and one antibody or antibody derivative comprising an antigen recognition domain against a tumor antigen.

In various embodiments, the present multispecific PD-1 or PD-L1 binding agents have two or more targeting moieties that target different antigens or receptors, and one targeting moiety may be attenuated for its antigen or receptor, *e.g.* the targeting moiety binds its antigen or receptor with a low affinity or avidity (including, for example, at an affinity or avidity that is less than the affinity or avidity the other targeting moiety has for its antigen or receptor, for instance the difference between the binding affinities may be about 10-fold, or 25-fold, or 50-fold, or 100-fold, or 300-fold, or 500-fold, or 1000-fold, or 5000-fold; for instance the lower affinity or avidity targeting moiety may bind its antigen or receptor at a K_D in the mid- to high-nM or low- to mid- μ M range while the higher affinity or avidity targeting moiety may bind its antigen or receptor at a K_D in the mid- to high-pM or low- to mid-nM range). For instance, in some embodiments, the present multispecific PD-1 or PD-L1 binding agents comprises an attenuated targeting moiety that is directed against a promiscuous antigen or receptor, which may improve targeting to a cell of interest (*e.g.* via the other targeting moiety) and prevent effects across multiple types of cells, including those not being targeted for therapy (*e.g.* by binding promiscuous antigen or receptor at a higher affinity than what is provided in these embodiments).

The multispecific PD-1 or PD-L1 binding agent of the invention may be constructed using methods known in the art, see for example, U.S. Patent No. 9,067,991, U.S. Patent Publication No. 20110262348 and WO 2004/041862, the entire contents of which are hereby incorporated by reference. In an illustrative embodiment, the multispecific PD-1 or PD-L1 binding agent of the invention comprising two or more targeting moieties may be constructed by chemical crosslinking, for example, by reacting amino acid residues with an organic derivatizing agent as described by Blattler *et al.*, Biochemistry 24,1517-1524 and EP294703, the entire contents of which are hereby incorporated by reference. In another illustrative embodiment, the multispecific PD-1 or PD-L1 binding agent comprising two or

more targeting moieties is constructed by genetic fusion, *i.e.*, constructing a single polypeptide which includes the polypeptides of the individual targeting moieties. For example, a single polypeptide construct may be formed which encodes a first VHH with an antigen recognition domain against PD-1 or PD-L1 and a second antibody or antibody derivative with an antigen recognition domain against a tumor antigen. A method for producing bivalent or multivalent VHH polypeptide constructs is disclosed in PCT patent application WO 96/34103, the entire contents of which is hereby incorporated by reference. In a further illustrative embodiment, the multispecific PD-1 or PD-L1 binding agent of the invention may be constructed by using linkers. For example, the carboxy-terminus of a first VHH with an antigen recognition domain against PD-1 or PD-L1 may be linked to the amino-terminus of a second antibody or antibody derivative with an antigen recognition domain against a tumor antigen (or vice versa). Exemplary linkers that may be used are described herein. In some embodiments, the components of the multispecific PD-1 or PD-L1 binding agent of the invention are directly linked to each other without the use of linkers.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention recognizes and binds to PD-1 or PD-L1 and one or more antigens found on one or more immune cells, which can include, without limitation, megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, monocytes, macrophages, natural killer cells, T lymphocytes (*e.g.*, cytotoxic T lymphocytes, T helper cells, natural killer T cells), B lymphocytes, plasma cells, dendritic cells, or subsets thereof. In some embodiments, the PD-1 or PD-L1 binding agent specifically binds to an antigen of interest and effectively directly or indirectly recruits one or more immune cells.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention recognizes and binds to PD-1 or PD-L1 and one or more antigens found on tumor cells. In these embodiments, the present PD-1 or PD-L1 binding agents may directly or indirectly recruit an immune cell to a tumor cell or the tumor microenvironment. In some embodiments, the present PD-1 or PD-L1 binding agents may directly or indirectly recruit an immune cell, *e.g.* an immune cell that can kill and/or suppress a tumor cell (*e.g.*, a CTL), to a site of action (such as, by way of non-limiting example, the tumor microenvironment).

In some embodiments, the present PD-1 or PD-L1 binding agents are capable of, or find use in methods involving, shifting the balance of immune cells in favor of immune attack of a tumor. For instance, the present PD-1 or PD-L1 binding agents can shift the ratio of immune cells at a site of clinical importance in favor of cells that can kill and/or suppress a tumor (*e.g.* T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, anti-tumor macrophages (*e.g.* M1 macrophages), neutrophils, B cells, dendritic cells or subsets thereof and in opposition to cells that protect tumors (*e.g.* myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs); tumor associated neutrophils (TANs), M2 macrophages, tumor associated macrophages (TAMs), or subsets thereof). In some embodiments, the present PD-1 or PD-L1 binding agent is capable of increasing a ratio of effector T cells to regulatory T cells.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to an antigen associated with tumor cells. In some embodiments, the targeting moiety directly or indirectly recruits tumor cells. For instance, in some embodiments, the recruitment of the tumor cell is to one or more effector cell (e.g. an immune cell as described herein) that can kill and/or suppress the tumor cell. In some embodiments, the targeting moiety directly or indirectly recruits T cells to a tumor cell, for example, by virtue of the two targeting moieties interacting with their respective antigens on a tumor and CD8-positive immune cell (e.g. T cell).

Tumor cells, or cancer cells refer to an uncontrolled growth of cells or tissues and/or an abnormal increased in cell survival and/or inhibition of apoptosis which interferes with the normal functioning of bodily organs and systems. For example, tumor cells include benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Illustrative tumor cells include, but are not limited to cells of: basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (e.g. that associated with brain tumors), and Meigs' syndrome.

Tumor cells, or cancer cells also include, but are not limited to, carcinomas, e.g. various subtypes, including, for example, adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcomas (including, for example, bone and soft tissue), leukemias (including, for example, acute myeloid, acute lymphoblastic, chronic myeloid, chronic lymphocytic, and hairy cell), lymphomas and myelomas (including, for example, Hodgkin and non-Hodgkin lymphomas, light chain, non-secretory, MGUS, and plasmacytomas), and central nervous system cancers (including, for example, brain (e.g. gliomas (e.g. astrocytoma, oligodendroglioma,

and ependymoma), meningioma, pituitary adenoma, and neuromas, and spinal cord tumors (e.g. meningiomas and neurofibroma).

Illustrative tumor antigens include, but are not limited to, MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-0017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α -fetoprotein, E-cadherin, α -catenin, β -catenin and γ -catenin, p120ctn, gp100 Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, Imp-1, NA, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 CT-7, c-erbB-2, CD19, CD20, CD22, CD30, CD33, CD37, CD56, CD70, CD74, CD138, AGS16, MUC1, GPNMB, Ep-CAM, PD-L1, PD-L2, PMSA, and BCMA (TNFRSF17).. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these tumor antigens.

In some embodiments, the present multi-specific PD-1 or PD-L1 binding agent recognizes and binds to PD-1 or PD-L1 as well as an antigen on a tumor cell.

In various embodiments, the present multi-specific PD-1 or PD-L1 binding agent has targeting moieties which target two different cells (e.g. to make a synapse) or the same cell (e.g. to get a more concentrated signaling agent effect).

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with T cells. In some embodiments, the targeting moiety directly or indirectly recruits T cells. In an embodiment, the antigen recognition domains specifically bind to effector T cells. In some embodiments, the antigen recognition domain directly or indirectly recruits effector T cells, e.g., in some embodiments, to a therapeutic site (e.g. a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative effector T cells include cytotoxic T cells (e.g. $\alpha\beta$ TCR, CD3⁺, CD8⁺, CD45RO⁺); CD4⁺ effector T cells (e.g. $\alpha\beta$ TCR, CD3⁺, CD4⁺, CCR7⁺, CD62L^{hi}, IL-7R/CD127⁺); CD8⁺ effector T cells (e.g. $\alpha\beta$ TCR, CD3⁺, CD8⁺, CCR7⁺, CD62L^{hi}, IL-7R/CD127⁺); effector memory T cells (e.g. CD62L^{low}, CD44⁺, TCR, CD3⁺, IL-7R/CD127⁺, IL-15R⁺, CCR7^{low}); central memory T cells (e.g. CCR7⁺, CD62L⁺, CD27⁺; or CCR7^{hi}, CD44⁺, CD62L^{hi}, TCR, CD3⁺, IL-7R/CD127⁺, IL-15R⁺); CD62L⁺ effector T cells; CD8⁺ effector memory T cells (TEM) including early effector memory T cells (CD27⁺ CD62L⁻) and

late effector memory T cells (CD27⁻ CD62L⁻) (TemE and TemL, respectively); CD127(+)CD25(low/-) effector T cells; CD127(-)CD25(-) effector T cells; CD8⁺ stem cell memory effector cells (TSCM) (e.g. CD44(low)CD62L(high)CD122(high)sca(+)); TH1 effector T-cells (e.g. CXCR3⁺, CXCR6⁺ and CCR5⁺; or αβ TCR, CD3⁺, CD4⁺, IL-12R⁺, IFNγR⁺, CXCR3⁺), TH2 effector T cells (e.g. CCR3⁺, CCR4⁺ and CCR8⁺; or αβ TCR, CD3⁺, CD4⁺, IL-4R⁺, IL-33R⁺, CCR4⁺, IL-17RB⁺, CRTH2⁺); TH9 effector T cells (e.g. αβ TCR, CD3⁺, CD4⁺); TH17 effector T cells (e.g. αβ TCR, CD3⁺, CD4⁺, IL-23R⁺, CCR6⁺, IL-1R⁺); CD4⁺CD45RO⁺CCR7⁺ effector T cells, ICOS⁺ effector T cells; CD4⁺CD45RO⁺CCR7(-) effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN-γ.

Illustrative T cell antigens of interest include, for example (and inclusive of the extracellular domains, where applicable): CD8, CD3, SLAMF4, IL-2Rα, 4-1BB/TNFRSF9, IL-2 R β, ALCAM, B7-1, IL-4 R, B7-H3, BLAME/SLAMFS, CEACAM1, IL-6 R, CCR3, IL-7 Rα, CCR4, CXCR1/IL-S RA, CCR5, CCR6, IL-10R α, CCR 7, IL-10 R β, CCRS, IL-12 R β 1, CCR9, IL-12 R β 2, CD2, IL-13 R α 1, IL-13, CD3, CD4, ILT2/CDS5j, ILT3/CDS5k, ILT4/CDS5d, ILT5/CDS5a, Integrin α 4/CD49d, CDS, Integrin α E/CD103, CD6, Integrin α M/CD 11 b, CDS, Integrin α X/CD11c, Integrin β 2/CD18, KIR/CD15S, CD27/TNFRSF7, KIR2DL1, CD2S, KIR2DL3, CD30/TNFRSFS, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, CD40 Ligand/TNFSF5, LAG-3, CD43, LAIR1, CD45, LAIR2, CDS3, Leukotriene B4-R1, CDS4/SLAMF5, NCAM-L1, CD94, NKG2A, CD97, NKG2C, CD229/SLAMF3, NKG2D, CD2F-10/SLAMF9, NT-4, CD69, NTB-A/SLAMF6, Common γ Chain/IL-2 R γ, Osteopontin, CRACC/SLAMF7, PD-1, CRTAM, PSGL-1, CTLA-4, RANK/TNFRSF11A, CX3CR1, CX3CL1, L-Selectin, CXCR3, SIRP β 1, CXCR4, SLAM, CXCR6, TCCR/WSX-1, DNAM-1, Thymopoietin, EMMPRIN/CD147, TIM-1, EphB6, TIM-2, Fas/TNFRSF6, TIM-3, Fas Ligand/TNFSF6, TIM-4, Fcγ RIII/CD16, TIM-6, TNFR1/TNFRSF1A, Granulysin, TNF RIII/TNFRSF1B, TRAIL RI/TNFRSF10A, ICAM-1/CD54, TRAIL R2/TNFRSF10B, ICAM-2/CD102, TRAILR3/TNFRSF10C, IFN-γR1, TRAILR4/TNFRSF10D, IFN-γ R2, TSLP, IL-1 R1 and TSLP R. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative T cell antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety against CD8 which is a VHH comprising a single amino acid chain having four “framework regions” or FRs and three “complementary determining regions” or CDRs. As used herein, “framework region” or “FR” refers to a region in the variable domain which is located between the CDRs. As used herein, “complementary determining region” or “CDR” refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a VHH against CD8 having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences.

In some embodiments, the CDR1 sequence is selected from SEQ ID NO: 342 or SEQ ID NO: 343.

In some embodiments, the CDR2 sequence is selected from SEQ ID NO: 344 or SEQ ID NO: 345.

In some embodiments, the CDR3 sequence is selected from SEQ ID NO: 346 or SEQ ID NO: 347 or SEQ ID NO: 348.

In various embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from the following sequences: R3HCD27 (SEQ ID NO: 349) or R3HCD129 (SEQ ID NO: 350) or R2HCD26 (SEQ ID NO: 351).

In various embodiments, the CD8 targeting moiety comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences as described below.

In some embodiments, the CDR1 sequence is selected from SEQ ID NO: 352 to SEQ ID NO: 420.

In some embodiments, the CDR2 sequence is selected from SEQ ID NO: 421 to SEQ ID NO: 489.

In some embodiments, the CDR3 sequence is selected from SEQ ID NO: 490 to SEQ ID NO: 558.

In various embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from the following sequences: 1CDA 7 (SEQ ID NO: 559) or 1CDA 12 (SEQ ID NO: 560) or 1CDA 14 (SEQ ID NO: 561) or 1CDA 15 (SEQ ID NO: 562) or 1CDA 17 (SEQ ID NO: 563) or 1CDA 18 (SEQ ID NO: 564) or 1CDA 19 (SEQ ID NO: 565) or 1CDA 24 (SEQ ID NO: 566) or 1CDA 26 (SEQ ID NO: 567) or 1CDA 28 (SEQ ID NO: 568) or 1CDA 37 (SEQ ID NO: 569) or 1CDA 43 (SEQ ID NO: 570) or 1CDA 45 (SEQ ID NO: 571) or 1CDA 47 (SEQ ID NO: 572) or 1CDA 48 (SEQ ID NO: 573) or 1CDA 58 (SEQ ID NO: 574) or 1CDA 65 (SEQ ID NO: 575) or 1CDA 68 (SEQ ID NO: 576) or 1CDA 73 (SEQ ID NO: 577) or 1CDA 75 (SEQ ID NO: 578) or 1CDA 86 (SEQ ID NO: 579) or 1CDA 87 (SEQ ID NO: 580) or 1CDA 88 (SEQ ID NO: 581) or 1CDA 89 (SEQ ID NO: 582) or 1CDA 92 (SEQ ID NO: 583) or 1CDA 93 (SEQ ID NO: 584) or 2CDA 1 (SEQ ID NO: 585) or 2CDA 5 (SEQ ID NO: 586) or 2CDA 22 (SEQ ID NO: 587) or 2CDA 28 (SEQ ID NO: 588) or 2CDA 62 (SEQ ID NO: 589) or 2CDA 68 (SEQ ID NO: 590) or 2CDA 73 (SEQ ID NO: 591) or 2CDA 74 (SEQ ID NO: 592) or 2CDA 75 (SEQ ID NO: 593) or 2CDA 77 (SEQ ID NO: 594) or 2CDA 81 (SEQ ID NO: 595) or 2CDA 87 (SEQ ID NO: 596) or 2CDA 88 (SEQ ID NO: 597) or 2CDA 89 (SEQ ID NO: 598) or 2CDA 91 (SEQ ID NO: 599) or 2CDA 92 (SEQ ID NO: 600) or 2CDA 93 (SEQ ID NO: 601) or 2CDA 94 (SEQ ID NO: 602) or 2CDA 95 (SEQ ID NO: 603) or 3CDA 3 (SEQ ID NO: 604) or 3CDA 8 (SEQ ID NO: 605) or 3CDA 11 (SEQ ID NO: 606) or 3CDA 18 (SEQ ID NO: 607) or 3CDA 19 (SEQ ID NO: 608) or 3CDA 21 (SEQ ID NO: 609) or 3CDA 24 (SEQ ID NO: 610) or 3CDA 28 (SEQ ID NO: 611) or 3CDA 29 (SEQ ID NO: 612) or 3CDA 31 (SEQ ID NO: 613) or 3CDA 32 (SEQ ID NO: 614) or 3CDA 33 (SEQ ID NO: 615) or 3CDA 37 (SEQ ID NO: 616) or 3CDA 40 (SEQ ID NO: 617) or 3CDA 41 (SEQ ID NO: 618) or 3CDA 48 (SEQ ID NO: 619) or 3CDA 57 (SEQ ID NO: 620) or 3CDA 65 (SEQ ID NO: 621) or 3CDA 70 (SEQ ID NO: 622) or 3CDA 73 (SEQ ID NO: 623) or 3CDA 83 (SEQ ID NO: 624) or 3CDA 86 (SEQ ID NO: 625) or 3CDA 88 (SEQ ID NO: 626) or 3CDA 90 (SEQ ID NO: 627).

In various exemplary embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from any one of the above sequences without the terminal histidine tag sequence (*i.e.*, HHHHHH; SEQ ID NO: 84).

In some embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from SEQ ID Nos: 559-627 (provided above) without the HA tag (*i.e.*, YPYDVPDYGS; SEQ ID NO: 85).

In some embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from SEQ ID Nos: 559-627 (provided above) without the AAA linker.

In some embodiments, the CD8 targeting moiety comprises an amino acid sequence selected from SEQ ID Nos: 559-627 (provided above) without the AAA linker, HA tag, and terminal histidine tag sequence (*i.e.*, AAAYPYDVDPDYGSHHHHHH; SEQ ID NO: 86). In various embodiments, the CD8 targeting moiety comprises an amino acid sequence described in US Patent Publication No. 2014/0271462, the entire contents of which are incorporated by reference. In various embodiments, the CD8 targeting moiety comprises an amino acid sequence described in Table 0.1, Table 0.2, Table 0.3, and/or Figures 1A-12I of US Patent Publication No. 2014/0271462, the entire contents of which are incorporated by reference. In various embodiments, the CD8 targeting moiety comprises a HCDR1 of a HCDR1 of SEQ ID NO: 22 or 23 and/or a HCDR2 of HCDR1 of SEQ ID NO: 22 or 23 and/or a HCDR3 of HCDR1 of SEQ ID NO: 22 or 23 and/or a LCDR1 of LCDR1 of SEQ ID NO: 24 and/or a LCDR2 of LCDR1 of SEQ ID NO: 24 and/or a LCDR3 of LCDR1 of SEQ ID NO: 24, as provided in SEQ ID NO: 628, SEQ ID NO: 629, or SEQ ID NO: 630.

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as “analogs”) of the targeting moiety directed against CD8 as described herein. In various embodiments, the amino acid sequence of the targeting moiety directed against CD8 further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with B cells. In some embodiments, the targeting moiety directly or indirectly recruits B cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative B cell antigens of interest include, for example, CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD38, CD39, CD40, CD70, CD72, CD73, CD74, CDw75, CDw76, CD77, CD78, CD79a/b, CD80, CD81, CD82, CD83, CD84, CD85, CD86, CD89, CD98, CD126, CD127, CDw130, CD138, CDw150, and B-cell maturation antigen (BCMA). In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative B cell antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically bind to a target (*e.g.* antigen, receptor) associated with Natural Killer cells. In some embodiments, the targeting moiety directly or indirectly recruits Natural Killer cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative Natural Killer cell antigens of interest include, for example TIGIT, 2B4/SLAMF4, KIR2DS4, CD155/PVR, KIR3DL1, CD94, LMIR1/CD300A, CD69, LMIR2/CD300c, CRACC/SLAMF7, LMIR3/CD300LF, Kir1alpha, DNAM-1, LMIR5/CD300LB, Fc-epsilon RII, LMIR6/CD300LE, Fc-gamma RI/CD64, MICA, Fc-gamma RIIB/CD32b, MICB, Fc-gamma RIIC/CD32c, MULT-1, Fc-gamma RIIA/CD32a, Nectin-2/CD112, Fc-gamma RIII/CD16, NKG2A, FcRH1/IRTA5, NKG2C, FcRH2/IRTA4, NKG2D, FcRH4/IRTA1, NKp30, FcRH5/IRTA2, NKp44, Fc-Receptor-like 3/CD16-2, NKp46/NCR1, NKp80/KLRP1, NTB-A/SLAMF6, Rae-1, Rae-1 α , Rae-1 β , Rae-1 delta, H60, Rae-1 epsilon, ILT2/CD85j, Rae-1 γ , ILT3/CD85k, TREM-1, ILT4/CD85d, TREM-2, ILT5/CD85a, TREM-3, KIR/CD158,

TREML1/TLT-1, KIR2DL1, ULBP-1, KIR2DL3, ULBP-2, KIR2DL4/CD158d and ULBP-3. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative NK cell antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with macrophages/monocytes. In some embodiments, the targeting moiety directly or indirectly recruits macrophages/monocytes, e.g., in some embodiments, to a therapeutic site (e.g. a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative macrophages/monocyte antigens of interest include, for example SIRP1a, B7-1/CD80, ILT4/CD85d, B7-H1, ILT5/CD85a, Common β Chain, Integrin α 4/CD49d, BLAME/SLAMF8, Integrin α X/CD11c, CCL6/C10, Integrin β 2/CD18, CD155/PVR, Integrin β 3/CD61, CD31/PECAM-1, Latexin, CD36/SR-B3, Leukotriene B4 R1, CD40/TNFRSF5, LIMP1/CD300A, CD45, LMIR2/CD300c, CD68, LMIR3/CD300LF, CD84/SLAMF5, LMIR5/CD300LB, CD97, LMIR6/CD300LE, CD163, LRP-1, CD2F-10/SLAMF9, MARCO, CRACC/SLAMF7, MD-1, ECF-L, MD-2, EMMPRIN/CD147, MGL2, Endoglin/CD105, Osteoactivin/GPNMB, Fc- γ RI/CD64, Osteopontin, Fc- γ RIIB/CD32b, PD-L2, Fc- γ RIIC/CD32c, Siglec-3/CD33, Fc- γ RIIA/CD32a, SIGNR1/CD209, Fc- γ RIII/CD16, SLAM, GM-CSF R α , TCCR/WSX-1, ICAM-2/CD102, TLR3, IFN- γ RI, TLR4, IFN- γ R2, TREM-1, IL-1 RII, TREM-2, ILT2/CD85j, TREM-3, ILT3/CD85k, TREML1/TLT-1, 2B4/SLAMF 4, IL-10 R α , ALCAM, IL-10 R β , AminopeptidaseN/ANPEP, ILT2/CD85j, Common β Chain, ILT3/CD85k, Clq R1/CD93, ILT4/CD85d, CCR1, ILT5/CD85a, CCR2, CD206, Integrin α 4/CD49d, CCR5, Integrin α M/CD11b, CCR8, Integrin α X/CD11c, CD155/PVR, Integrin β 2/CD18, CD14, Integrin β 3/CD61, CD36/SR-B3, LAIR1, CD43, LAIR2, CD45, Leukotriene B4-R1, CD68, LIMP1/CD300A, CD97, LMIR2/CD300c, CD163, LMIR3/CD300LF, Coagulation Factor III/Tissue Factor, LMIR5/CD300LB, CX3CR1, CX3CL1, LMIR6/CD300LE, CXCR4, LRP-1, CXCR6, M-CSF R, DEP-1/CD148, MD-1, DNAM-1, MD-2, EMMPRIN/CD147, MMR, Endoglin/CD105, NCAM-L1, Fc- γ RI/CD64, PSGL-1, Fc- γ RIIICD16, RP105, G-CSF R, L-Selectin, GM-CSF R α , Siglec-3/CD33, HVEM/TNFRSF14, SLAM, ICAM-1/CD54, TCCR/WSX-1, ICAM-2/CD102, TREM-1, IL-6 R, TREM-2, CXCR1/IL-8 RA, TREM-3 and TREML1/TLT-1. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative macrophage/monocyte antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with dendritic cells. In some embodiments, the targeting moiety directly or indirectly recruits dendritic cells, e.g., in some embodiments, to a therapeutic site (e.g. a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative dendritic cell antigens of interest include, for example, Clec9A, XCR1, RANK, CD36/SRB3, LOX-1/SR-E1, CD68, MARCO, CD163, SR-A1/MSR, CD5L, SREC-1, CL-PI/COLEC12, SREC-II, LIMP1/CD300A, RP105, TLR4, TLR1, TLR5, TLR2, TLR6, TLR3, TLR9, 4-1BB Ligand/TNFSF9, IL-12/IL-23 p40, 4-Amino-1,8-naphthalimide, ILT2/CD85j, CCL21/6CKine, ILT3/CD85k, 8-oxo-dG, ILT4/CD85d, 8D6A, ILT5/CD85a, A2B5, Integrin α 4/CD49d, Aag, Integrin β 2/CD18, AMICA, Langerin, B7-2/CD86, Leukotriene B4 RI, B7-H3,

LMIR1/CD300A, BLAME/SLAMF8, LMIR2/CD300c, Clq R1/CD93, LMIR3/CD300LF, CCR6, LMIR5/CD300LB, CCR7, LMIR6/CD300LE, CD40/TNFRSF5, MAG/Siglec-4-a, CD43, MCAM, CD45, MD-1, CD68, MD-2, CD83, MDL-1/CLEC5A, CD84/SLAMF5, MMR, CD97, NCAMLI, CD2F-10/SLAMF9, Osteoactivin GPNMB, Chern 23, PD-L2, CLEC-1, RP105, CLEC-2, CLEC-8, Siglec-2/CD22, CRACC/SLAMF7, Siglec-3/CD33, DC-SIGN, DEC-205, Siglec-5, DC-SIGNR/CD299, Siglec-6, DCAR, Siglec-7, DCIR/CLEC4A, Siglec-9, DEC-205, Siglec-10, Dectin-1/CLEC7A, Siglec-F, Dectin-2/CLEC6A, SIGNR1/CD209, DEP-1/CD148, SIGNR4, DLEC, SLAM, EMMPRIN/CD147, TCCR/WSX-1, Fc-γ R1/CD64, TLR3, Fc-γ RIIB/CD32b, TREM-1, Fc-γ RIIC/CD32c, TREM-2, Fc-γ RIIA/CD32a, TREM-3, Fc-γ RIIB/CD16, TREML1/TLT-1, ICAM-2/CD102, DEC205, and Vanilloid R1. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative DC antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety against Clec9A which is a VHH comprising a single amino acid chain having four “framework regions” or FRs and three “complementary determining regions” or CDRs. As used herein, “framework region” or “FR” refers to a region in the variable domain which is located between the CDRs. As used herein, “complementary determining region” or “CDR” refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a VHH against Clec9A having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences.

In exemplary embodiments, the CDR1 sequence is selected from SEQ ID NO: 631 to SEQ ID NO: 650.

In exemplary embodiments, the CDR2 sequence is selected from SEQ ID NO: 651 to SEQ ID NO: 672.

In exemplary embodiments, the CDR3 sequence is selected from SEQ ID NO: 673 to SEQ ID NO: 687; or LGR; or VIK.

In various embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from the following sequences: R2CHCL8 (SEQ ID NO: 688); R1CHCL50 (SEQ ID NO: 689); R1CHCL21 (SEQ ID NO: 690); R2CHCL87 (SEQ ID NO: 691); R2CHCL24 (SEQ ID NO: 692); R2CHCL38 (SEQ ID NO: 693); R1CHCL16 (SEQ ID NO: 694); R2CHCL10 (SEQ ID NO: 695); R1CHCL34 (SEQ ID NO: 696); R1CHCL82 (SEQ ID NO: 697); R2CHCL3 (SEQ ID NO: 698); R2CHCL69 (SEQ ID NO: 699); R1CHCL56 (SEQ ID NO: 700); R2CHCL32 (SEQ ID NO: 701); R2CHCL49 (SEQ ID NO: 702); R2CHCL53 (SEQ ID NO: 703); R2CHCL22 (SEQ ID NO: 704); R2CHCL25 (SEQ ID NO: 705); R2CHCL18 (SEQ ID NO: 706); R1CHCL23 (SEQ ID NO: 707); R1CHCL27 (SEQ ID NO: 708); R2CHCL13 (SEQ ID NO: 709); R2CHCL14 (SEQ ID NO: 710); R2CHCL42 (SEQ ID NO: 711); R2CHCL41 (SEQ ID NO: 712); R2CHCL94 (SEQ ID NO: 713); or R2CHCL27 (SEQ ID NO: 714) .

In various embodiments, the Clec9A targeting moiety comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences as described below.

In some embodiments, the CDR1 sequence is selected from: SEQ ID NO: 715 to SEQ ID NO: 780.

In some embodiments, the CDR2 sequence is selected from: SEQ ID NO: 781 to SEQ ID NO: 846.

In some embodiments, the CDR3 sequence is selected from: SEQ ID NO: 847 to SEQ ID NO: 912.

In various exemplary embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from the following sequences: 1LEC 7 (SEQ ID NO: 913).

In various exemplary embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from any one of the sequences above without the terminal histidine tag sequence (*i.e.*, HHHHHH; SEQ ID NO: 84).

In some embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from SEQ ID Nos: 913-978 (provided above) without the HA tag (*i.e.*, YPYDVPDYGS; SEQ ID NO: 85).

In some embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from SEQ ID Nos: 913-978 (provided above) without the AAA linker.

In some embodiments, the Clec9A targeting moiety comprises an amino acid sequence selected from SEQ ID Nos: 913-978 (provided above) without the AAA linker, HA tag, and terminal histidine tag sequence (*i.e.*, AAAYPYDVPDYGSHHHHHH; SEQ ID NO: 86).

In an embodiment, the targeting moiety comprises the anti-Clec9A antibody as disclosed in Tullett et al., JCI Insight. 2016;1(7):e87102, the entire disclosures of which are hereby incorporated by reference.

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analog") of the targeting moiety directed against Clec9A as described herein. In various embodiments, the amino acid sequence of the targeting moiety directed against Clec9A further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids

In various embodiments, the present chimeric protein comprises a targeting moiety comprising an amino acid sequence that is at least 60% identical to any one of the sequences disclosed herein. For example, the chimeric protein may comprise a targeting moiety comprising an amino acid sequence that is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any one of the sequences disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about

87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, about 99% or about 100% sequence identity to any one of the sequences disclosed herein).

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds a target (e.g. antigen, receptor) on immune cells selected from, but not limited to, megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, and eosinophils. In some embodiments, the antigen recognition domains directly or indirectly recruit megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, and eosinophil, e.g., in some embodiments, to a therapeutic site (e.g. a locus with one or more disease cell or cell to be modulated for a therapeutic effect).

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with megakaryocytes and/or thrombocytes. Illustrative megakaryocyte and/or thrombocyte antigens of interest include, for example, GP IIb/IIIa, GPIb, vWF, PF4, and TSP. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative megakaryocyte and/or thrombocyte antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with erythrocytes. Illustrative erythrocyte antigens of interest include, for example, CD34, CD36, CD38, CD41a (platelet glycoprotein IIb/IIIa), CD41b (GPIIb), CD71 (transferrin receptor), CD105, glycophorin A, glycophorin C, c-kit, HLA-DR, H2 (MHC-II), and Rhesus antigens. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these illustrative erythrocyte antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with mast cells. Illustrative mast cells antigens of interest include, for example, SCFR/CD117, Fc ϵ RI, CD2, CD25, CD35, CD88, CD203c, C5R1, CMAI, FCERIA, FCER2, TPSAB1. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these mast cell antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with basophils. Illustrative basophils antigens of interest include, for example, Fc ϵ RI, CD203c, CD123, CD13, CD107a, CD107b, and CD164. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these basophil antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with neutrophils. Illustrative neutrophils antigens of interest include, for example, 7D5, CD10/CALLA, CD13, CD16

(FcRIII), CD18 proteins (LFA-1, CR3, and p150, 95), CD45, CD67, and CD177. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these neutrophil antigens.

In some embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (e.g. antigen, receptor) associated with eosinophils. Illustrative eosinophils antigens of interest include, for example, CD35, CD44 and CD69. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these eosinophil antigens.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to any appropriate antigen or receptor or cell surface markers known by the skilled artisan. In some embodiments, the antigen or cell surface marker is a tissue-specific marker. Illustrative tissue-specific markers include, but are not limited to, endothelial cell surface markers such as ACE, CD14, CD34, CDH5, ENG, ICAM2, MCAM, NOS3, PECAMI, PROCR, SELE, SELP, TEK, THBD, VCAMI, VWF; smooth muscle cell surface markers such as ACTA2, MYH10, MYH11, MYH9, MYOCD; fibroblast (stromal) cell surface markers such as ALCAM, CD34, COL1A1, COL1A2, COL3A1, FAP, PH-4; epithelial cell surface markers such as CDID, K6IRS2, KRT10, KRT13, KRT17, KRT18, KRT19, KRT4, KRT5, KRT8, MUC1, TACSTD1; neovasculature markers such as CD13, TFNA, Alpha-v beta-3 ($\alpha_v\beta_3$), E-selectin; and adipocyte surface markers such as ADIPOQ, FABP4, and RETN. In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that binds one or more of these antigens.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a checkpoint marker expressed on a T cell, e.g. one or more of PD-1, CD28, CTLA4, ICOS, BTLA, KIR, LAG3, CD137, OX40, CD27, CD40L, TIM3, and A2aR.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a checkpoint marker, e.g. one or more of PD-1/PD-L1 or PD-L2, CD28/CD80 or CD86, CTLA4/CD80 or CD86, ICOS/ICOSL or B7RP1, BTLA/HVEM, KIR, LAG3, CD137/CD137L, OX40/OX40L, CD27, CD40L, TIM3/Gal9, and A2aR.

By way of non-limiting example, in various embodiments, the present multispecific PD-1 or PD-L1 binding agent comprises a targeting moiety directed against (i) CD8; (ii) a checkpoint marker expressed on a T cell, e.g. one or more of PD-1, CD28, CTLA4, ICOS, BTLA, KIR, LAG3, CD137, OX40, Cd27, CD40L, TIM3, and A2aR and/or (iii) a targeting moiety is directed against a tumor cell, along with any of the modified (e.g. mutant) signaling agents described herein.

In various embodiments, the present multi-specific PD-1 or PD-L1 binding agent comprises one or more targeting moieties directed against PD-1 in addition to the PD-1 binding VHHs disclosed elsewhere herein. In some embodiments, the PD-1 or PD-L1 binding agent has one or more targeting moieties which selectively bind a PD-1 polypeptide. In some embodiments, the PD-1 or PD-L1 binding agent comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-1 polypeptide. In

some embodiments, the PD-1 binding agent comprises one or more of the PD-1 binding agents disclosed below along with one or more signaling agents disclosed herein.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody pembrolizumab (aka MK-3475, KEYTRUDA), or fragments thereof. Pembrolizumab and other humanized anti-PD-1 antibodies are disclosed in Hamid, et al. (2013) New England Journal of Medicine 369 (2): 134-44, US 8,354,509, and WO 2009/114335, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, pembrolizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 979; and/or a light chain comprising the amino acid sequence of SEQ ID NO: 980.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody, nivolumab (aka BMS-936558, MDX-1106, ONO-4538, OPDIVO), or fragments thereof. Nivolumab (clone 5C4) and other human monoclonal antibodies that specifically bind to PD-1 are disclosed in US 8,008,449 and WO 2006/121168, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, nivolumab or an antigen-binding fragment thereof comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 981; and/or a light chain comprising the amino acid sequence of SEQ ID NO: 982.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody pidilizumab (aka CT-011, hBAT or hBAT-1), or fragments thereof. Pidilizumab and other humanized anti-PD-1 monoclonal antibodies are disclosed in US 2008/0025980 and WO 2009/101611, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the anti-PD-1 antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a light chain variable regions comprising an amino acid sequence selected from SEQ ID NOS: 15-18 of US 2008/0025980: SEQ ID No: 15 of US 2008/0025980 (SEQ ID NO: 983); SEQ ID No: 16 of US 2008/0025980 (SEQ ID NO: 984); SEQ ID No: 17 of US 2008/0025980 (SEQ ID NO: 985); SEQ ID No: 18 of US 2008/0025980 (SEQ ID NO: 986); and/or a heavy chain comprising an amino acid sequence selected from SEQ ID NOS: 20-24 of US 2008/0025980: SEQ ID No: 20 of US 2008/0025980 (SEQ ID NO: 987); SEQ ID No: 21 of US 2008/0025980 (SEQ ID NO: 988); SEQ ID No: 22 of US 2008/0025980 (SEQ ID NO: 989); SEQ ID No: 23 of US 2008/0025980 (SEQ ID NO: 990); or SEQ ID No: 24 of US 2008/0025980 (SEQ ID NO: 991).

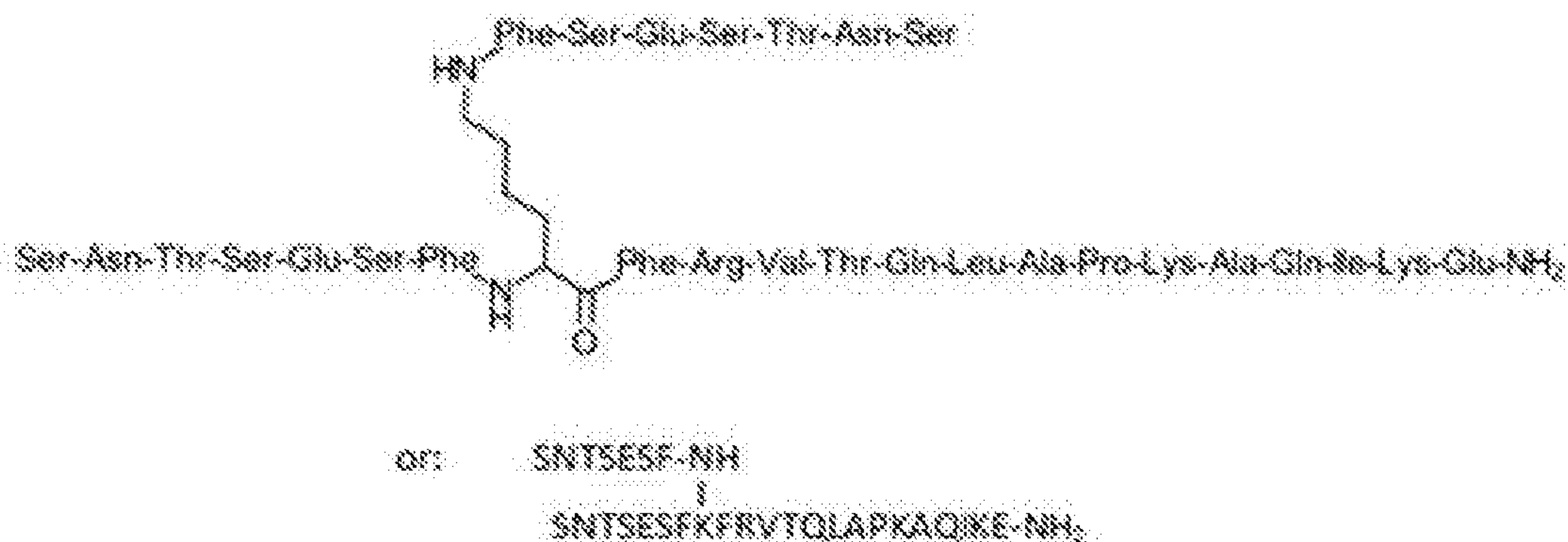
In an embodiment, the targeting moiety comprises a light chain comprising SEQ ID NO:18 of US 2008/0025980 and a heavy chain comprising SEQ ID NO:22 of US 2008/0025980.

In an embodiment, the targeting moiety comprises AMP-514 (aka MEDI-0680).

In an embodiment, the targeting moiety comprises the PD-L2-Fc fusion protein AMP-224, which is disclosed in WO2010/027827 and WO 2011/066342, the entire disclosures of which are hereby incorporated by reference. In such an embodiment, the targeting moiety may include a targeting domain which comprises SEQ ID NO:4 of WO2010/027827 (SEQ ID NO: 992); and/or the B7-DC fusion protein which comprises SEQ ID NO:83 of WO2010/027827 (SEQ ID NO: 993).

In an embodiment, the targeting moiety comprises the peptide AUNP 12 or any of the other peptides disclosed in US 2011/0318373 or 8,907,053. For example, the targeting moiety may comprise AUNP 12 (*i.e.*, Compound 8 or SEQ ID NO:49 of US 2011/0318373) which has the sequence of SEQ ID NO: 994:

SNTSESEFK (SNTSESF) FRVTQLAPKAQIKE-NH₂



In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1E3, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 995; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 996.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1E8, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 997; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 998.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1H3, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1H3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 999; and/or light chain variable region comprising the amino acid sequence of SEQ ID NO: 1000.

In an embodiment, the targeting moiety comprises a VHH directed against PD-1 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-1 comprise SEQ ID NOS: 347-351 of US 8,907,065: SEQ ID No: 347 of US 8,907,065 (SEQ ID NO: 1001); SEQ ID No: 348 of US 8,907,065 (SEQ ID NO: 1002); SEQ ID No: 349

of US 8,907,065 (SEQ ID NO: 1003); SEQ ID No: 350 of US 8,907,065 (SEQ ID NO: 1004); or SEQ ID No: 351 of US 8,907,065 (SEQ ID NO: 1005).

In some embodiments, the PD-1 targeting moiety comprising an amino acid sequence selected from SEQ ID NOs: 1001-1005 having one or more substitutions at positions 11, 37, 44, 45, 47, 83, 84, 103, 104, and 108 (according to Kabat numbering). In some embodiments, the amino acid at position 11 is L, M, S, V, or W. In some embodiments, the amino acid at position 37 is F, Y, H, I, L, or V. In some embodiments, the amino acid at position 44 is G, E, A, D, Q, R, S, or L. In some embodiments, the amino acid at position 45 is L, R, C, I, L, P, Q, or V. In some embodiments, the amino acid at position 47 is W, L, F, A, G, I, M, R, S, V or Y. In some embodiments, the amino acid at position 83 is R, K, N, E, G, I, M, Q or T. In some embodiments, the amino acid at position 84 is P, A, L, R, S, T, D, or V. In some embodiments, the amino acid at position 103 is W, P, R, or S; 104- G or D. In some embodiments, the amino acid at position 108 is Q, L, or R.

In an embodiment, the targeting moiety comprises any one of the anti-PD-1 antibodies, or fragments thereof, as disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID NOS: 25-29 of US2011/0271358: SEQ ID No: 25 of US2011/0271358 (SEQ ID NO: 1006); SEQ ID No: 26 of US2011/0271358 (SEQ ID NO: 1007); SEQ ID No: 27 of US2011/0271358 (SEQ ID NO: 1008); SEQ ID No: 28 of US2011/0271358 (SEQ ID NO: 1009); SEQ ID No: 29 of US2011/0271358 (SEQ ID NO: 1010); and/or a light chain comprising an amino acid sequence selected from SEQ ID NOS: 30-33 of US2011/0271358: SEQ ID No: 30 of US2011/0271358 (SEQ ID NO: 1011); SEQ ID No: 31 of US2011/0271358 (SEQ ID NO: 1012); SEQ ID No: 32 of US2011/0271358 (SEQ ID NO: 1013); or SEQ ID No: 33 of US2011/0271358 (SEQ ID NO: 1014).

In various embodiments, the present multi-specific PD-1 or PD-L1 binding agent comprises one or more antibodies directed against PD-1, or antibody fragments thereof, selected from TSR-042 (Tesaro, Inc.), REGN2810 (Regeneron Pharmaceuticals, Inc.), PDR001 (Novartis Pharmaceuticals), and BGB-A317 (BeiGene Ltd.)

In various embodiments, the present multi-specific PD-1 or PD-L1 binding agent has one or more targeting moieties directed against PD-L1. In some embodiments, the PD-1 or PD-L1 binding agent has one or more targeting moieties which selectively bind a PD-L1 polypeptide. In some embodiments, the PD-1 or PD-L1 binding agent comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-L1 polypeptide. In some embodiments, the PD-L1 binding agent comprises one or more of the PD-1 binding agents disclosed below along with one or more signaling agents disclosed herein.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody MEDI4736 (aka durvalumab), or fragments thereof. MEDI4736 is selective for PD-L1 and blocks the binding of PD-L1 to the PD-1 and CD80 receptors. MEDI4736 and antigen-binding fragments thereof for use in the methods provided herein comprises a heavy chain and a light chain or a heavy chain variable region and a light chain variable region. The sequence of

MEDI4736 is disclosed in WO/2016/06272, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, MEDI4736 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 1015; and/or a light chain comprising the amino acid sequence of SEQ ID NO: 1016.

In illustrative embodiments, the MEDI4736 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:4 of WO/2016/06272 (SEQ ID NO: 1017); and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO:3 of WO/2016/06272 (SEQ ID NO: 1018).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody atezolizumab (aka MPDL3280A, RG7446), or fragments thereof. In illustrative embodiments, atezolizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 1019; and/or a light chain comprising the amino acid sequence of SEQ ID NO: 1020.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody avelumab (aka MSB0010718C), or fragments thereof. In illustrative embodiments, avelumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 1021; and/or a light chain comprising the amino acid sequence of SEQ ID NO: 1022.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody BMS-936559 (aka 12A4, MDX-1105), or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, BMS-936559 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1023; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1024.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3G10, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3G10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1025; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1026.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 10A5, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 10A5 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1027; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1028.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 5F8, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 5F8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1029; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1030.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 10H10, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 10H10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1031; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1032.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1B12, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1B12 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1033; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1034.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 7H1, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 7H1 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1035; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1036.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 11E6, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 11E6 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1037; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1038.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 12B7, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 12B7 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1039; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1040.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 13G4, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by

reference. In illustrative embodiments, 13G4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1041; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1042.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1E12, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E12 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1043; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1044.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1F4, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1F4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1045; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1046.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2G11, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2G11 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1047; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1048.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3B6, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3B6 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1049; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1050.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3D10, or fragments thereof, as disclosed in US 2014/0044738 and WO2012/145493, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3D10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1051; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1052.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 34-38 of

US2011/0271358: SEQ ID No: 34 of US2011/0271358 (SEQ ID NO: 1053); SEQ ID No: 35 of US2011/0271358 (SEQ ID NO: 1054); SEQ ID No: 36 of US2011/0271358 (SEQ ID NO: 1055); SEQ ID No: 37 of US2011/0271358 (SEQ ID NO: 1056); SEQ ID No: 38 of US2011/0271358 (SEQ ID NO: 1057); and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 39-42 of US2011/0271358: SEQ ID No: 39 of US2011/0271358 (SEQ ID NO: 1058); SEQ ID No: 40 of US2011/0271358 (SEQ ID NO: 1059); SEQ ID No: 41 of US2011/0271358 (SEQ ID NO: 1060); or SEQ ID No: 42 of US2011/0271358 (SEQ ID NO: 1061).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.7A4, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.7A4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of: SEQ ID No: 2 of WO 2011/066389 (SEQ ID NO: 1062); and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1063.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.9D10, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.9D10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of: SEQ ID No: 12 of WO 2011/066389 (SEQ ID NO: 1064); and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1065.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.14H9, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.14H9 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of: SEQ ID No: 22 of WO 2011/066389 (SEQ ID NO: 1066); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 27 of WO 2011/066389 (SEQ ID NO: 1067).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.20A8, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.20A8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of: SEQ ID No: 32 of WO 2011/066389 (SEQ ID NO: 1068); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 37 of WO 2011/066389 (SEQ ID NO: 1069).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3.15G8, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3.15G8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 42 of WO 2011/066389 (SEQ ID NO: 1070); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 47 of WO 2011/066389 (SEQ ID NO: 1071).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3.18G1, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3.18G1 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID No: 52 of WO 2011/066389 (SEQ ID NO: 1072); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 57 of WO 2011/066389 (SEQ ID NO: 1073).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.7A4OPT, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.7A4OPT or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID No: 62 of WO 2011/066389 (SEQ ID NO: 1074); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 67 of WO 2011/066389 (SEQ ID NO: 1075).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.14H9OPT, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.14H9OPT or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of: SEQ ID No: 72 of WO 2011/066389 (SEQ ID NO: 1076); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 77 of WO 2011/066389 (SEQ ID NO: 1077).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2016/061142, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 18, 30, 38, 46, 50, 54, 62, 70, and 78 of WO2016/061142: SEQ ID No: 18 of WO2016/061142 (SEQ ID NO: 1078); SEQ ID No: 30 of WO2016/061142 (SEQ ID NO: 1079); SEQ ID No: 38 of WO2016/061142 (SEQ ID NO: 1080); SEQ ID No: 46 of WO2016/061142 (SEQ ID NO: 1081); SEQ ID No: 50 of WO2016/061142 (SEQ ID NO: 1082); SEQ ID No: 54 of WO2016/061142 (SEQ ID NO: 1083); SEQ ID No: 62 of WO2016/061142 (SEQ ID NO: 1084); SEQ ID No: 70 of WO2016/061142 (SEQ ID NO: 1085); SEQ ID No: 78 of WO2016/061142 (SEQ ID NO: 1086); and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 22, 26, 34, 42, 58, 66, 74, 82, and 86 of WO2016/061142: SEQ ID No: 22 of WO2016/061142 (SEQ ID NO: 1087); SEQ ID No: 26 of WO2016/061142 (SEQ ID NO: 1088); SEQ ID No: 34 of WO2016/061142 (SEQ ID NO: 1089); SEQ ID No: 42 of WO2016/061142 (SEQ ID NO: 1090); SEQ ID No: 58 of WO2016/061142 (SEQ ID NO: 1091); SEQ ID No: 66 of WO2016/061142

(SEQ ID NO: 1092); SEQ ID No: 74 of WO2016/061142 (SEQ ID NO: 1093); SEQ ID No: 82 of WO2016/061142 (SEQ ID NO: 1094); or SEQ ID No: 86 of WO2016/061142 (SEQ ID NO: 1095).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2016/022630, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 2, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, and 46 of WO2016/022630: SEQ ID No: 2 of WO2016/022630 (SEQ ID NO: 1096); SEQ ID No: 6 of WO2016/022630 (SEQ ID NO: 1097); SEQ ID No: 10 of WO2016/022630 (SEQ ID NO: 1098); SEQ ID No: 14 of WO2016/022630 (SEQ ID NO: 1099); SEQ ID No: 18 of WO2016/022630 (SEQ ID NO: 1100); SEQ ID No: 22 of WO2016/022630 (SEQ ID NO: 1101); SEQ ID No: 26 of WO2016/022630 (SEQ ID NO: 1102); SEQ ID No: 30 of WO2016/022630 (SEQ ID NO: 1103); SEQ ID No: 34 of WO2016/022630 (SEQ ID NO: 1104); SEQ ID No: 38 of WO2016/022630 (SEQ ID NO: 1105); SEQ ID No: 42 of WO2016/022630 (SEQ ID NO: 1106); SEQ ID No: 46 of WO2016/022630 (SEQ ID NO: 1107); and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 of WO2016/022630: SEQ ID No: 4 of WO2016/022630 (SEQ ID NO: 1108); SEQ ID No: 8 of WO2016/022630 (SEQ ID NO: 1109); SEQ ID No: 12 of WO2016/022630 (SEQ ID NO: 1110); SEQ ID No: 16 of WO2016/022630 (SEQ ID NO: 1111); SEQ ID No: 20 of WO2016/022630 (SEQ ID NO: 1112); SEQ ID No: 24 of WO2016/022630 (SEQ ID NO: 1113); SEQ ID No: 28 of WO2016/022630 (SEQ ID NO: 1114); SEQ ID No: 32 of WO2016/022630 (SEQ ID NO: 1115); SEQ ID No: 36 of WO2016/022630 (SEQ ID NO: 1116); SEQ ID No: 40 of WO2016/022630 (SEQ ID NO: 1117); SEQ ID No: 44 of WO2016/022630 (SEQ ID NO: 1118); or SEQ ID No: 48 of WO2016/022630 (SEQ ID NO: 1119).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2015/112900, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 38, 50, 82, and 86 of WO 2015/112900: SEQ ID No: 38 of WO2015/112900 (SEQ ID NO: 1120); SEQ ID No: 50 of WO 2015/112900 (SEQ ID NO: 1121); SEQ ID No: 82 of WO 2015/112900 (SEQ ID NO: 1122); SEQ ID No: 86 of WO 2015/112900 (SEQ ID NO: 1123); and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 42, 46, 54, 58, 62, 66, 70, 74, and 78 of WO 2015/112900: SEQ ID No: 42 of WO2015/112900 (SEQ ID NO: 1124); SEQ ID No: 46 of WO 2015/112900 (SEQ ID NO: 1125); SEQ ID No: 54 of WO 2015/112900 (SEQ ID NO: 1126); SEQ ID No: 58 of WO 2015/112900 (SEQ ID NO: 1127); SEQ ID No: 62 of WO 2015/112900 (SEQ ID NO: 1128); SEQ ID No: 66 of WO 2015/112900 (SEQ ID NO: 1129); SEQ ID No: 70 of WO 2015/112900 (SEQ ID NO: 1130); SEQ ID No: 74 of WO 2015/112900 (SEQ ID NO: 1131); or SEQ ID No: 78 of WO 2015/112900 (SEQ ID NO: 1132).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO 2010/077634 and US 8,217,149, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the anti-PD-L1 antibody or an antigen-binding fragment thereof for use in the methods provided

herein comprises a heavy chain region comprising the amino acid sequence of: SEQ ID No: 20 of WO 2010/077634 (SEQ ID NO: 1133); and/or a light chain variable region comprising the amino acid sequence of SEQ ID No: 21 of WO 2010/077634 (SEQ ID NO: 1134).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies obtainable from the hybridoma accessible under CNCM deposit numbers CNCM I-4122, CNCM I-4080 and CNCM I-4081 as disclosed in US 20120039906, the entire disclosures of which are hereby incorporated by reference.

In an embodiment, the targeting moiety comprises a VHH directed against PD-L1 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-L1 comprise SEQ ID NOS: 394-399 of US 8,907,065: SEQ ID No: 394 of US 8,907,065 (SEQ ID NO: 1135); SEQ ID No: 395 of US 8,907,065 (SEQ ID NO: 1136); SEQ ID No: 396 of US 8,907,065 (SEQ ID NO: 1137); SEQ ID No: 397 of US 8,907,065 (SEQ ID NO: 1138); SEQ ID No: 398 of US 8,907,065 (SEQ ID NO: 1139); or SEQ ID No: 399 of US 8,907,065 (SEQ ID NO: 1140).

In some embodiments, the PD-L1 targeting moiety comprising an amino acid sequence selected from SEQ ID NOs: 1135-1140 having one or more substitutions at positions 11, 37, 44, 45, 47, 83, 84, 103, 104, and 108 (according to Kabat numbering). In some embodiments, the amino acid at position 11 is L, M, S, V, or W. In some embodiments, the amino acid at position 37 is F, Y, H, I, L, or V. In some embodiments, the amino acid at position 44 is G, E, A, D, Q, R, S, or L. In some embodiments, the amino acid at position 45 is L, R, C, I, L, P, Q, or V. In some embodiments, the amino acid at position 47 is W, L, F, A, G, I, M, R, S, V or Y. In some embodiments, the amino acid at position 83 is R, K, N, E, G, I, M, Q or T. In some embodiments, the amino acid at position 84 is P, A, L, R, S, T, D, or V. In some embodiments, the amino acid at position 103 is W, P, R, or S; 104- G or D. In some embodiments, the amino acid at position 108 is Q, L, or R.

In various embodiments, the present multi-specific PD-1 or PD-L1 binding agent has one or more targeting moieties directed against PD-L2. In some embodiments, the PD-1 or PD-L1 binding agent has one or more targeting moieties which selectively bind a PD-L2 polypeptide. In some embodiments, the PD-1 or PD-L1 binding agent comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-L2 polypeptide.

In an embodiment, the targeting moiety comprises a VHH directed against PD-L2 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-1 comprise SEQ ID Nos: 449-455 of US 8,907,065: SEQ ID No: 449 of US 8,907,065 (SEQ ID NO: 1141); SEQ ID No: 450 of US 8,907,065 (SEQ ID NO: 1142); SEQ ID No: 451 of US 8,907,065 (SEQ ID NO: 1143); SEQ ID No: 452 of US 8,907,065 (SEQ ID NO: 1144); SEQ ID No: 453 of US 8,907,065 (SEQ ID NO: 1145); SEQ ID No: 454 of US 8,907,065 (SEQ ID NO: 1146); or SEQ ID No: 455 of US 8,907,065 (SEQ ID NO: 1147).

In some embodiments, the PD-L2 targeting moiety comprising an amino acid sequence selected from SEQ ID NOs: 1141-1147 having one or more substitutions at positions 11, 37, 44, 45, 47, 83, 84, 103, 104, and 108 (according to Kabat numbering). In some embodiments, the amino acid at position 11 is L, M, S, V, or W. In some embodiments, the amino acid at position 37 is F, Y, H, I, L, or V. In some embodiments, the amino acid at position 44 is G, E, A, D, Q, R, S, or L. In some embodiments, the amino acid at position 45 is L, R, C, I, L, P, Q, or V. In some embodiments, the amino acid at position 47 is W, L, F, A, G, I, M, R, S, V or Y. In some embodiments, the amino acid at position 83 is R, K, N, E, G, I, M, Q or T. In some embodiments, the amino acid at position 84 is P, A, L, R, S, T, D, or V. In some embodiments, the amino acid at position 103 is W, P, R, or S; 104- G or D. In some embodiments, the amino acid at position 108 is Q, L, or R.

In various embodiments, the PD-L2 targeting moiety comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences. In various embodiments, the PD-L2 binding agent comprises a VHH having a variable region comprising at least one FR1, FR2, FR3, and FR4 sequences.

In some embodiments, the PD-L2 CDR1 sequence is selected from SEQ ID NO: 1148 to SEQ ID NO: 1154.

In some embodiments, the PD-L2 CDR2 sequence is selected from SEQ ID NO: 1155 to SEQ ID NO: 1161.

In some embodiments, the PD-L2 CDR3 sequence is selected from SEQ ID NO: 1162 to SEQ ID NO: 1168.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L2 antibodies disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 43-47 of US2011/0271358: SEQ ID No: 43 of US2011/0271358 (SEQ ID NO: 1169); SEQ ID No: 44 of US2011/0271358 (SEQ ID NO: 1170); SEQ ID No: 45 of US2011/0271358 (SEQ ID NO: 1171); SEQ ID No: 46 of US2011/0271358 (SEQ ID NO: 1172); SEQ ID No: 47 of US2011/0271358 (SEQ ID NO: 1173); and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 48-51 of US2011/0271358: SEQ ID No: 48 of US2011/0271358 (SEQ ID NO: 1174); SEQ ID No: 49 of US2011/0271358 (SEQ ID NO: 1175); SEQ ID No: 50 of US2011/0271358 (SEQ ID NO: 1176); or SEQ ID No: 51 of US2011/0271358 (SEQ ID NO: 1177).

In various embodiments, the targeting moieties of the invention may comprise a sequence that targets PD-1, PD-L1, and/or PD-L2 which is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any of the sequences disclosed herein (e.g. about 60%, or

about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, about 99% or about 100% sequence identity with any of the sequences disclosed herein).

In various embodiments, the targeting moieties of the invention may comprise any combination of heavy chain, light chain, heavy chain variable region, light chain variable region, complementarity determining region (CDR), and framework region sequences that target PD-1, PD-L1, and/or PD-L2 as disclosed herein.

Additional antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind or target PD-1, PD-L1 and/or PD-L2 are disclosed in WO 2011/066389, US 2008/0025980, US 2013/0034559, US 8,779,108, US 2014/0356353, US 8,609,089, US 2010/028330, US 2012/0114649, WO 2010/027827, WO 2011/066342, US 8,907,065, WO 2016/062722, WO 2009/101611, WO2010/027827, WO 2011/066342, WO 2007/005874, WO 2001/014556, US2011/0271358, WO 2010/036959, WO 2010/077634, US 8,217,149, US 2012/0039906, WO 2012/145493, US 2011/0318373, U.S. Patent No. 8,779,108, US 20140044738, WO 2009/089149, WO 2007/00587, WO 2016061142, WO 2016,02263, WO 2010/077634, and WO 2015/112900, the entire disclosures of which are hereby incorporated by reference.

In various embodiments, the multispecific PD-1 or PD-L1 binding agent of the present technology comprises a targeting moiety against signal regulatory protein α -1 (SIRP1 α). SIRP1 α (also known as SIRP α) belongs to a family of cell immune receptors encompassing inhibitory (SIRP α), activating (SIRP β), nonsignaling (SIRP γ) and soluble (SIRP δ) members. SIRP1 α is expressed primarily on myeloid cells, including macrophages, granulocytes, myeloid dendritic cells (DCs), mast cells, and their precursors, including hematopoietic stem cells. SIRP1 α acts as an inhibitory receptor that interacts with a broadly expressed transmembrane glycoprotein CD47 to regulate phagocytosis. In particular, the binding of SIRP1 α on macrophages by CD47 expressed on target cells, generates an inhibitory signal that negatively regulates phagocytosis of the target cell.

In various embodiments, the SIRP1 α targeting moiety is a targeting moiety that specifically recognizes and binds SIRP1 α on macrophages.

In various embodiments, the SIRP1 α targeting moiety is a targeting moiety that specifically recognizes and binds SIRP1 α on monocytes.

In various embodiments, the SIRP1 α targeting moiety is a targeting moiety that specifically recognizes and binds SIRP1 α on TAMs (Tumor Associated Macrophages).

In various embodiments, the SIRP1 α targeting moiety is a targeting moiety that specifically recognizes and binds SIRP1 α on dendritic cells, including without limitation cDC2 and pDC.

In various embodiments, the SIRP1 α targeting moiety comprises a targeting moiety having a recognition domain that recognizes SIRP1 α . In an embodiment, the recognition domain recognizes one or more linear epitopes present on SIRP1 α . As used herein, a linear epitope refers to any continuous sequence of amino acids present on SIRP1 α . In another embodiment, the recognition domain recognizes one or more conformational epitopes present on SIRP1 α . As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In some embodiments, the SIRP1 α targeting moiety may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of SIRP1 α . In an embodiment, the SIRP1 α is human SIRP1 α . In various embodiments, the SIRP1 α targeting moiety may bind to any forms of the human SIRP1 α , including monomeric, dimeric, heterodimeric, multimeric and associated forms. In an embodiment, the SIRP1 α targeting moiety binds to the monomeric form of SIRP1 α . In another embodiment, the SIRP1 α targeting moiety binds to a dimeric form of SIRP1 α .

In an embodiment, the SIRP1 α targeting moiety comprises a recognition domain that recognizes one or more epitopes present on human SIRP1 α . In an embodiment, the SIRP1 α targeting moiety comprises a recognition domain that recognizes human SIRP1 α with a signal peptide sequence. An exemplary human SIRP1 α polypeptide with a signal peptide sequence is SEQ ID NO: 1178.

In an embodiment, the SIRP1 α targeting moiety comprises a recognition domain that recognizes human SIRP1 α without a signal peptide sequence. An exemplary human SIRP1 α polypeptide without a signal peptide sequence is SEQ ID NO: 1179.

In an embodiment, the SIRP1 α targeting moiety comprises a recognition domain that recognizes a polypeptide encoding human SIRP1 α isoform 2 is SEQ ID NO: 1180.

In an embodiment, the SIRP1 α targeting moiety comprises a recognition domain that recognizes a polypeptide encoding human SIRP1 α isoform 4 is SEQ ID NO: 1181.

In various embodiments, the SIRP1 α targeting moieties may be any protein-based agent capable of specific binding, such as an antibody or derivatives thereof. In an embodiment, the SIRP1 α targeting moiety comprises an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains. Each heavy chain includes one variable region (e.g., V_H) and at least three constant regions (e.g., CH₁, CH₂ and CH₃), and each light chain includes one variable region (V_L) and one constant region (C_L). The variable regions determine the specificity of the antibody. Each variable region comprises three hypervariable regions also known as complementarity determining regions (CDRs) flanked by four relatively conserved framework regions (FRs). The three CDRs, referred to as CDR1, CDR2, and CDR3, contribute to the antibody binding specificity. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the SIRP1 α targeting moiety comprises antibody derivatives or formats. In some embodiments, the SIRP1 α targeting moiety is a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; a Microbody; a peptide aptamer; an alterase; a plastic antibodies; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; Affimers, a DuoBody, a Fv, a Fab, a Fab', a F(ab')₂, a peptide mimetic molecule, or a synthetic molecule, as described in US Patent Nos. or Patent Publication Nos. US 7,417,130, US 2004/132094, US 5,831,012, US 2004/023334, US 7,250,297, US 6,818,418, US 2004/209243, US 7,838,629, US 7,186,524, US 6,004,746, US 5,475,096, US 2004/146938, US 2004/157209, US 6,994,982, US 6,794,144, US 2010/239633, US 7,803,907, US 2010/119446, and/or US 7,166,697, the contents of which are hereby incorporated by reference in their entireties. See also, Storz MABs. 2011 May-Jun; 3(3): 310–317.

In one embodiment, the SIRP1 α targeting comprises a single-domain antibody, such as VHH from, for example, an organism that produces VHH antibody such as a camelid, a shark, or a designed VHH. VHHs are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH2 and CH3).

In an embodiment, the SIRP1 α targeting moiety comprises a VHH. In some embodiments, the VHH is a humanized VHH or camelized VHH.

In some embodiments, the VHH comprises a fully human V_H domain, e.g. a HUMABODY (Crescendo Biologics, Cambridge, UK). In some embodiments, fully human V_H domain, e.g. a HUMABODY is monovalent, bivalent, or trivalent. In some embodiments, the fully human V_H domain, e.g. a HUMABODY is mono- or multi-specific such as monospecific, bispecific, or trispecific. Illustrative fully human V_H domains, e.g. a HUMABODIES are described in, for example, WO 2016/113555 and WO2016/113557, the entire disclosure of which is incorporated by reference.

For example, in some embodiments, the SIRP1 α targeting moiety comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, VHHs, or fusion proteins that selectively bind SIRP1 α . In some embodiments, the SIRP1 α targeting moiety comprises an antibody or derivative thereof that specifically binds to SIRP1 α . In some embodiments, the SIRP1 α targeting moiety is a camelid heavy chain antibody (VHH) that specifically binds to SIRP1 α .

In various embodiments, the SIRP1 α targeting moieties may comprise any combination of heavy chain, light chain, heavy chain variable region, light chain variable region, complementarity determining region (CDR), and framework region sequences that is known to recognize and bind to SIRP1 α .

In various embodiments, the present technology contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as “analog”) of the SIRP1 α targeting

moiety described herein. In various embodiments, the amino acid sequence of the SIRP1 α targeting moiety further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In various embodiments, the SIRP1 α targeting moieties comprise an amino acid sequence having one or more amino acid mutations with respect to any targeting moiety sequence that is known to recognize and bind to SIRP1 α . In various embodiments, the SIRP1 α targeting moiety comprises an amino acid sequence having one, or two, or three, or four, or five, or six, or seven, or eight, or nine, or ten, or fifteen, twenty, thirty, forty, or fifty amino acid mutations with respect to any targeting moiety sequence, which is known to recognize and bind to SIRP1 α . In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

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

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

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

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

In various embodiments, the substitutions may also include non-classical amino acids. Exemplary non-classical amino acids include, but are not limited to, selenocysteine, pyrrolysine, *N*-formylmethionine β -alanine, GABA and δ -Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α -methyl amino acids, N α -methyl amino acids, and amino acid analogs in general.

In various embodiments, the amino acid mutation may be in the CDRs of the targeting moiety (e.g., the CDR1, CDR2 or CDR3 regions). In another embodiment, amino acid alteration may be in the framework regions (FRs) of the targeting moiety (e.g., the FR1, FR2, FR3, or FR4 regions).

Modification of the amino acid sequences may be achieved using any known technique in the art *e.g.*, site-directed mutagenesis or PCR based mutagenesis. Such techniques are described, for example, in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., 1989 and Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1989.

In various embodiments, the mutations do not substantially reduce the SIRP1 α targeting moiety's capability to specifically recognize and bind to SIRP1 α . In various embodiments, the mutations do not substantially reduce the SIRP1 α targeting moiety's capability to specifically bind to SIRP1 α and without functionally modulating (*e.g.*, partially or fully neutralizing) SIRP1 α .

In various embodiments, the SIRP1 α targeting moiety binds but does not functionally modulate the antigen of interest, *i.e.*, SIRP1 α . For instance, in various embodiments, the SIRP1 α targeting moiety simply targets the antigen but does not substantially functionally modulate (*e.g.* substantially inhibit, reduce or neutralize) a biological effect that the antigen has. In various embodiments, the SIRP1 α targeting moiety binds an epitope that is physically separate from an antigen site that is important for its biological activity (*e.g.* an antigen's active site).

In other embodiments, the SIRP1 α targeting moiety binds and functionally modulates the antigen of interest, *i.e.*, SIRP1 α . For instance, in various embodiments, the SIRP1 α targeting moiety targets the antigen, *i.e.*, SIRP1 α , and functionally modulates (*e.g.* inhibit, reduce or neutralize) a biological effect that the antigen has. Such binding along with functional modulation may find use in various embodiments of the present invention including methods in which the present chimeric protein is used to directly or indirectly recruit active immune cells to a site of need via an effector antigen.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to XCR1, *e.g.* on DCs. In various embodiments, the multi-specific PD-1 or PD-L1 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that comprise all of or part of XCL1.

In various embodiments, the multi-specific PD-1 or PD-L1 binding agents have targeting moieties having recognition domains which specifically bind to a target (*e.g.* antigen, receptor) which is part of a non-cellular structure. In some embodiments, the antigen or receptor is not an integral component of an intact cell or cellular structure. In some embodiments, the antigen or receptor is an extracellular antigen or receptor. In some embodiments, the target is a non-proteinaceous, non-cellular marker, including, without limitation, nucleic acids, inclusive of DNA or RNA, such as, for example, DNA released from necrotic tumor cells or extracellular deposits such as cholesterol.

In some embodiments, the target (*e.g.* antigen, receptor) of interest is part of the non-cellular component of the stroma or the extracellular matrix (ECM) or the markers associated therewith. As used herein, stroma refers to the connective and supportive framework of a tissue or organ. Stroma may include a compilation of cells such as fibroblasts/myofibroblasts, glial, epithelia, fat, immune, vascular, smooth muscle, and immune cells along with the extracellular matrix (ECM) and extracellular molecules. In various embodiments, the target (*e.g.* antigen, receptor)

of interest is part of the non-cellular component of the stroma such as the extracellular matrix and extracellular molecules. As used herein, the ECM refers to the non-cellular components present within all tissues and organs. The ECM is composed of a large collection of biochemically distinct components including, without limitation, proteins, glycoproteins, proteoglycans, and polysaccharides. These components of the ECM are usually produced by adjacent cells and secreted into the ECM via exocytosis. Once secreted, the ECM components often aggregate to form a complex network of macromolecules. In various embodiments, the chimeric protein of the invention comprises a targeting moiety that recognizes a target (*e.g.*, an antigen or receptor or non-proteinaceous molecule) located on any component of the ECM. Illustrative components of the ECM include, without limitation, the proteoglycans, the non-proteoglycan polysaccharides, fibers, and other ECM proteins or ECM non-proteins, *e.g.* polysaccharides and/or lipids, or ECM associated molecules (*e.g.* proteins or non-proteins, *e.g.* polysaccharides, nucleic acids and/or lipids).

In some embodiments, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on ECM proteoglycans. Proteoglycans are glycosylated proteins. The basic proteoglycan unit includes a core protein with one or more covalently attached glycosaminoglycan (GAG) chains. Proteoglycans have a net negative charge that attracts positively charged sodium ions (Na⁺), which attracts water molecules via osmosis, keeping the ECM and resident cells hydrated. Proteoglycans may also help to trap and store growth factors within the ECM. Illustrative proteoglycans that may be targeted by the chimeric proteins of the invention include, but are not limited to, heparan sulfate, chondroitin sulfate, and keratan sulfate. In an embodiment, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on non-proteoglycan polysaccharides such as hyaluronic acid.

In some embodiments, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on ECM fibers. ECM fibers include collagen fibers and elastin fibers. In some embodiments, the targeting moiety recognizes one or more epitopes on collagens or collagen fibers. Collagens are the most abundant proteins in the ECM. Collagens are present in the ECM as fibrillar proteins and provide structural support to resident cells. In one or more embodiments, the targeting moiety recognizes and binds to various types of collagens present within the ECM including, without limitation, fibrillar collagens (types I, II, III, V, XI), facit collagens (types IX, XII, XIV), short chain collagens (types VIII, X), basement membrane collagens (type IV), and/or collagen types VI, VII, or XIII. Elastin fibers provide elasticity to tissues, allowing them to stretch when needed and then return to their original state. In some embodiments, the target moiety recognizes one or more epitopes on elastins or elastin fibers.

In some embodiments, the targeting moiety recognizes one or more ECM proteins including, but not limited to, a tenascin, a fibronectin, a fibrin, a laminin, or a nidogen/entactin.

In an embodiment, the targeting moiety recognizes and binds to tenascin. The tenascin (TN) family of glycoproteins includes at least four members, tenascin-C, tenascin-R, tenascin-X, and tenascin W. The primary structures of tenascin proteins include several common motifs ordered in the same consecutive sequence: amino-terminal heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III domain repeats, and a carboxyl-terminal fibrinogen-like globular domain. Each protein member is associated with typical variations in the number

and nature of EGF-like and fibronectin type III repeats. Isoform variants also exist particularly with respect to tenascin-C. Over 27 splice variants and/or isoforms of tenascin-C are known. In a particular embodiment, the targeting moiety recognizes and binds to tenascin-CA1. Similarly, tenascin-R also has various splice variants and isoforms. Tenascin-R usually exists as dimers or trimers. Tenascin-X is the largest member of the tenascin family and is known to exist as trimers. Tenascin-W exists as trimers. In some embodiments, the targeting moiety recognizes one or more epitopes on a tenascin protein. In some embodiments, the targeting moiety recognizes the monomeric and/or the dimeric and/or the trimeric and/or the hexameric forms of a tenascin protein.

In an embodiment, the targeting moieties recognize and bind to fibronectin. Fibronectins are glycoproteins that connect cells with collagen fibers in the ECM, allowing cells to move through the ECM. Upon binding to integrins, fibronectins unfolds to form functional dimers. In some embodiments, the targeting moiety recognizes the monomeric and/or the dimeric forms of fibronectin. In some embodiments, the targeting moiety recognizes one or more epitopes on fibronectin. In illustrative embodiments, the targeting moiety recognizes fibronectin extracellular domain A (EDA) or fibronectin extracellular domain B (EDB). Elevated levels of EDA are associated with various diseases and disorders including psoriasis, rheumatoid arthritis, diabetes, and cancer. In some embodiments, the targeting moiety recognizes fibronectin that contains the EDA isoform and may be utilized to target the chimeric protein to diseased cells including cancer cells. In some embodiments, the targeting moiety recognizes fibronectin that contains the EDB isoform. In various embodiments, such targeting moieties may be utilized to target the chimeric protein to tumor cells including the tumor neovasculature.

In an embodiment, the targeting moiety recognizes and binds to fibrin. Fibrin is another protein substance often found in the matrix network of the ECM. Fibrin is formed by the action of the protease thrombin on fibrinogen which causes the fibrin to polymerize. In some embodiments, the targeting moiety recognizes one or more epitopes on fibrin. In some embodiments, the targeting moiety recognizes the monomeric as well as the polymerized forms of fibrin.

In an embodiment, the targeting moiety recognizes and binds to laminin. Laminin is a major component of the basal lamina, which is a protein network foundation for cells and organs. Laminins are heterotrimeric proteins that contain an α -chain, a β -chain, and a γ -chain. In some embodiments, the targeting moiety recognizes one or more epitopes on laminin. In some embodiments, the targeting moiety recognizes the monomeric, the dimeric as well as the trimeric forms of laminin.

In an embodiment, the targeting moiety recognizes and binds to a nidogen or entactin. Nidogens/entactins are a family of highly conserved, sulfated glycoproteins. They make up the major structural component of the basement membranes and function to link laminin and collagen IV networks in basement membranes. Members of this family include nidogen-1 and nidogen-2. In various embodiments, the targeting moiety recognizes an epitope on nidogen-1 and/or nidogen-2.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes an epitope present on any of the targets (e.g., ECM proteins) described herein. In an embodiment, the antigen-recognition

domain recognizes one or more linear epitopes present on the protein. As used herein, a linear epitope refers to any continuous sequence of amino acids present on the protein. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on the protein. As used herein, a conformational epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the targeting moiety may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of any of the targets (e.g., ECM proteins) described herein. In various embodiments, the targeting moiety may bind to any forms of the proteins described herein, including monomeric, dimeric, trimeric, tetrameric, heterodimeric, multimeric and associated forms. In various embodiments, the targeting moiety may bind to any post-translationally modified forms of the proteins described herein, such as glycosylated and/or phosphorylated forms.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes extracellular molecules such as DNA. In some embodiments, the targeting moiety comprises an antigen recognition domain that recognizes DNA. In an embodiment, the DNA is shed into the extracellular space from necrotic or apoptotic tumor cells or other diseased cells.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes one or more non-cellular structures associated with atherosclerotic plaques. Two types of atherosclerotic plaques are known. The fibro-lipid (fibro-fatty) plaque is characterized by an accumulation of lipid-laden cells underneath the intima of the arteries. Beneath the endothelium there is a fibrous cap covering the atheromatous core of the plaque. The core includes lipid-laden cells (macrophages and smooth muscle cells) with elevated tissue cholesterol and cholesterol ester content, fibrin, proteoglycans, collagen, elastin, and cellular debris. In advanced plaques, the central core of the plaque usually contains extracellular cholesterol deposits (released from dead cells), which form areas of cholesterol crystals with empty, needle-like clefts. At the periphery of the plaque are younger foamy cells and capillaries. A fibrous plaque is also localized under the intima, within the wall of the artery resulting in thickening and expansion of the wall and, sometimes, spotty localized narrowing of the lumen with some atrophy of the muscular layer. The fibrous plaque contains collagen fibers (eosinophilic), precipitates of calcium (hematoxylinophilic) and lipid-laden cells. In some embodiments, the targeting moiety recognizes and binds to one or more of the non-cellular components of these plaques such as the fibrin, proteoglycans, collagen, elastin, cellular debris, and calcium or other mineral deposits or precipitates. In some embodiments, the cellular debris is a nucleic acid, e.g. DNA or RNA, released from dead cells.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes one or more non-cellular structures found in the brain plaques associated with neurodegenerative diseases. In some embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures located in the

amyloid plaques found in the brains of patients with Alzheimer's disease. For example, the targeting moiety may recognize and bind to the peptide amyloid beta, which is a major component of the amyloid plaques. In some embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures located in the brains plaques found in patients with Huntington's disease. In various embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures found in plaques associated with other neurodegenerative or musculoskeletal diseases such as Lewy body dementia and inclusion body myositis. Linkers and Functional Groups

In various embodiments, the PD-1 or PD-L1 binding agent may include one or more functional groups, residues, or moieties. In various embodiments, the one or more functional groups, residues, or moieties are attached or genetically fused to any of the signaling agents or targeting moieties described herein. In some embodiments, such functional groups, residues or moieties confer one or more desired properties or functionalities to the PD-1 or PD-L1 binding agent of the invention. Examples of such functional groups and of techniques for introducing them into the PD-1 or PD-L1 binding agent are known in the art, for example, see *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Pa. (1980).

In various embodiments, the PD-1 or PD-L1 binding agent may be conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In some embodiments, the PD-1 or PD-L1 binding agent may be fused or conjugated with one or more of PEG, XTEN (e.g., as rPEG), polysialic acid (POLYXEN), albumin (e.g., human serum albumin or HAS), elastin-like protein (ELP), PAS, HAP, GLK, CTP, transferrin, and the like. In some embodiments, the PD-1 or PD-L1 binding agent may be fused or conjugated with an antibody or an antibody fragment such as an Fc fragment. For example, the chimeric protein may be fused to either the N-terminus or the C-terminus of the Fc domain of human immunoglobulin (Ig) G. In various embodiments, each of the individual chimeric proteins is fused to one or more of the agents described in BioDrugs (2015) 29:215–239, the entire contents of which are hereby incorporated by reference.

In some embodiments, the functional groups, residues, or moieties comprise a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). In some embodiments, attachment of the PEG moiety increases the half-life and/or reduces the immunogenicity of the PD-1 or PD-L1 binding protein. Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to single domain antibodies such as VHHs); see, for example, Chapman, *Nat. Biotechnol.*, 54, 531-545 (2002); by Veronese and Harris, *Adv. Drug Deliv. Rev.* 54, 453-456 (2003), by Harris and Chess, *Nat. Rev. Drug. Discov.*, 2, (2003) and in WO04060965, the entire contents of which are hereby incorporated by reference. Various reagents for pegylation of proteins are also commercially available, for example, from Nektar Therapeutics, USA. In some embodiments, site-directed pegylation is used, in particular via a cysteine-residue (see, for example, Yang *et al.*, *Protein Engineering*, 16, 10, 761-770 (2003), the entire contents of which is hereby incorporated by reference). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in the PD-1 or PD-L1 binding agent of the invention. In some embodiments, the PD-1 or PD-L1 binding agent of the

invention is modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the amino- and/or carboxy-terminus of the PD-1 or PD-L1 binding agent, using techniques known in the art.

In some embodiments, the functional groups, residues, or moieties comprise N-linked or O-linked glycosylation. In some embodiments, the N-linked or O-linked glycosylation is introduced as part of a co-translational and/or post-translational modification.

In some embodiments, the functional groups, residues, or moieties comprise one or more detectable labels or other signal-generating groups or moieties. Suitable labels and techniques for attaching, using and detecting them are known in the art and, include, but are not limited to, fluorescent labels (such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine and fluorescent metals such as Eu or others metals from the lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, therrromatic acridinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes, metals, metals chelates or metallic cations or other metals or metallic cations that are particularly suited for use in in vivo, in vitro or in situ diagnosis and imaging, as well as chromophores and enzymes (such as malate dehydrogenase, staphylococcal nuclease, delta- V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels include moieties that can be detected using NMR or ESR spectroscopy. Such labeled VHHs and polypeptides of the invention may, for example, be used for in vitro, in vivo or in situ assays (including immunoassays known per se such as ELISA, RIA, EIA and other "sandwich assays," etc.) as well as in vivo diagnostic and imaging purposes, depending on the choice of the specific label.

In some embodiments, the functional groups, residues, or moieties comprise a tag that is attached or genetically fused to the PD-1 or PD-L1 binding agent. In some embodiments, the PD-1 or PD-L1 binding agent may include a single tag or multiple tags. The tag for example is a peptide, sugar, or DNA molecule that does not inhibit or prevent binding of the PD-1 or PD-L1 binding agent to PD-1 or PD-L1 or any other antigen of interest such as tumor antigens. In various embodiments, the tag is at least about: three to five amino acids long, five to eight amino acids long, eight to twelve amino acids long, twelve to fifteen amino acids long, or fifteen to twenty amino acids long. Illustrative tags are described for example, in U.S. Patent Publication No. US2013/0058962. In some embodiment, the tag is an affinity tag such as glutathione-S-transferase (GST) and histidine (His) tag. In an embodiment, the PD-1 or PD-L1 binding agent comprises a His tag.

In some embodiments, the functional groups, residues, or moieties comprise a chelating group, for example, to chelate one of the metals or metallic cations. Suitable chelating groups, for example, include, without limitation, diethyl-enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

In some embodiments, the functional groups, residues, or moieties comprise a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the PD-1 or PD-L1 binding agent of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, *i.e.*, through formation of the binding pair. For example, a PD-1 or PD-L1 binding agent of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated PD-1 or PD-L1 binding agent may be used as a reporter, for example, in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may, for example, also be used to bind the PD-1 or PD-L1 binding agent to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh, *Journal of Drug Targeting*, 8, 4, 257 (2000). Such binding pairs may also be used to link a therapeutically active agent to the PD-1 or PD-L1 binding agent of the invention.

In some embodiments, the present PD-1 or PD-L1 binding agent optionally comprises one or more linkers. In some embodiments, the PD-1 or PD-L1 binding agent includes a linker that connects each binding region and/or targeting moieties. In some embodiments, the PD-1 or PD-L1 binding agent includes a linker that connects each signaling agent and targeting moiety (or, if more than one targeting moiety, a signaling agent to one of the targeting moieties). In some embodiments, the linker may be utilized to link various functional groups, residues, or moieties as described herein to the PD-1 or PD-L1 binding agent. In some embodiments, the linker is a single amino acid or a plurality of amino acids that does not affect or reduce the stability, orientation, binding, neutralization, and/or clearance characteristics of the binding regions and the binding protein. In various embodiments, the linker is selected from a peptide, a protein, a sugar, or a nucleic acid.

In some embodiments, the present PD-1 or PD-L1 binding agent comprises a linker connecting the targeting moiety and the signaling agent. In some embodiments, the present chimeric protein comprises a linker within the signaling agent (*e.g.* in the case of single chain TNF, which can comprise two linkers to yield a trimer).

The invention contemplates the use of a variety of linker sequences. In various embodiments, the linker may be derived from naturally-occurring multi-domain proteins or are empirical linkers as described, for example, in Chichili *et al.*, (2013), *Protein Sci.* 22(2):153-167, Chen *et al.*, (2013), *Adv Drug Deliv Rev.* 65(10):1357-1369, the entire contents of which are hereby incorporated by reference. In some embodiments, the linker may be designed using linker designing databases and computer programs such as those described in Chen *et al.*, (2013), *Adv Drug Deliv Rev.* 65(10):1357-1369 and Crasto *et al.*, (2000), *Protein Eng.* 13(5):309-312, the entire contents of which are hereby incorporated by reference. In various embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present PD-1 or PD-L1 binding agent.

In some embodiments, the linker is a polypeptide. In some embodiments, the linker is less than about 100 amino acids long. For example, the linker may be less than about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20,

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

In some embodiments, the linker length allows for efficient binding of a targeting moiety and the signaling agent to their receptors. For instance, in some embodiments, the linker length allows for efficient binding of one of the targeting moieties and the signaling agent to receptors on the same cell as well as the efficient binding of the other targeting moiety to another cell. Illustrative pairs of cells are provided elsewhere herein.

In some embodiments the linker length is at least equal to the minimum distance between the binding sites of one of the targeting moieties and the signaling agent to receptors on the same cell. In some embodiments the linker length is at least twice, or three times, or four times, or five times, or ten times, or twenty times, or 25 times, or 50 times, or one hundred times, or more the minimum distance between the binding sites of one of the targeting moieties and the signaling agent to receptors on the same cell.

In some embodiments, a linker connects the two targeting moieties to each other and this linker has a short length and a linker connects a targeting moiety and a signaling agent this linker is longer than the linker connecting the two targeting moieties. For example, the difference in amino acid length between the linker connecting the two targeting moieties and the linker connecting a targeting moiety and a signaling agent may be about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids. In some embodiments, the linker is flexible. In another embodiment, the linker is rigid.

In various embodiments, the linker is substantially comprised of glycine and serine residues (e.g. about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97% glycines and serines). For example, in some embodiments, the linker is $(\text{Gly}_4\text{Ser})_n$, where n is from about 1 to about 8, e.g. 1, 2, 3, 4, 5, 6, 7, or 8 (SEQ ID NOs: 1182-1189). In an embodiment, the linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO: 1190). Additional illustrative linkers include, but are not limited to, linkers having the sequence LE, GGGGS (SEQ ID NO: 1182), $(\text{GGGGS})_n$ ($n=1-4$) (SEQ ID NOs: 1182-1185), $(\text{Gly})_8$ (SEQ ID NO: 1191), $(\text{Gly})_6$ (SEQ ID NO: 1192), $(\text{EAAAK})_n$ ($n=1-3$) (SEQ ID NOs: 1193-1195); $\text{A}(\text{EAAAK})_n\text{A}$ ($n = 2-5$) (SEQ ID NOs: 1196-1199), AEAAAKEAAKA (SEQ ID NO: 1196), $\text{A}(\text{EAAAK})_4\text{ALEA}(\text{EAAAK})_4\text{A}$ (SEQ ID NO: 1200), PAPAP (SEQ ID NO: 1201), $\text{KESGSVSSEQLAQFRSLD}$ (SEQ ID NO: 1202), EGKSSGSGSESKST (SEQ ID NO: 1203), GSAGSAAGSGEF (SEQ ID NO: 1204), and $(\text{XP})_n$, with X designating any amino acid, e.g., Ala,

Lys, or Glu. In various embodiments, the linker is (GGG)_n (n=1-20) (SEQ ID NO: 1205 - SEQ ID NO: 1224). In some embodiments, the linker is G. In some embodiments, the linker is AAA. In some embodiments, the linker is (GGGGG)_n (n=9-20) (SEQ ID NOs: 1225-1236).

In some embodiments, the linker is one or more of GGGSE (SEQ ID NO: 1237), GSESG (SEQ ID NO: 1238), GSEGS (SEQ ID NO: 1239), GEGSGEGSSGEGSSSEGGGSEGGGSEGGGSEGGGS (SEQ ID NO: 1240), and a linker of randomly placed G, S, and E every 4 amino acid intervals.

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

According to crystallographic studies, the immunoglobulin hinge region can be further subdivided functionally into three regions: the upper hinge region, the core region, and the lower hinge region. See Shin *et al.*, 1992 *Immunological Reviews* 130:87. The upper hinge region includes amino acids from the carboxyl end of C_{H1} to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the C_{H2} domain and includes residues in C_{H2}. *Id.* The core hinge region of wild-type human IgG1 contains the sequence SEQ ID NO: 1241, which, when dimerized by disulfide bond formation, results in a cyclic octapeptide believed to act as a pivot, thus conferring flexibility. In various embodiments, the present linker comprises, one, or two, or three of the upper hinge region, the core region, and the lower hinge region of any antibody (e.g., of IgG, IgA, IgD, and IgE, inclusive of subclasses (e.g. IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region may also contain one or more glycosylation sites, which include a number of

structurally distinct types of sites for carbohydrate attachment. For example, IgA1 contains five glycosylation sites within a 17-amino-acid segment of the hinge region, conferring resistance of the hinge region polypeptide to intestinal proteases, considered an advantageous property for a secretory immunoglobulin. In various embodiments, the linker of the present invention comprises one or more glycosylation sites. In various embodiments, the linker is a hinge-CH2-CH3 domain of a human IgG4 antibody.

If desired, the present PD-1 or PD-L1 binding agent can be linked to an antibody Fc region, comprising one or both of C_H2 and C_H3 domains, and optionally a hinge region. For example, vectors encoding the present PD-1 or PD-L1 binding agents linked as a single nucleotide sequence to an Fc region can be used to prepare such polypeptides.

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

In various embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present PD-1 or PD-L1 binding agent. In another example, the linker may function to target the PD-1 or PD-L1 binding agent to a particular cell type or location.

Modifications and Production of PD-1 or PD-L1 binding agents

In various embodiments, the PD-1 or PD-L1 binding agent comprises a targeting moiety that is a VHH. In various embodiments, the VHH is not limited to a specific biological source or to a specific method of preparation. For example, the VHH can generally be obtained: (1) by isolating the V_HH domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring V_HH domain; (3) by "humanization" of a naturally occurring V_HH domain or by expression of a nucleic acid encoding a such humanized V_HH domain; (4) by "camelization" of a naturally occurring VH domain from any animal species, such as from a mammalian species, such as from a human being, or by expression of a nucleic acid encoding such a camelized VH domain; (5) by "camelization" of a "domain antibody" or "Dab" as described in the art, or by expression of a nucleic acid encoding such a camelized VH domain; (6) by using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences known in the art; (7) by preparing a nucleic acid encoding a VHH using techniques for nucleic acid synthesis known in the art, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination of one or more of the foregoing.

In an embodiment, the PD-1 or PD-L1 binding agent comprises a VHH that corresponds to the V_HH domains of naturally occurring heavy chain antibodies directed against human PD-1 or PD-L1. In some embodiments, such V_HH sequences can generally be generated or obtained by suitably immunizing a species of Camelid with a PD-1 or PD-L1 molecule, (*i.e.*, so as to raise an immune response and/or heavy chain antibodies directed against PD-1 or PD-L1), by obtaining a suitable biological sample from the Camelid (such as a blood sample, or any sample of B-cells), and by generating V_HH sequences directed against PD-1 or PD-L1, starting from the sample, using any suitable known techniques. In some embodiments, naturally occurring V_HH domains against PD-1 or PD-L1 can be obtained from naive libraries of Camelid V_HH sequences, for example, by screening such a library using PD-1

or PD-L1 or at least one part, fragment, antigenic determinant or epitope thereof using one or more screening techniques known in the art. Such libraries and techniques are, for example, described in WO9937681, WO0190190, WO03025020 and WO03035694, the entire contents of which are hereby incorporated by reference. In some embodiments, improved synthetic or semi-synthetic libraries derived from naive V_HH libraries may be used, such as V_HH libraries obtained from naive V_HH libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example, described in WO0043507, the entire contents of which are hereby incorporated by reference. In some embodiments, another technique for obtaining V_HH sequences directed against a PD-1 or PD-L1 involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (*i.e.*, so as to raise an immune response and/or heavy chain antibodies directed against PD-1 or PD-L1), obtaining a suitable biological sample from the transgenic mammal (such as a blood sample, or any sample of B-cells), and then generating V_HH sequences directed against PD-1 or PD-L1 starting from the sample, using any suitable known techniques. For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO02085945 and in WO04049794 (the entire contents of which are hereby incorporated by reference) can be used.

In an embodiment, the PD-1 or PD-L1 binding agent comprises a VHH that has been “humanized” *i.e.*, by replacing one or more amino acid residues in the amino acid sequence of the naturally occurring V_HH sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional 4-chain antibody from a human being. This can be performed using humanization techniques known in the art. In some embodiments, possible humanizing substitutions or combinations of humanizing substitutions may be determined by methods known in the art, for example, by a comparison between the sequence of a VHH and the sequence of a naturally occurring human VH domain. In some embodiments, the humanizing substitutions are chosen such that the resulting humanized VHHs still retain advantageous functional properties. Generally, as a result of humanization, the VHHs of the invention may become more “human-like,” while still retaining favorable properties such as a reduced immunogenicity, compared to the corresponding naturally occurring V_HH domains. In various embodiments, the humanized VHHs of the invention can be obtained in any suitable manner known in the art and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_HH domain as a starting material.

In an embodiment, the PD-1 or PD-L1 binding agent comprises a VHH that has been “camelized,” *i.e.*, by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring VH domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_HH domain of a heavy chain antibody of a camelid. In some embodiments, such “camelizing” substitutions are inserted at amino acid positions that form and/or are present at the VH-VL interface, and/or at the so-called Camelidae hallmark residues (see, for example, WO9404678, the entire contents of which are hereby incorporated by reference). In some embodiments, the VH sequence that is used as a starting material or starting point for generating or designing the camelized VHH is a VH sequence from a mammal, for example, the VH sequence of a human being, such as a VH3 sequence. In various embodiments, the camelized VHHs can be obtained in any

suitable manner known in the art (*i.e.*, as indicated under points (1)-(8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring VH domain as a starting material.

In various embodiments, both “humanization” and “camelization” can be performed by providing a nucleotide sequence that encodes a naturally occurring V_HH domain or VH domain, respectively, and then changing, in a manner known in the art, one or more codons in the nucleotide sequence in such a way that the new nucleotide sequence encodes a “humanized” or “camelized” VHH, respectively. This nucleic acid can then be expressed in a manner known in the art, so as to provide the desired VHH of the invention. Alternatively, based on the amino acid sequence of a naturally occurring V_HH domain or VH domain, respectively, the amino acid sequence of the desired humanized or camelized VHH of the invention, respectively, can be designed and then synthesized *de novo* using techniques for peptide synthesis known in the art. Also, based on the amino acid sequence or nucleotide sequence of a naturally occurring V_HH domain or VH domain, respectively, a nucleotide sequence encoding the desired humanized or camelized VHH, respectively, can be designed and then synthesized *de novo* using techniques for nucleic acid synthesis known in the art, after which the nucleic acid thus obtained can be expressed in a manner known in the art, so as to provide the desired VHH of the invention. Other suitable methods and techniques for obtaining the VHHs of the invention and/or nucleic acids encoding the same, starting from naturally occurring VH sequences or V_HH sequences, are known in the art, and may, for example, comprise combining one or more parts of one or more naturally occurring VH sequences (such as one or more FR sequences and/or CDR sequences), one or more parts of one or more naturally occurring V_HH sequences (such as one or more FR sequences or CDR sequences), and/or one or more synthetic or semi-synthetic sequences, in a suitable manner, so as to provide a VHH of the invention or a nucleotide sequence or nucleic acid encoding the same.

Methods for producing the PD-1 or PD-L1 binding agents of the invention are described herein. For example, DNA sequences encoding the PD-1 or PD-L1 binding agents of the invention can be chemically synthesized using methods known in the art. Synthetic DNA sequences can be ligated to other appropriate nucleotide sequences, including, *e.g.*, expression control sequences, to produce gene expression constructs encoding the desired PD-1 or PD-L1 binding agents. Accordingly, in various embodiments, the present invention provides for isolated nucleic acids comprising a nucleotide sequence encoding the PD-1 or PD-L1 binding agent of the invention.

Nucleic acids encoding the PD-1 or PD-L1 binding agent of the invention can be incorporated (ligated) into expression vectors, which can be introduced into host cells through transfection, transformation, or transduction techniques. For example, nucleic acids encoding the PD-1 or PD-L1 binding agent of the invention can be introduced into host cells by retroviral transduction. Illustrative host cells are *E.coli* cells, Chinese hamster ovary (CHO) cells, human embryonic kidney 293 (HEK 293) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), and myeloma cells. Transformed host cells can be grown under conditions that permit the host cells to express the genes that encode the PD-1 or PD-L1 binding agent of the invention. Accordingly, in various embodiments, the present invention provides expression

vectors comprising nucleic acids that encode the PD-1 or PD-L1 binding agent of the invention. In various embodiments, the present invention additionally provides host cells comprising such expression vectors.

Specific expression and purification conditions will vary depending upon the expression system employed. For example, if a gene is to be expressed in *E. coli*, it is first cloned into an expression vector by positioning the engineered gene downstream from a suitable bacterial promoter, e.g., Trp or Tac, and a prokaryotic signal sequence. In another example, if the engineered gene is to be expressed in eukaryotic host cells, e.g., CHO cells, it is first inserted into an expression vector containing for example, a suitable eukaryotic promoter, a secretion signal, enhancers, and various introns. The gene construct can be introduced into the host cells using transfection, transformation, or transduction techniques.

The PD-1 or PD-L1 binding agent of the invention can be produced by growing a host cell transfected with an expression vector encoding the PD-1 or PD-L1 binding agent under conditions that permit expression of the protein. Following expression, the protein can be harvested and purified using techniques well known in the art, e.g., affinity tags such as glutathione-S-transferase (GST) and histidine (His) tags or by chromatography. In an embodiment, the PD-1 or PD-L1 binding agent comprises a His tag. In an embodiment, the PD-1 or PD-L1 binding agent comprises a His tag and a proteolytic site to allow cleavage of the His tag.

Accordingly, in various embodiments, the present invention provides for a nucleic acid encoding a PD-1 or PD-L1 binding agent of the present invention. In various embodiments, the present invention provides for a host cell comprising a nucleic acid encoding a PD-1 or PD-L1 binding agent of the present invention.

In various embodiments, the present PD-1 or PD-L1 binding agent or chimeric protein comprising the same may be expressed *in vivo*, for instance, in a patient. For example, in various embodiments, the present PD-1 or PD-L1 binding agent or chimeric protein comprising the same may be administered in the form of nucleic acid which encodes the present PD-1 or PD-L1 binding agents or chimeric proteins comprising the same. In various embodiments, the nucleic acid is DNA or RNA. In some embodiments, present PD-1 or PD-L1 binding agent or chimeric protein comprising the same is encoded by a modified mRNA, *i.e.* an mRNA comprising one or more modified nucleotides. In some embodiments, the modified mRNA comprises one or more modifications found in U.S. Patent No. 8,278,036, the entire contents of which are hereby incorporated by reference. In some embodiments, the modified mRNA comprises one or more of m5C, m5U, m6A, s2U, Ψ, and 2'-O-methyl-U. In some embodiments, the present invention relates to administering a modified mRNA encoding one or more of the present chimeric proteins. In some embodiments, the present invention relates to gene therapy vectors comprising the same. In some embodiments, the present invention relates to gene therapy methods comprising the same. In various embodiments, the nucleic acid is in the form of an oncolytic virus, e.g. an adenovirus, reovirus, measles, herpes simplex, Newcastle disease virus or vaccinia.

Pharmaceutically Acceptable Salts and Excipients

The PD-1 or PD-L1 binding agents (and/or any other therapeutic agents) described herein can possess a sufficiently basic functional group, which can react with an inorganic or organic acid, or a carboxyl group, which

can react with an inorganic or organic base, to form a pharmaceutically acceptable salt. A pharmaceutically acceptable acid addition salt is formed from a pharmaceutically acceptable acid, as is well known in the art. Such salts include the pharmaceutically acceptable salts listed in, for example, *Journal of Pharmaceutical Science*, 66, 2-19 (1977) and *The Handbook of Pharmaceutical Salts; Properties, Selection, and Use*. P. H. Stahl and C. G. Wermuth (eds.), Verlag, Zurich (Switzerland) 2002, which are hereby incorporated by reference in their entirety.

Pharmaceutically acceptable salts include, by way of non-limiting example, sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, camphorsulfonate, pamoate, phenylacetate, trifluoroacetate, acrylate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, isobutyrate, phenylbutyrate, α -hydroxybutyrate, butyne-1,4-dicarboxylate, hexyne-1,4-dicarboxylate, caprate, caprylate, cinnamate, glycollate, heptanoate, hippurate, malate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, phthalate, teraphthalate, propiolate, propionate, phenylpropionate, sebacate, suberate, p-bromobenzenesulfonate, chlorobenzenesulfonate, ethylsulfonate, 2-hydroxyethylsulfonate, methylsulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, naphthalene-1,5-sulfonate, xylenesulfonate, and tartarate salts.

The term "pharmaceutically acceptable salt" also refers to a salt of the compositions of the present invention having an acidic functional group, such as a carboxylic acid functional group, and a base. Suitable bases include, but are not limited to, hydroxides of alkali metals such as sodium, potassium, and lithium; hydroxides of alkaline earth metal such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia, and organic amines, such as unsubstituted or hydroxy-substituted mono-, di-, or tri-alkylamines, dicyclohexylamine; tributyl amine; pyridine; N-methyl, N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-OH-lower alkylamines), such as mono-, bis-, or tris-(2-hydroxyethyl)amine, 2-hydroxy-tert-butylamine, or tris-(hydroxymethyl)methylamine, N,N-di-lower alkyl-N-(hydroxyl-lower alkyl)-amines, such as N,N-dimethyl-N-(2-hydroxyethyl)amine or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; and amino acids such as arginine, lysine, and the like.

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

Pharmaceutical Compositions and Formulations

In various embodiments, the present invention pertains to pharmaceutical compositions comprising the PD-1 or PD-L1 binding agents (and/or any other therapeutic agents) described herein and a pharmaceutically acceptable carrier or excipient. In some embodiments, the present invention pertains to pharmaceutical compositions comprising the present PD-1 or PD-L1 binding agents. In another embodiment, the present invention pertains to pharmaceutical compositions comprising any other therapeutic agents described herein. In a further embodiment, the present invention pertains to pharmaceutical compositions comprising a combination of the present PD-1 or

PD-L1 binding agents and any other therapeutic agents described herein. Any pharmaceutical compositions described herein can be administered to a subject as a component of a composition that comprises a pharmaceutically acceptable carrier or vehicle. Such compositions can optionally comprise a suitable amount of a pharmaceutically acceptable excipient so as to provide the form for proper administration.

In various embodiments, pharmaceutical excipients can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical excipients can be, for example, saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one embodiment, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, specifically for injectable solutions. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Any agent described herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents. Other examples of suitable pharmaceutical excipients are described in *Remington's Pharmaceutical Sciences* 1447-1676 (Alfonso R. Gennaro eds., 19th ed. 1995), incorporated herein by reference.

The present invention includes the described pharmaceutical compositions (and/or additional therapeutic agents) in various formulations. Any inventive pharmaceutical composition (and/or additional therapeutic agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, gelatin capsules, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, lyophilized powder, frozen suspension, desiccated powder, or any other form suitable for use. In one embodiment, the composition is in the form of a capsule. In another embodiment, the composition is in the form of a tablet. In yet another embodiment, the pharmaceutical composition is formulated in the form of a soft-gel capsule. In a further embodiment, the pharmaceutical composition is formulated in the form of a gelatin capsule. In yet another embodiment, the pharmaceutical composition is formulated as a liquid.

Where necessary, the inventive pharmaceutical compositions (and/or additional agents) can also include a solubilizing agent. Also, the agents can be delivered with a suitable vehicle or delivery device as known in the art. Combination therapies outlined herein can be co-delivered in a single delivery vehicle or delivery device.

The formulations comprising the inventive pharmaceutical compositions (and/or additional agents) of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the therapeutic agents into association with a carrier, which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired

formulation (e.g., wet or dry granulation, powder blends, *etc.*, followed by tableting using conventional methods known in the art).

In various embodiments, any pharmaceutical compositions (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for a mode of administration described herein.

Routes of administration include, for example: oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically. Administration can be local or systemic. In some embodiments, the administering is effected orally. In another embodiment, the administration is by parenteral injection. The mode of administration can be left to the discretion of the practitioner, and depends in-part upon the site of the medical condition. In most instances, administration results in the release of any agent described herein into the bloodstream.

In one embodiment, the PD-1 or PD-L1 binding agent described herein is formulated in accordance with routine procedures as a composition adapted for oral administration. Compositions for oral delivery can be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions can comprise one or more agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving any PD-1 or PD-L1 binding agents described herein are also suitable for orally administered compositions. In these latter platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time-delay material such as glycerol monostearate or glycerol stearate can also be useful. Oral compositions can include standard excipients such as mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, and magnesium carbonate. In one embodiment, the excipients are of pharmaceutical grade. Suspensions, in addition to the active compounds, may contain suspending agents such as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth, *etc.*, and mixtures thereof.

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

glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose.

For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). The carrier should be stable under the conditions of manufacture and storage, and should be preserved against microorganisms. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol), and suitable mixtures thereof.

The compositions provided herein, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Any inventive pharmaceutical compositions (and/or additional agents) described herein can be administered by controlled-release or sustained-release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,556, each of which is incorporated herein by reference in its entirety. Such dosage forms can be useful for providing controlled- or sustained-release of one or more active ingredients using, for example, hydropropyl cellulose, hydropropylmethyl cellulose, polyvinylpyrrolidone, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Suitable controlled- or sustained-release formulations known to those skilled in the art, including those described herein, can be readily selected for use with the active ingredients of the agents described herein. The invention thus provides single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled- or sustained-release.

Controlled- or sustained-release of an active ingredient can be stimulated by various conditions, including but not limited to, changes in pH, changes in temperature, stimulation by an appropriate wavelength of light, concentration or availability of enzymes, concentration or availability of water, or other physiological conditions or compounds.

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

Pharmaceutical formulations preferably are sterile. Sterilization can be accomplished, for example, by filtration through sterile filtration membranes. Where the composition is lyophilized, filter sterilization can be conducted prior to or following lyophilization and reconstitution.

Administration and Dosage

It will be appreciated that the actual dose of the PD-1 or PD-L1 binding agent and/or any therapeutic agents described herein to be administered according to the present invention will vary according to the particular dosage form, and the mode of administration. Many factors that may modify the action of the PD-1 or PD-L1 binding agent (e.g., body weight, gender, diet, time of administration, route of administration, rate of excretion, condition of the subject, drug combinations, genetic disposition and reaction sensitivities) can be taken into account by those skilled in the art. Administration can be carried out continuously or in one or more discrete doses within the maximum tolerated dose. Optimal administration rates for a given set of conditions can be ascertained by those skilled in the art using conventional dosage administration tests.

In some embodiments, a suitable dosage of the PD-1 or PD-L1 binding agent and/or any therapeutic agents described herein is in a range of about 0.01 mg/kg to about 10 g/kg of body weight of the subject, about 0.01 mg/kg to about 1 g/kg of body weight of the subject, about 0.01 mg/kg to about 100 mg/kg of body weight of the subject, about 0.01 mg/kg to about 10 mg/kg of body weight of the subject, for example, about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5 mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, about 1.9 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg body weight, about 100 mg/kg body weight, about 1 g/kg of body weight, about 10 g/kg of body weight, inclusive of all values and ranges therebetween.

Individual doses of the PD-1 or PD-L1 binding agent and/or any therapeutic agents described herein can be administered in unit dosage forms containing, for example, from about 0.01 mg to about 100 g, from about 0.01 mg to about 75 g, from about 0.01 mg to about 50 g, from about 0.01 mg to about 25 g, about 0.01 mg to about 10 g, about 0.01 mg to about 7.5 g, about 0.01 mg to about 5 g, about 0.01 mg to about 2.5 g, about 0.01 mg to about 1 g, about 0.01 mg to about 100 mg, from about 0.1 mg to about 100 mg, from about 0.1 mg to about 90 mg, from about 0.1 mg to about 80 mg, from about 0.1 mg to about 70 mg, from about 0.1 mg to about 60 mg, from about 0.1 mg to about 50 mg, from about 0.1 mg to about 40 mg active ingredient, from about 0.1 mg to about 30 mg, from about 0.1 mg to about 20 mg, from about 0.1 mg to about 10 mg, from about 0.1 mg to about 5 mg, from about 0.1 mg to about 3 mg, from about 0.1 mg to about 1 mg per unit dosage form, or from about 5 mg to about 80 mg per unit dosage form. For example, a unit dosage form can be about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05 mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg,

about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

In one embodiment, the PD-1 or PD-L1 binding agent and/or any therapeutic agents described herein are administered at an amount of from about 0.01 mg to about 100 g daily, from about 0.01 mg to about 75 g daily, from about 0.01 mg to about 50 g daily, from about 0.01 mg to about 25 g daily, from about 0.01 mg to about 10 g daily, from about 0.01 mg to about 7.5 g daily, from about 0.01 mg to about 5 g daily, from about 0.01 mg to about 2.5 g daily, from about 0.01 mg to about 1 g daily, from about 0.01 mg to about 100 mg daily, from about 0.1 mg to about 100 mg daily, from about 0.1 mg to about 95 mg daily, from about 0.1 mg to about 90 mg daily, from about 0.1 mg to about 85 mg daily, from about 0.1 mg to about 80 mg daily, from about 0.1 mg to about 75 mg daily, from about 0.1 mg to about 70 mg daily, from about 0.1 mg to about 65 mg daily, from about 0.1 mg to about 60 mg daily, from about 0.1 mg to about 55 mg daily, from about 0.1 mg to about 50 mg daily, from about 0.1 mg to about 45 mg daily, from about 0.1 mg to about 40 mg daily, from about 0.1 mg to about 35 mg daily, from about 0.1 mg to about 30 mg daily, from about 0.1 mg to about 25 mg daily, from about 0.1 mg to about 20 mg daily, from about 0.1 mg to about 15 mg daily, from about 0.1 mg to about 10 mg daily, from about 0.1 mg to about 5 mg daily, from about 0.1 mg to about 3 mg daily, from about 0.1 mg to about 1 mg daily, or from about 5 mg to about 80 mg daily. In various embodiments, the PD-1 or PD-L1 binding agent is administered at a daily dose of about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05 mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 7.5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

In accordance with certain embodiments of the invention, the pharmaceutical composition comprising the PD-1 or PD-L1 binding agent and/or any therapeutic agents described herein may be administered, for example, more than once daily (e.g., about two times, about three times, about four times, about five times, about six times, about seven times, about eight times, about nine times, or about ten times daily), about once per day, about every other day, about every third day, about once a week, about once every two weeks, about once every month, about once every two months, about once every three months, about once every six months, or about once every year.

Combination Therapy and Additional Therapeutic Agents

In various embodiments, the pharmaceutical composition of the present invention is co-administered in conjunction with additional therapeutic agent(s). Co-administration can be simultaneous or sequential.

In one embodiment, the additional therapeutic agent and the PD-1 or PD-L1 binding agent of the present invention are administered to a subject simultaneously. The term “simultaneously” as used herein, means that the additional

therapeutic agent and the PD-1 or PD-L1 binding agent are administered with a time separation of no more than about 60 minutes, such as no more than about 30 minutes, no more than about 20 minutes, no more than about 10 minutes, no more than about 5 minutes, or no more than about 1 minute. Administration of the additional therapeutic agent and the PD-1 or PD-L1 binding agent can be by simultaneous administration of a single formulation (e.g., a formulation comprising the additional therapeutic agent and the PD-1 or PD-L1 binding agent) or of separate formulations (e.g., a first formulation including the additional therapeutic agent and a second formulation including the PD-1 or PD-L1 binding agent).

Co-administration does not require the therapeutic agents to be administered simultaneously, if the timing of their administration is such that the pharmacological activities of the additional therapeutic agent and the PD-1 or PD-L1 binding agent overlap in time, thereby exerting a combined therapeutic effect. For example, the additional therapeutic agent and the PD-1 or PD-L1 binding agent can be administered sequentially. The term “sequentially” as used herein means that the additional therapeutic agent and the PD-1 or PD-L1 binding agent are administered with a time separation of more than about 60 minutes. For example, the time between the sequential administration of the additional therapeutic agent and the PD-1 or PD-L1 binding agent can be more than about 60 minutes, more than about 2 hours, more than about 5 hours, more than about 10 hours, more than about 1 day, more than about 2 days, more than about 3 days, more than about 1 week, or more than about 2 weeks, or more than about one month apart. The optimal administration times will depend on the rates of metabolism, excretion, and/or the pharmacodynamic activity of the additional therapeutic agent and the PD-1 or PD-L1 binding agent being administered. Either the additional therapeutic agent or the PD-1 or PD-L1 binding agent cell may be administered first.

Co-administration also does not require the therapeutic agents to be administered to the subject by the same route of administration. Rather, each therapeutic agent can be administered by any appropriate route, for example, parenterally or non-parenterally.

In some embodiments, the PD-1 or PD-L1 binding agent described herein acts synergistically when co-administered with another therapeutic agent. In such embodiments, the PD-1 or PD-L1 binding agent and the additional therapeutic agent may be administered at doses that are lower than the doses employed when the agents are used in the context of monotherapy.

In some embodiments, the present invention pertains to chemotherapeutic agents as additional therapeutic agents. For example, without limitation, such combination of the present PD-1 or PD-L1 binding agents and chemotherapeutic agent find use in the treatment of cancers, as described elsewhere herein. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and CYTOXAN cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (e.g., bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan);

bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (e.g., cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall (see, e.g., Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994))); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino- doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanol, testolactone; anti-adrenals such as minoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (e.g., T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumburg, 111.), and TAXOTERE doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE. vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib

(Tykerb); inhibitors of PKC- α , Raf, H-Ras, EGFR (e.g., erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation. In addition, the methods of treatment can further include the use of photodynamic therapy.

Accordingly, in some embodiments, the present invention relates to combination therapies using the PD-1 or PD-L1 binding agent and a chemotherapeutic agent. In some embodiments, the present invention relates to administration of the PD-1 or PD-L1 binding agent to a patient undergoing treatment with a chemotherapeutic agent. In some embodiments, the chemotherapeutic agent is a DNA-intercalating agent such as, without limitation, doxorubicin, cisplatin, daunorubicin, and epirubicin. In an embodiment, the DNA-intercalating agent is doxorubicin.

In illustrative embodiments, the PD-1 or PD-L1 binding agent acts synergistically when co-administered with doxorubicin. In an illustrative embodiment, the PD-1 or PD-L1 binding agent acts synergistically when co-administered with doxorubicin for use in treating tumor or cancer. For example, co-administration of the PD-1 or PD-L1 binding agent and doxorubicin may act synergistically to reduce or eliminate the tumor or cancer, or slow the growth and/or progression and/or metastasis of the tumor or cancer. In illustrative embodiments, the combination of the PD-1 or PD-L1 binding agent and doxorubicin may exhibit improved safety profiles when compared to the agents used alone in the context of monotherapy. In illustrative embodiments, the PD-1 or PD-L1 binding agent and doxorubicin may be administered at doses that are lower than the doses employed when the agents are used in the context of monotherapy. In some embodiments, the PD-1 or PD-L1 binding agent comprises a mutated interferon such as a mutated IFN α . In illustrative embodiments, the mutated IFN α comprises one or more mutations at positions 148, 149, and 153 with reference to SEQ ID NO: 317 or SEQ ID NO: 318, such as the substitutions M148A, R149A, and L153A.

In some embodiments, the present invention relates to combination therapy with one or more immune-modulating agents, for example, without limitation, agents that modulate immune checkpoint. In various embodiments, the immune-modulating agent targets one or more of PD-1, PD-L1, and PD-L2. In various embodiments, the immune-modulating agent is PD-1 inhibitor. In various embodiments, the immune-modulating agent is an antibody specific for one or more of PD-1, PD-L1, and PD-L2. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, nivolumab, (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), MPDL3280A (ROCHE). In some embodiments, the immune-modulating agent targets one or more of CD137 or CD137L. In various embodiments, the immune-modulating agent is an antibody specific for one or more of CD137 or CD137L. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, urelumab (also known as BMS-663513 and anti-4-1BB antibody). In some embodiments, the present chimeric protein is combined with urelumab (optionally with one or more of nivolumab, lirilumab, and urelumab) for the treatment of solid tumors and/or B-cell non-Hodgkins lymphoma and/or head and neck cancer and/or multiple myeloma. In some embodiments, the immune-modulating agent is an agent that targets one or more of CTLA-4, AP2M1, CD80, CD86, SHP-2, and

PPP2R5A. In various embodiments, the immune-modulating agent is an antibody specific for one or more of CTLA-4, AP2M1, CD80, CD86, SHP-2, and PPP2R5A. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, ipilimumab (MDX-010, MDX-101, Yervoy, BMS) and/or tremelimumab (Pfizer). In some embodiments, the present chimeric protein is combined with ipilimumab (optionally with bavituximab) for the treatment of one or more of melanoma, prostate cancer, and lung cancer. In various embodiments, the immune-modulating agent targets CD20. In various embodiments, the immune-modulating agent is an antibody specific CD20. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, Ofatumumab (GENMAB), obinutuzumab (GAZYVA), AME-133v (APPLIED MOLECULAR EVOLUTION), Ocrelizumab (GENENTECH), TRU-015 (TRUBION/EMERGENT), veltuzumab (IMMU-106).

In some embodiments, the present invention relates to combination therapy using the PD-1 or PD-L1 binding agent and a checkpoint inhibitor. In some embodiments, the present invention relates to administration of the PD-1 or PD-L1 binding agent to a patient undergoing treatment with a checkpoint inhibitor. In some embodiments, the checkpoint inhibitor is an agent that targets one or more of PD-1, PD-L1, PD-L2, and CTLA-4 (including any of the anti-PD-1, anti-PD-L1, anti-PD-L2, and anti-CTLA-4 agents described herein). In some embodiment, the checkpoint inhibitor is one or more of nivolumab, (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), MPDL3280A (ROCHE), ipilimumab (MDX-010, MDX-101, Yervoy, BMS) and tremelimumab (Pfizer). In an embodiment, the checkpoint inhibitor is an antibody against PD-L1.

In illustrative embodiments, the PD-1 or PD-L1 binding agent acts synergistically when co-administered with the anti-PD-L1 antibody. In an illustrative embodiment, the PD-1 or PD-L1 binding agent acts synergistically when co-administered with the anti-PD-L1 antibody for use in treating tumor or cancer. For example, co-administration of the PD-1 or PD-L1 binding agent and the anti-PD-L1 antibody may act synergistically to reduce or eliminate the tumor or cancer, or slow the growth and/or progression and/or metastasis of the tumor or cancer. In some embodiments, the combination of the PD-1 or PD-L1 binding agent and the anti-PD-L1 antibody may exhibit improved safety profiles when compared to the agents used alone in the context of monotherapy. In some embodiments, the PD-1 or PD-L1 binding agent and the anti-PD-L1 antibody may be administered at doses that are lower than the doses employed when the agents are used in the context of monotherapy. In some embodiments, the PD-1 or PD-L1 binding agent comprises a mutated interferon such as a mutated IFN α . In illustrative embodiments, the mutated IFN α comprises one or more mutations at positions 148, 149, and 153 with reference to SEQ ID NO: 317 or SEQ ID NO: 318, such as the substitutions M148A, R149A, and L153A.

In some embodiments, the present invention relates to combination therapies using the PD-1 or PD-L1 binding agent and an immunosuppressive agent. In some embodiments, the present invention relates to administration of the PD-1 or PD-L1 binding agent to a patient undergoing treatment with an immunosuppressive agent. In an embodiment, the immunosuppressive agent is TNF.

In illustrative embodiments, the PD-1 or PD-L1 binding agent acts synergistically when co-administered with TNF. In an illustrative embodiment, the PD-1 or PD-L1 binding agent acts synergistically when co-administered with TNF for use in treating tumor or cancer. For example, co-administration of the PD-1 or PD-L1 binding agent and TNF may act synergistically to reduce or eliminate the tumor or cancer, or slow the growth and/or progression and/or metastasis of the tumor or cancer. In some embodiments, the combination of the PD-1 or PD-L1 binding agent and TNF may exhibit improved safety profiles when compared to the agents used alone in the context of monotherapy. In some embodiments, the PD-1 or PD-L1 binding agent and TNF may be administered at doses that are lower than the doses employed when the agents are used in the context of monotherapy. In some embodiments, the PD-1 or PD-L1 binding agent comprises a mutated interferon such as a mutated IFN α . In illustrative embodiments, the mutated IFN α comprises one or more mutations at positions 148, 149, and 153 with reference to SEQ ID NO: 317 or SEQ ID NO: 318, such as the substitutions M148A, R149A, and L153A.

In some embodiments, the PD-1 or PD-L1 binding agent acts synergistically when used in combination with Chimeric Antigen Receptor (CAR) T-cell therapy. In an illustrative embodiment, the PD-1 or PD-L1 binding agent acts synergistically when used in combination with CAR T-cell therapy in treating tumor or cancer. In an embodiment, the PD-1 or PD-L1 binding agent acts synergistically when used in combination with CAR T-cell therapy in treating blood-based tumors. In an embodiment, the PD-1 or PD-L1 binding agent acts synergistically when used in combination with CAR T-cell therapy in treating solid tumors. For example, use of the PD-1 or PD-L1 binding agent and CAR T-cells may act synergistically to reduce or eliminate the tumor or cancer, or slow the growth and/or progression and/or metastasis of the tumor or cancer. In various embodiments, the PD-1 or PD-L1 binding agent of the invention induces CAR T-cell division. In various embodiments, the PD-1 or PD-L1 binding agent of the invention induces CAR T-cell proliferation. In various embodiments, the PD-1 or PD-L1 binding agent of the invention prevents anergy of the CAR T cells.

In various embodiments, the CAR T-cell therapy comprises CAR T cells that target antigens (e.g., tumor antigens) such as, but not limited to, carbonic anhydrase IX (CAIX), 5T4, CD19, CD20, CD22, CD30, CD33, CD38, CD47, CS1, CD138, Lewis-Y, L1-CAM, MUC16, ROR-1, IL13R α 2, gp100, prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), B-cell maturation antigen (BCMA), human papillomavirus type 16 E6 (HPV-16 E6), CD171, folate receptor alpha (FR- α), GD2, human epidermal growth factor receptor 2 (HER2), mesothelin, EGFRvIII, fibroblast activation protein (FAP), carcinoembryonic antigen (CEA), and vascular endothelial growth factor receptor 2 (VEGF-R2), as well as other tumor antigens well known in the art. Additional illustrative tumor antigens include, but are not limited to MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-0017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor

antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α -fetoprotein, E-cadherin, α -catenin, β -catenin and γ -catenin, p120ctn, gp100 Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, Imp-1, NA, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 CT-7, c-erbB-2, CD19, CD37, CD56, CD70, CD74, CD138, AGS16, MUC1, GPNMB, Ep-CAM, PD-L1, and PD-L2.

Exemplary CAR T-cell therapy include, but are not limited to, JCAR014 (Juno Therapeutics), JCAR015 (Juno Therapeutics), JCAR017 (Juno Therapeutics), JCAR018 (Juno Therapeutics), JCAR020 (Juno Therapeutics), JCAR023 (Juno Therapeutics), JCAR024 (Juno Therapeutics), CTL019 (Novartis), KTE-C19 (Kite Pharma), BPX-401 (Bellicum Pharmaceuticals), BPX-501 (Bellicum Pharmaceuticals), BPX-601 (Bellicum Pharmaceuticals), bb2121 (Bluebird Bio), CD-19 Sleeping Beauty cells (Ziopharm Oncology), UCART19 (Collectis), UCART123 (Collectis), UCART38 (Collectis), UCARTCS1 (Collectis), OXB-302 (Oxford BioMedica), MB-101 (Mustang Bio) and CAR T-cells developed by Innovative Cellular Therapeutics.

In some embodiments, the PD-1 or PD-L1 binding agent is used in a method of treating multiple sclerosis (MS) in combination with one or more MS therapeutics including, but not limited to, 3-interferons, glatiramer acetate, T-interferon, IFN- β -2 (U. S. Patent Publication No. 2002/0025304), spirogermaniums (e.g., N-(3-dimethylaminopropyl)-2-aza-8,8-dimethyl-8-germaspiro [4:5] decane, N-(3-dimethylaminopropyl)-2-aza-8,8-diethyl-8-germaspiro [4:5] decane, N-(3-dimethylaminopropyl)-2-aza-8,8-dipropyl-8-germaspiro [4:5] decane, and N-(3-dimethylaminopropyl)-2-aza-8,8-dibutyl-8-germaspiro [4:5] decane), vitamin D analogs (e.g., 1,25 (OH) 2D3, (see, e.g., U.S. Patent No. 5,716,946)), prostaglandins (e.g., latanoprost, brimonidine, PGE1, PGE2 and PGE3, see, e.g., U. S. Patent Publication No. 2002/0004525), tetracycline and derivatives (e.g., minocycline and doxycycline, see, e.g., U.S. Patent Publication No. 20020022608), a VLA-4 binding antibody (see, e.g., U.S. Patent Publication No. 2009/0202527), adrenocorticotrophic hormone, corticosteroid, prednisone, methylprednisone, 2-chlorodeoxyadenosine, mitoxantrone, sulphasalazine, methotrexate, azathioprine, cyclophosphamide, cyclosporin, fumarate, anti-CD20 antibody (e.g., rituximab), and tizanidine hydrochloride.

In some embodiments, the PD-1 or PD-L1 binding agent is used in combination with one or more therapeutic agents that treat one or more symptoms or side effects of MS. Such agents include, but are not limited to, amantadine, baclofen, papaverine, meclizine, hydroxyzine, sulfamethoxazole, ciprofloxacin, docusate, pemoline, dantrolene, desmopressin, dexamethasone, tolterodine, phenyloin, oxybutynin, bisacodyl, venlafaxine, amitriptyline, methenamine, clonazepam, isoniazid, vardenafil, nitrofurantoin, psyllium hydrophilic mucilloid, alprostadil, gabapentin, nortriptyline, paroxetine, propantheline bromide, modafinil, fluoxetine, phenazopyridine, methylprednisolone, carbamazepine, imipramine, diazepam, sildenafil, bupropion, and sertraline.

In some embodiments, the PD-1 or PD-L1 binding agent is used in a method of treating multiple sclerosis in combination with one or more of the disease modifying therapies (DMTs) described herein (e.g. the agents of Table A). In some embodiments, the present invention provides an improved therapeutic effect as compared to use of one or more of the DMTs described herein (e.g. the agents listed in the Table below) without the one or more disclosed binding agent. In an embodiment, the combination of the PD-1 or PD-L1 binding agent and the one or more DMTs produces synergistic therapeutic effects.

Illustrative Disease Modifying Therapies

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
teriflunomide	AUBAGIO (GENZYME)	Every day; pill taken orally; 7 mg or 14 mg.
interferon beta-1a	AVONEX (BIOGEN IDEC)	Once a week; intramuscular (into the muscle) injection; 30 mcg
interferon beta-1b	BETASERON (BAYER HEALTHCARE PHARMACEUTICALS, INC.)	Every other day; subcutaneous (under the skin) injection; 250 mcg.
glatiramer acetate	COPAXONE (TEVA NEUROSCIENCE)	Every day; subcutaneous (under the skin) injection; 20 mg (20,000 mcg) OR Three times a week; subcutaneous (under the skin) injection; 40 mg (40,000 mcg)
interferon beta-1b	EXTAVIA (NOVARTIS PHARMACEUTICALS CORP.)	Every other day; subcutaneous (under the skin) injection; 250 mcg.
fingolimod	GILENYA (NOVARTIS PHARMACEUTICALS CORP.)	Every day; capsule taken orally; 0.5 mg.
Alemtuzumab (anti-CD52 monoclonal antibody)	LEMTRADA (GENZYME)	Intravenous infusion on five consecutive days, followed by intravenous infusion on three consecutive days one year later (12 mg)
mitoxantrone	NOVANTRONE (EMD SERONO)	Four times a year by IV infusion in a medical facility. Lifetime cumulative dose limit of approximately 8–12 doses over 2–3 years (140 mg/m ²).
pegylated interferon beta-1a	PLEGRIDY (BIOGEN IDEC)	Every 14 days; subcutaneous (under the skin) injection; 125 mcg
interferon beta-1a	REBIF (EMD SERONO, INC.)	Three times a week; subcutaneous (under the skin) injection; 44 mcg
dimethyl fumarate (BG-12)	TECFIDERA (BIOGEN IDEC)	Twice a day; capsule taken orally; 120 mg for one week and 240 mg thereafter
Natalizumab (humanized monoclonal antibody VLA-4 antagonist)	TYSABRI (BIOGEN IDEC)	Every four weeks by IV infusion in a registered infusion facility; 300 mg
DMTs in Development		
Amiloride (targets Acid-sensing ion channel-1 Epithelial sodium channel Na ⁺ /H ⁺ exchanger)	PAR PHARMACEUTICAL, PERRIGO COMPANY, SIGMAPHARM LABORATORIES	Oral
ATX-MS-1467 (targets Major histocompatibility complex class II T cell responses to myelin basic protein)	APITOPE / MERCK SERONO	Intradermal Subcutaneous
BAF312 (targets Sphingosine 1-phosphate (S1P) receptor subtypes	NOVARTIS PHARMA	Oral

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
S1P1 and S1P5 B cell distribution T cell distribution)		
BGC20-0134 (targets Proinflammatory and anti-inflammatory cytokines)	BTG PLC	Oral
BLIB033 (targets LINGO-1 ("leucine-rich repeat and immunoglobulin-like domain-containing, Nogo receptor-interacting protein"))	BIOGEN	Intravenous infusion used in Phase I and Phase II trials Subcutaneous injection used in Phase I trial
Cladribine (targets CD4+ T cells DNA synthesis and repair E-selectin Intracellular adhesion molecule-1 Pro-inflammatory cytokines interleukin 2 and interleukin 2R Pro-inflammatory cytokines interleukin 8 and RANTES Cytokine secretion Monocyte and lymphocyte migration)	MERCK SERONO	Oral
Cyclophosphamide (targets T cells, particularly CD4+ helper T cells B cells)	BAXTER HEALTHCARE CORPORATION	Oral, monthly intravenous pulses
Daclizumab (humanized monoclonal antibody targeting CD25 Immune modulator of T cells)	BIOGEN IDEC/ABBVIE BIOTHERAPEUTICS	Projected to be IM injection once monthly
Dalfampridine (targets Voltage-gated potassium channels Degenerin/epithelial sodium channels L-type calcium channels that contain subunit Cavbeta3)	ACORDA THERAPEUTICS / BIOGEN IDEC	One tablet every 12 hours (extended release), 10 mg twice a day
Dronabinol (targets Cannabinoid receptor CB1 Cannabinoid receptor CB2)	ABBVIE INC.	Oral
Firategrast (targets Alpha4beta1 integrin)	GLAXOSMITHKLINE	Oral
GNbAC1MSRV-Env (targets envelope protein of the MS-associated retrovirus)	GENEURO SA / SERVIER	Intravenous infusion
Idebenone (targets Reactive oxygen species)	SANTHERA PHARMACEUTICALS	Oral Dose in clinical trial for PPMS is 2250 mg per day (750 mg dose, 3 times per day)
Imilecleucel-T (targets Myelin-specific, autoreactive T cells)	OPEXA THERAPEUTICS / MERCK SERONO	Subcutaneous Given 5 times per year, according to information from the manufacturer
Laquinimod	TEVA	Projected to be 0.6 mg or 1.2 mg oral tablet taken daily
Masitinib (targets KIT (a stem cell factor, also called	AB SCIENCE	Oral

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
c-KIT) receptor as well as select other tyrosine kinases Mast cells)		
MEDI-551 (targets CD19, a B cell-specific antigen that is part of the B cell receptor complex and that functions in determining the threshold for B cell activation B cells Plasmablasts, B cells that express CD19 (but not CD20) and that secrete large quantities of antibodies; depletion of plasmablasts may be useful in autoimmune diseases involving pathogenic autoantibodies)	MEDIMMUNE	Intravenous Subcutaneous
Minocycline (targets T cells Microglia Leukocyte migration Matrix metalloproteinases)	VARIOUS	Oral Available as pellet-filled capsules and an oral suspension
MIS416 (targets Innate immune system Pathogen-associated molecular pattern recognition receptors of the innate immune system Myeloid cells of the innate immune system, which might be able to remodel the deregulated immune system activity that occurs in SPMS)	INNATE IMMUNOTHERAPEUTICS	Intravenous
Mycophenolate mofetil (targets Purine synthesis)	MANUFACTURED BY GENENTECH	Oral
Naltrexone (targets Opioid receptors Toll-like receptor 4)	VARIOUS	Given at low doses (3 to 4.5 mg per day) in oral form as "Low-dose naltrexone" (or "LDN")
Ocrelizumab and Ofatumumab (humanized monoclonal antibodies targeting CD20 B cell suppression)	ROCHE / GSK	Projected to be IV infusion
ONO-4641 (targets Sphingosine 1-phosphate receptor)	ONO PHARMACEUTICAL CO.	Oral
Phenytoin (targets Sodium channels)	PFIZER	Intravenous Intramuscular (less favored option) Oral
Ponesimod	ACTELION	To be determined
Raltegravir (targets Retroviral integrase Herpesvirus DNA packaging terminase)	MERCK	Oral 400 mg tablet twice daily, according to information from the manufacturer

Generic Name	Branded Name/Company	Frequency/Route of Delivery/Usual Dose
RHB-104	REDHILL BIOPHARMA LIMITED	95 mg clarithromycin, 45 mg rifabutin, and 10 mg clofazimine
Riluzole (targets Glutamatergic neurotransmission Glutamate uptake and release Voltage-gated sodium channels Protein kinase C)	COVIS PHARMA / SANOFI	Oral

MS disease progression may be most intensive, and most damaging, at the earliest stages of disease progression. Accordingly, counter to many reimbursement policies and physician practice in light of, for example, costs and side effect mitigation, it may be most beneficial for a patient's long term disease status to begin treatment with the most intensive DMTs, for instance so-called second-line therapies. In some embodiments, a patient is treated with a regimen of the PD-1 or PD-L1 binding agent in combination with a second-line therapy. Such a combination is used to reduce the side effect profile of one or more second-line therapies. In some embodiments, the combination is used to reduce dose of frequency of administration of one or more second-line therapies. For example, the doses of agents listed in the Table provided above may be reduced by about 50%, or about 40%, or about 30%, or about 25% in the context of the combination and the/or the frequency of dosing may be decreased to be half as often, or a third as often or may be reduced from, for example, daily to every other day or weekly, every other day to weekly or bi-weekly, weekly to bi-weekly or monthly, *etc.* Accordingly, in some embodiments, the PD-1 or PD-L1 binding agent increase patient adherence by allowing for more convenient treatment regimens. Further, some DMTs have a suggested lifetime dose limitation *e.g.* for mitoxantrone, the lifetime cumulative dose should be strictly limited to 140 mg/m², or 2 to 3 years of therapy. In some embodiments, supplementation with the PD-1 or PD-L1 binding agent preserves patient's access to mitoxantrone by allowing for lower or less frequent dosing with this DMT.

In some embodiments, the patient is a naive patient, who has not received treatment with one or more DMTs, and the PD-1 or PD-L1 binding agent is used to buffer the side effects of a second-line therapy. Accordingly, the naive patient is able to benefit from the long-term benefits of a second-line therapy at disease outset. In some embodiments, the PD-1 or PD-L1 binding agent is used as an entry therapy that precedes the use of a second-line therapy. For example, the PD-1 or PD-L1 binding agent may be administered for an initial treatment period of about 3 months to stabilize disease and then the patient may be transitioned to a maintenance therapy of a second line agent.

It is generally believed that naive patients are more likely to respond to therapy as compared to patients that have received, and perhaps failed one or more DMT. In some embodiments, the PD-1 or PD-L1 binding agent finds use in patients that have received, and perhaps failed one or more DMT. For example, in some embodiments, the PD-1 or PD-L1 binding agent increases the therapeutic effect in patients that have received, and perhaps failed one or more DMT and may allow these patients to respond like naive patients.

In some embodiments, the patient has received or is receiving treatment with one or more DMTs and is not responding well. For example, the patient may be refractory or poorly responsive to one or more DMTs. In some embodiments, the patient is refractory, or poorly responsive to one or more of teriflunomide (AUBAGIO (GENZYME)); interferon beta-1a (AVONEX (BIOGEN IDEC)); interferon beta-1b (BETASERON (BAYER HEALTHCARE PHARMACEUTICALS, INC.)); glatiramer acetate (COPAXONE (TEVA NEUROSCIENCE)); interferon beta-1b (EXTAVIA (NOVARTIS PHARMACEUTICALS CORP.)); fingolimod (GILENYA (NOVARTIS PHARMACEUTICALS CORP.)); alemtuzumab (LEMTRADA (GENZYME)); mitoxantrone (NOVANTRONE (EMD SERONO)); pegylated interferon beta-1a (PLEGRIDY (BIOGEN IDEC)); interferon beta-1a (REBIF (EMD SERONO, INC.)); dimethyl fumarate (BG-12) (TECFIDERA (BIOGEN IDEC)); and natalizumab (TYSABRI (BIOGEN IDEC)). In some embodiments, the one or more disclosed binding agent results in a therapeutic benefit of one or more DMTs in the patient and therefore reduces or eliminates the non-responsiveness to the DMT. For instance, this may spare the patient therapy with one or more DMTs at a higher dosing or frequency.

In patients with more aggressive disease, one approach is an induction treatment model, where a therapy with strong efficacy but strong safety concerns would be given first, followed by a maintenance therapy. An example of such a model might include initial treatment with alemtuzumab, followed by IFN- β , GA, or BG-12. In some embodiments, the one or more disclosed binding agent is used to prevent the need to switch therapies for maintenance. In some embodiments, the one or more disclosed binding agent is used to as maintenance therapy to one or more DMTs, including second line therapies. In some embodiments, the one or more disclosed binding agent is used to as first therapy in an induction, followed by another DMT as a maintenance therapy- such as, for example, a first line therapy.

In some embodiments, the one or more disclosed binding agent may be administered for an initial treatment period of about 3 months to stabilize disease and then the patient may be transitioned to a maintenance therapy of a first line agent.

In various embodiments, the one or more disclosed binding agent is used to reduce one or more side effects of a DMT, including without limitation any agent disclosed herein. For example, the one or more disclosed binding agent may be used in a regimen that allows dose sparing for one or more DMTs and therefore results in fewer side effects. For example, in some embodiments, the one or more disclosed binding agent may reduce one or more side effects of AUBAGIO or related agents, which may include hair thinning, diarrhea, flu, nausea, abnormal liver tests and unusual numbness or tingling in the hands or feet (paresthesias), levels of white blood cells, which can increase the risk of infections; increase in blood pressure; and severe liver damage. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of AVONEX or related agents which include flu-like symptoms following injection, depression, mild anemia, liver abnormalities, allergic reactions, and heart problems. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of BETASERON or related agents which include flu-like symptoms following injection, injection site reactions, allergic reactions, depression, liver abnormalities, and low white blood cell counts. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of COPAXONE or

related agents which include injection site reactions, vasodilation (dilation of blood vessels); chest pain; a reaction immediately after injection, which includes anxiety, chest pain, palpitations, shortness of breath, and flushing. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of EXTAVIA or related agents which include flu-like symptoms following injection, injection site reactions, allergic reactions, depression, liver abnormalities, and low white blood cell counts. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of GILENYA or related agents which include headache, flu, diarrhea, back pain, liver enzyme elevations, cough, slowed heart rate following first dose, infections, and swelling in the eye. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of LEMTRADA or related agents which include rash, headache, fever, nasal congestion, nausea, urinary tract infection, fatigue, insomnia, upper respiratory tract infection, hives, itching, thyroid gland disorders, fungal infection, pain in joints, extremities and back, diarrhea, vomiting, flushing, and infusion reactions (including nausea, hives, itching, insomnia, chills, flushing, fatigue, shortness of breath, changes in the sense of taste, indigestion, dizziness, pain). In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of NOVANTRONE or related agents which include blue-green urine 24 hours after administration; infections, bone marrow suppression (fatigue, bruising, low blood cell counts), nausea, hair thinning, bladder infections, mouth sores, and serious liver and heart damage. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of PLEGRIDY or related agents which include flu-like symptoms following injection, injection site reactions, depression, mild anemia, liver abnormalities, allergic reactions, and heart problems. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of REBIF or related agents which include flu-like symptoms following injection, injection site reactions, liver abnormalities, depression, allergic reactions, and low red or white blood cell counts. In some embodiments, one or more disclosed binding agent may reduce one or more side effects of TECFIDERA or related agents which include flushing (sensation of heat or itching and a blush on the skin), gastrointestinal issues (nausea, diarrhea, abdominal pain), rash, protein in the urine, elevated liver enzymes; and reduction in blood lymphocyte (white blood cell) counts. In some embodiments, the one or more disclosed binding agent may reduce one or more side effects of TYSABRI or related agents which include headache, fatigue, urinary tract infections, depression, respiratory tract infections, joint pain, upset stomach, abdominal discomfort, diarrhea, vaginitis, pain in the arms or legs, rash, allergic or hypersensitivity reactions within two hours of infusion (dizziness, fever, rash, itching, nausea, flushing, low blood pressure, difficulty breathing, chest pain).

In some embodiments, the present invention relates to combination therapy with one or more chimeric agents described in WO 2013/10779, WO 2015/007536, WO 2015/007520, WO 2015/007542, and WO 2015/007903, the entire contents of which are hereby incorporated by reference in their entireties.

In some embodiments, inclusive of, without limitation, infectious disease applications, the present invention pertains to anti-infectives as additional therapeutic agents. In some embodiments, the anti-infective is an anti-viral agent including, but not limited to, Abacavir, Acyclovir, Adefovir, Amprenavir, Atazanavir, Cidofovir, Darunavir, Delavirdine, Didanosine, Docosanol, Efavirenz, Elvitegravir, Emtricitabine, Enfuvirtide, Etravirine, Famciclovir, and

Foscarnet. In some embodiments, the anti-infective is an anti-bacterial agent including, but not limited to, cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); monobactam antibiotics (aztreonam); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem). In some embodiments, the anti-infectives include anti-malarial agents (e.g., chloroquine, quinine, mefloquine, primaquine, doxycycline, artemether/lumefantrine, atovaquone/proguanil and sulfadoxine/pyrimethamine), metronidazole, tinidazole, ivermectin, pyrantel pamoate, and albendazole.

In some embodiments, inclusive, without limitation, of autoimmune applications, the additional therapeutic agent is an immunosuppressive agent. In some embodiments, the immunosuppressive agent is an anti-inflammatory agent such as a steroidal anti-inflammatory agent or a non-steroidal anti-inflammatory agent (NSAID). Steroids, particularly the adrenal corticosteroids and their synthetic analogues, are well known in the art. Examples of corticosteroids useful in the present invention include, without limitation, hydroxyltriamcinolone, alpha-methyl dexamethasone, beta-methyl betamethasone, beclomethasone dipropionate, betamethasone benzoate, betamethasone dipropionate, betamethasone valerate, clobetasol valerate, desonide, desoxymethasone, dexamethasone, diflorasone diacetate, diflucortolone valerate, fluadrenolone, flucorolone acetonide, flumethasone pivalate, fluosinolone acetonide, fluocinonide, flucortine butylester, fluocortolone, fluprednidene (fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, clocortelone, clescinolone, dichlorisone, difluprednate, flucoronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate. (NSAIDS) that may be used in the present invention, include but are not limited to, salicylic acid, acetyl salicylic acid, methyl salicylate, glycol salicylate, salicylmides, benzyl-2,5-diacetoxybenzoic acid, ibuprofen, fulindac, naproxen, ketoprofen, etofenamate, phenylbutazone, and indomethacin. In some embodiments, the immunosuppressive agent may be cytostatics such as alkylating agents, antimetabolites (e.g., azathioprine, methotrexate), cytotoxic antibiotics, antibodies (e.g., basiliximab, daclizumab, and muromonab), anti-immunophilins (e.g., cyclosporine, tacrolimus, sirolimus), inteferons, opioids, TNF binding proteins, mycophenolates, and small biological agents (e.g., fingolimod, myriocin). Additional anti-inflammatory agents are described, for example, in U.S. Patent No. 4,537,776, the entire contents of which is incorporated by reference herein.

In some embodiments, the PD-1 or PD-L1 binding agent described herein, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the composition such that covalent attachment does not prevent the activity of the composition. For example, but not by way of limitation, derivatives include composition that have been modified by, *inter alia*, glycosylation, lipidation, acetylation, pegylation, phosphorylation, amidation,

derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, *etc.*

In still other embodiments, the PD-1 or PD-L1 binding agent described herein further comprise a cytotoxic agent, comprising, in illustrative embodiments, a toxin, a chemotherapeutic agent, a radioisotope, and an agent that causes apoptosis or cell death. Such agents may be conjugated to a composition described herein.

The PD-1 or PD-L1 binding agent described herein may thus be modified post-translationally to add effector moieties such as chemical linkers, detectable moieties such as for example fluorescent dyes, enzymes, substrates, bioluminescent materials, radioactive materials, and chemiluminescent moieties, or functional moieties such as for example streptavidin, avidin, biotin, a cytotoxin, a cytotoxic agent, and radioactive materials.

Illustrative cytotoxic agents include, but are not limited to, methotrexate, aminopterin, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine; alkylating agents such as mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU), mitomycin C, lomustine (CCNU), 1-methylnitrosourea, cyclophosphamide, mechlorethamine, busulfan, dibromomannitol, streptozotocin, mitomycin C, cis-dichlorodiamine platinum (II) (DDP) cisplatin and carboplatin (paraplatin); anthracyclines include daunorubicin (formerly daunomycin), doxorubicin (adriamycin), detorubicin, carminomycin, idarubicin, epirubicin, mitoxantrone and bisantrene; antibiotics include dactinomycin (actinomycin D), bleomycin, calicheamicin, mithramycin, and anthramycin (AMC); and antimetabolic agents such as the vinca alkaloids, vincristine and vinblastine. Other cytotoxic agents include paclitaxel (taxol), ricin, pseudomonas exotoxin, gemcitabine, cytochalasin B, gramicidin D, ethidium bromide, emetine, etoposide, teniposide, colchicin, dihydroxy anthracin dione, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, procarbazine, hydroxyurea, asparaginase, corticosteroids, mytotane (O,P'-(DDD)), interferons, and mixtures of these cytotoxic agents.

Further cytotoxic agents include, but are not limited to, chemotherapeutic agents such as carboplatin, cisplatin, paclitaxel, gemcitabine, calicheamicin, doxorubicin, 5-fluorouracil, mitomycin C, actinomycin D, cyclophosphamide, vincristine, bleomycin, VEGF antagonists, EGFR antagonists, platins, taxols, irinotecan, 5-fluorouracil, gemcitabine, leucovorine, steroids, cyclophosphamide, melphalan, vinca alkaloids (*e.g.*, vinblastine, vincristine, vindesine and vinorelbine), mustines, tyrosine kinase inhibitors, radiotherapy, sex hormone antagonists, selective androgen receptor modulators, selective estrogen receptor modulators, PDGF antagonists, TNF antagonists, IL-1 antagonists, interleukins (*e.g.* IL-12 or IL-2), IL-12R antagonists, Toxin conjugated monoclonal antibodies, tumor antigen specific monoclonal antibodies, Erbitux, Avastin, Pertuzumab, anti-CD20 antibodies, Rituxan, ocrelizumab, ofatumumab, DXL625, HERCEPTIN®, or any combination thereof. Toxic enzymes from plants and bacteria such as ricin, diphtheria toxin and Pseudomonas toxin may be conjugated to the therapeutic agents (*e.g.* antibodies) to generate cell-type-specific-killing reagents (Youle, *et al.*, Proc. Nat'l Acad. Sci. USA 77:5483 (1980); Gilliland, *et al.*, Proc. Nat'l Acad. Sci. USA 77:4539 (1980); Krolick, *et al.*, Proc. Nat'l Acad. Sci. USA 77:5419 (1980)).

Other cytotoxic agents include cytotoxic ribonucleases as described by Goldenberg in U.S. Pat. No. 6,653,104. Embodiments of the invention also relate to radioimmunoconjugates where a radionuclide that emits alpha or beta particles is stably coupled to the PD-1 or PD-L1 binding agent, with or without the use of a complex-forming agent. Such radionuclides include beta-emitters such as Phosphorus-32, Scandium-47, Copper-67, Gallium-67, Yttrium-88, Yttrium-90, Iodine-125, Iodine-131, Samarium-153, Lutetium-177, Rhenium-186 or Rhenium-188, and alpha-emitters such as Astatine-211, Lead-212, Bismuth-212, Bismuth-213 or Actinium-225.

Illustrative detectable moieties further include, but are not limited to, horseradish peroxidase, acetylcholinesterase, alkaline phosphatase, beta-galactosidase and luciferase. Further illustrative fluorescent materials include, but are not limited to, rhodamine, fluorescein, fluorescein isothiocyanate, umbelliferone, dichlorotriazinylamine, phycoerythrin and dansyl chloride. Further illustrative chemiluminescent moieties include, but are not limited to, luminol. Further illustrative bioluminescent materials include, but are not limited to, luciferin and aequorin. Further illustrative radioactive materials include, but are not limited to, Iodine-125, Carbon-14, Sulfur-35, Tritium and Phosphorus-32.

Methods of Treatment

Methods and compositions described herein have application to treating various diseases and disorders, including, but not limited to cancer, infections, immune disorders, and inflammatory diseases or conditions.

Further, any of the present agents may be for use in the treating, or the manufacture of a medicament for treating, various diseases and disorders, including, but not limited to cancer, infections, immune disorders, inflammatory diseases or conditions, and autoimmune diseases.

In some embodiments, the present invention relates to the treatment of, or a patient having cancer. As used herein, cancer refers to any uncontrolled growth of cells that may interfere with the normal functioning of the bodily organs and systems, and includes both primary and metastatic tumors. Primary tumors or cancers that migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. A metastasis is a cancer cell or group of cancer cells, distinct from the primary tumor location, resulting from the dissemination of cancer cells from the primary tumor to other parts of the body. Metastases may eventually result in death of a subject. For example, cancers can include benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases.

Illustrative cancers that may be treated include, but are not limited to, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer;

retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); acute myeloid leukemia (AML); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (e.g. that associated with brain tumors), and Meigs' syndrome.

In various embodiments, the present invention provides PD-1 or PD-L1 binding agents which are part of a chimera that further comprises modified signaling agents for the treatment of cancer. In some embodiments, the PD-1 or PD-L1 binding agents of the invention significantly reduce and/or eliminate tumors. In some embodiments, the present PD-1 or PD-L1 binding agents significantly reduce and/or eliminate tumors when administered to a subject in combination with other anti-cancer agents such as chemotherapeutic agents, checkpoint inhibitors, and immunosuppressive agents. In various embodiments, the combination of PD-1 or PD-L1 binding agents and other anti-cancer agents synergistically reduced tumor size and/or eliminated tumor cells.

In various embodiments, the present invention relates to cancer combination therapies with a PD-1 or PD-L1 binding agent that is part of a chimera comprising one or more targeting moieties and one or more modified signaling agents. Accordingly, the present invention provides for chimeric or fusion proteins that include, for example, a targeting moiety against PD-1 or PD-L1 and one or more signaling agents and uses thereof in combination with anti-cancer agents.

For instance, in various embodiments, the present invention pertains to combination therapies for cancer involving chimeras of a PD-1 or PD-L1 binding agent described herein and a modified signaling agent, including, without limitation a mutated human interferon, such as IFN alpha, including human interferon alpha 2.

In some embodiments, the present invention relates to the treatment of, or a patient having a microbial infection and/or chronic infection. Illustrative infections include, but are not limited to, HIV/AIDS, tuberculosis, osteomyelitis, hepatitis B, hepatitis C, Epstein-Barr virus or parvovirus, T cell leukemia virus, bacterial overgrowth syndrome, fungal or parasitic infections.

In various embodiments, the present compositions are used to treat or prevent one or more inflammatory diseases or conditions, such as inflammation, acute inflammation, chronic inflammation, respiratory disease, atherosclerosis, restenosis, asthma, allergic rhinitis, atopic dermatitis, septic shock, rheumatoid arthritis, inflammatory bowel disease, inflammatory pelvic disease, pain, ocular inflammatory disease, celiac disease, Leigh

Syndrome, Glycerol Kinase Deficiency, Familial eosinophilia (FE), autosomal recessive spastic ataxia, laryngeal inflammatory disease; Tuberculosis, Chronic cholecystitis, Bronchiectasis, Silicosis and other pneumoconioses.

In various embodiments, the present compositions are used to treat or prevent one or more autoimmune and/or neurodegenerative diseases or conditions, such as MS, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (e.g., prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, myasthenia gravis, Reiter's syndrome, Grave's disease, and other autoimmune diseases.

In various embodiments, the present invention is used to treat or prevent various autoimmune and/or neurodegenerative diseases. In some embodiments, the autoimmune and/or neurodegenerative diseases selected from MS (including without limitation the subtypes described herein), Alzheimer's disease (including, without limitation, Early-onset Alzheimer's, Late-onset Alzheimer's, and Familial Alzheimer's disease (FAD), Parkinson's disease and parkinsonism (including, without limitation, Idiopathic Parkinson's disease, Vascular parkinsonism, Drug-induced parkinsonism, Dementia with Lewy bodies, Inherited Parkinson's, Juvenile Parkinson's), Huntington's disease, Amyotrophic lateral sclerosis (ALS, including, without limitation, Sporadic ALS, Familial ALS, Western Pacific ALS, Juvenile ALS, Hiramaya Disease).

Kits

The present invention also provides kits for the administration of any PD-1 or PD-L1 binding agent described herein (e.g. with or without additional therapeutic agents). The kit is an assemblage of materials or components, including at least one of the inventive pharmaceutical compositions described herein. Thus, in some embodiments, the kit contains at least one of the pharmaceutical compositions described herein.

The exact nature of the components configured in the kit depends on its intended purpose. In one embodiment, the kit is configured for the purpose of treating human subjects.

Instructions for use may be included in the kit. Instructions for use typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired therapeutic outcome, such as to treat cancer. Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, bandaging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

The materials and components assembled in the kit can be provided to the practitioner stored in any convenience and suitable ways that preserve their operability and utility. For example, the components can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging materials. In various embodiments, the packaging material is constructed by well-known methods, preferably to provide a sterile,

contaminant-free environment. The packaging material may have an external label which indicates the contents and/or purpose of the kit and/or its components.

Definitions

As used herein, “a,” “an,” or “the” can mean one or more than one.

Further, the term “about” when used in connection with a referenced numeric indication means the referenced numeric indication plus or minus up to 10% of that referenced numeric indication. For example, the language “about 50” covers the range of 45 to 55.

An “effective amount,” when used in connection with medical uses is an amount that is effective for providing a measurable treatment, prevention, or reduction in the rate of pathogenesis of a disease of interest.

As used herein, something is “decreased” if a read-out of activity and/or effect is reduced by a significant amount, such as by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100%, in the presence of an agent or stimulus relative to the absence of such modulation. As will be understood by one of ordinary skill in the art, in some embodiments, activity is decreased and some downstream read-outs will decrease but others can increase.

Conversely, activity is “increased” if a read-out of activity and/or effect is increased by a significant amount, for example by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100% or more, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 50-fold, at least about 100-fold, in the presence of an agent or stimulus, relative to the absence of such agent or stimulus.

As referred to herein, all compositional percentages are by weight of the total composition, unless otherwise specified. As used herein, the word “include,” and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the compositions and methods of this technology. Similarly, the terms “can” and “may” and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present technology that do not contain those elements or features.

Although the open-ended term “comprising,” as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as “consisting of” or “consisting essentially of.”

As used herein, the words “preferred” and “preferably” refer to embodiments of the technology that afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or

other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the technology.

The amount of compositions described herein needed for achieving a therapeutic effect may be determined empirically in accordance with conventional procedures for the particular purpose. Generally, for administering therapeutic agents for therapeutic purposes, the therapeutic agents are given at a pharmacologically effective dose. A “pharmacologically effective amount,” “pharmacologically effective dose,” “therapeutically effective amount,” or “effective amount” refers to an amount sufficient to produce the desired physiological effect or amount capable of achieving the desired result, particularly for treating the disorder or disease. An effective amount as used herein would include an amount sufficient to, for example, delay the development of a symptom of the disorder or disease, alter the course of a symptom of the disorder or disease (*e.g.*, slow the progression of a symptom of the disease), reduce or eliminate one or more symptoms or manifestations of the disorder or disease, and reverse a symptom of a disorder or disease. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to about 50% of the population) and the ED50 (the dose therapeutically effective in about 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. In some embodiments, compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from *in vitro* assays, including, for example, cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 as determined in cell culture, or in an appropriate animal model. Levels of the described compositions in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

In certain embodiments, the effect will result in a quantifiable change of at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 70%, or at least about 90%. In some embodiments, the effect will result in a quantifiable change of about 10%, about 20%, about 30%, about 50%, about 70%, or even about 90% or more. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

As used herein, “methods of treatment” are equally applicable to use of a composition for treating the diseases or disorders described herein and/or compositions for use and/or uses in the manufacture of a medicaments for treating the diseases or disorders described herein.

EXAMPLES

The term “AcTaferon (AFN)” is occasionally used herein to reference an interferon-based chimera.

In the following examples, unless noted, mutations to IFN are relative to human IFN- α 2.

Example 1. Efficiency of Human PD-1 or PD-L1 Targeting of Mono-Specific Human Chimeras

Efficiency of targeting by human AcTaferon alpha comprising PD-1 or PD-L1 antibodies was examined by FACS-based quantification of STAT1 phosphorylation. Specifically, targeting was analyzed in primary peripheral blood mononuclear cells (PBMCs), transfected Hek293T cells (for PD-1 targeting), or the human breast-cancer cell-line MDA-MB-321 (for PD-L1 targeting). The following antibodies were used:

Anti-PD-1 Antibodies:

- Pem: Pembrolizumab/Keytruda (Merck)
- Niv: Nivolumab/Opdivo (BMS)

Anti-PD-L1 Antibodies:

- Ate: Atezolizumab/Tecentriq/MPDL3280 (Roche/Genetech)
- Dur: Durvalumab/MEDI4736 (Celgene/AstraZeneca)
- Ave: Avelumab/MSB0010718C (Merck/Pfizer)
- Bms: BMS-936559/MDX-1105 (BMS)

The sequences of these antibodies are described elsewhere herein. With respect to the anti-PD-L1 BMS-936559/MDX-1105 antibody particularly, the variable domains of this antibody was grafted on an univesal human IgG. Accordingly, BMS-936559 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 1242; and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO: 1243.

Following enfragment of the variable domains onto human IgG, the following heavy and light chain sequences are obtained:

Heavy: SEQ ID NO: 1244

Light: SEQ ID NO: 1245

The various antibody heavy chains were genetically fused to hIFNa2_R149 sequence (e.g., human IFN- α 2 having a R149A mutation) via a flexible 20*GGs linker in the pMTW expression-vector (resulting vector: pMTW-SlgK-heavy chain-(GGs)₂₀-hIFNa2_R149A-GGS-(His)₆). The light chains were cloned in the same vector (resulting vector: pMTW-SlgK-light chain). A schematic of the cloning strategy is provided in Figure 1. PD-L1 AFN's were produced by transient transfection of both plasmids in FreeStyle 293-F cells (ThermoFisher) with 25K PEI (polyethylenimine) according to standard protocols. Medium was harvested, cells removed by centrifugation and filter-sterilised. Recombinant proteins were purified using Ni Excel resin (GE Healthcare) according to the manufacturers instructions and imidazole removed from the samples with PD-10 columns (GE Healthcare).

Binding of PD-L1 Ab-AFN's to transfected Hek293T cells

Hek293T cells were transfected with the membrane-bound PD-1 or PD-L1 using calcium phosphate. Two days after transfection, cells were resuspended and incubated with PD-L1 Ab-AFN's (1 µg/ml in FACS buffer: PBS supplemented with 2% FBS; 0,5 mM EDTA) for two hours at 4°C. After two washes, cells were incubated with FITC-coupled THE HIS antibody (GenScript; 1 hr at 4°C). Binding was measured with a FACSCalibur instrument (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences). Data in Figure 2 clearly illustrate that PD-L1 AFN's selectively bound to PD-L1 transfected cells, while PD-1 AFN's only bound to PD-1 expressing cells.

PD-1-targeting: pSTAT1 in Peripheral blood mononuclear cells (PBMC's)

PBMCs from buffy coats of healthy donors were isolated using density gradient centrifugation with Lymphoprep (StemCell Technologies). Cells were washed twice with FACS buffer (2% FBS, 0,5 mM EDTA in PBS) and stained with FITC coupled anti-human PD-1 (clone PD-1.3.1.3; Miltenyi Biotec) for 20 minutes at 4°C. After two washes, cells were stimulated with a serial dilution PD-1 Ab-AFN's for 15 minutes at 37°C. After fixation (10 minutes, 37°C, Fix Buffer I; BD Biosciences) and permeabilisation (30 minutes, on ice, Perm III Buffer I; BD Biosciences) and washing, cells were stained with anti-STAT1 pY701 Ab (BD Biosciences). Samples were acquired with a FACSCalibur (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences). Data in Figure 3 show that Pembrolizumab and Nivolumab coupled AcTaferon's were able to phosphorylate STAT1 in PD-1 positive, but not PD-1 negative PBMC's.

PD-1-targeting: pSTAT1 in transfected Hek293T cells

Hek293T cells were transfected with human PD-1 or an empty vector using calcium phosphate. After two days, cells were resuspended and stimulated with a serial dilution PD-1 AFN's (15 min; 37°C). pSTAT1 was quantified as described above. Data in Figure 4 illustrate that PD-1 Ab AFN's efficiently induce STAT1 phosphorylation in PD-1 expressing Hek293T cells, but not in MOCK transfected cells.

PD-L1-targeting: pSTAT1 in MDA-MB-231 cells

MDA-MB-321 cells were stimulated with PD-(L)1 AFN's for 15 minutes at 37°C in DMEM medium supplemented with 10% FBS. After stimulation, pSTAT1 was quantified as described previously. PD-L1, but not PD-1, coupled AFN's were able to efficiently phosphorylate STAT1, thereby illustrating the PD-L1 targeting effect (Figure 5).

Example 2. Construction and Evaluation of VHHs Specific for Human PD-1 or PD-L1

ELISA assays were performed to evaluate the binding affinity of the various PD-1 VHHs as described elsewhere herein for the PD-1 antigen. The affinities of the PD-1 VHHs are presented in Table 1 below (see column A/control):

Table 1

Clone	Collection nr.	Family	ELISA A	ELISA control	A / control
2PD23	6418	1	0.9399	0.2061	4.56041

2PD26	6419	1	0.7177	0.1708	4.20199
2PD90	6423	1	1.3425	0.2566	5.23188
2PD106	6424	1	1.462	0.2118	6.90274
2PD16	6417	2	1.8255	0.475	8.84316
2PD71	6421	2	2.5085	0.6984	8.59178
2PD152	6425	2	2.3148	0.3628	6.38037
2PD12	6416	3	3.8075	0.1775	21.4507
3PD55	6427	3	3.578	0.0886	40.3837
3PD82	6428	3	4	0.0886	45.1467
2PD8	6415	4	0.7629	0.2521	3.03618
2PD27	6420	4	1.1358	0.329	3.45228
2PD82	6422	5	2.9666	0.6775	4.37875
3PD36	6426	6	3.9512	0.0957	41.2874

The binding affinities of the various PD-L1 VHHs as described herein for the PD-L1 antigen were also assessed by ELISA. The affinities of the PD-1 VHHs are presented in Table 2 below (see column A/control):

Table 2

Clone	Collection nr.	Family	ELISA A	ELISA control	A / control
2LIG2	6283	5	0.5099	0.1322	3.85703
2LIG3	6284	1	1.4632	0.15	9.75467
2LIG16	6285	3	3.0203	0.1144	26.4012
2LIG22	6286	6	0.4687	0.1031	4.54607
2LIG27	6287	1	0.4289	0.1134	3.78219
2LIG29	6288	2	3.772	0.109	34.6055
2LIG30	6289	7	1.1105	0.1158	9.58981
2LIG34	6290	1	0.5681	0.1581	3.5933
2LIG35	6291	2	0.8438	0.1031	8.18429
2LIG48	6292	3	4	0.1204	33.2226
2LIG65	6293	4	2.108	0.0892	23.6323
2LIG85	6294	10	2.6661	0.1182	22.5556
2LIG86	6295	9	3.803	0.1133	33.5658
2LIG89	6296	4	2.8804	0.106	27.1736
2LIG97	6297	1	1.0302	0.201	5.12537
2LIG99	6298	1	2.1105	0.2304	9.16016
2LIG109	6299	2	3.5455	0.1088	32.5673
2LIG127	6300	4	3.7303	0.1079	34.5718
2LIG139	6301	4	4	0.1202	33.2779
2LIG176	6302	11	0.4153	0.0983	4.22482
2LIG189	6303	7	2.2343	0.1726	12.945
3LIG3	6304	5	0.5797	0.081	7.15679
3LIG7	6305	2	3.6414	0.0968	37.6178
3LIG8	6306	1	0.4292	0.0978	4.38855
3LIG9	6307	2	3.6634	0.0972	37.6893

3LIG18	6308	4	3.505	0.0877	39.9658
3LIG20	6309	8	0.5131	0.1179	4.35199
3LIG28	6310	6	3.0276	0.0913	33.161
3LIG29	6311	2	3.9596	0.0967	40.9473
3LIG30	6312	3	4	0.0845	47.3373
3LIG33	6313	5	1.7581	0.0921	19.089

The binding of the PD-L1 VHHs to transfected Hek293T cells was also examined. Specifically, human PD-L1 transfected Hek293T cells were stained with PD-L1 VHH's (2 µg/ml; 2 hours at 4°C) and a FITC-coupled anti-His antibody (GenScript; 1 hour at 4°C). Binding was measured with a FACSCalibur instrument (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences). Results of the binding assays are shown in Figure 6.

Example 3. Characterization of Human PD-L1 VHH AcTaferons

After an initial screen of anti-human PD-L1 VHHs for their capacity to inhibit the interaction with PD-1, the following PD-L1 VHHs were selected for further characterization: 2LIG3 (SEQ ID NO: 287), 2LIG27 (SEQ ID NO: 290), 2LIG 97 (SEQ ID NO: 300), 2LIG99 (SEQ ID NO: 301), 3LIG8 (SEQ ID NO: 309), 2LIG189 (SEQ ID NO: 306), and 2LIG176 (SEQ ID NO: 305).

PD-L1 VHHs 2LIG3, 2LIG27, 2LIG97, 2LIG99, 3LIG8, 2LIG189, and 2LIG176 were cloned in the pHEN6C expression vector as follows: pHEN6C-PeIB-PD-L1 VHH-(His)₆. Proteins were produced overnight in *E. coli* upon IPTG stimulation and purified from periplasmic extracts using the TALON metal affinity resin (Clontech) according to the manufacturer's instructions.

The effect of the PD-L1 VHH's on the interaction between PD-1 and PD-L1 was tested in a plate binding assay. The recombinant extracellular domains of PD-L1 were immobilized overnight on MaxiSorp plates (Nunc) using its C-terminal FLAG-tag and the M2 anti-FLAG Ab (Sigma). Plates were block (PBS + 0,1% Casein) and incubated with hPD-1-hFc fusion-protein (SinoBiologicals) in the presence or absence of a serial dilution VHH's. After washing, bound PD-1 was measured using a HRP (horseradish peroxidase) coupled anti-human Ab (Jackson ImmunoResearch) and the TMB peroxidase substrate (KPL).

Affinities of the PD-L1 VHH's for human and cynomolgus PD-L1 were measured using bio-layer interferometry an Octet RED96 system (FortéBio): VHH's were immobilized on Penta-HIS (HIS1K) biosensors (FortéBio) and dipped in a serial dilution human PD-L1-Fc or cynomolgus PD-L1-Fc proteins (both from SinoBiological). Affinities were calculated with the Octet software (FortéBio).

Table 3 shows that all PD-L1 VHHs tested were able to potently inhibit the PD-L1/PD-1 interaction. In addition, all PD-L1 VHHs showed a similar binding affinity for human and cynomolgus PD-L1.

Table 3

Clone	IC ₅₀ neutralisation PD1-PDL1 (ng/ml)	Human PD-L1 affinity			Cyno PD-L1 affinity			EC50 luciferase in HL116 cells (ng/ml)
		KD (M)	k _{on} (1/Ms)	k _{dis} (1/s)	KD (M)	k _{on} (1/Ms)	k _{dis} (1/s)	
2LIG3	18.87	1.19E-09	3.73E+05	4.48E-04	8.90E-10	3.58E+05	3.18E-04	0.0220
2LIG27	9.57	7.51E-09	4.72E+05	3.50E-03	8.74E-09	3.21E+05	2.80E-03	1.0380
2LIG97	4.81	4.35E-09	4.23E+05	1.98E-03	9.16E-09	3.19E+05	2.93E-03	1.1100
2LIG99	6.21	8.75E-10	7.29E+05	6.34E-04	1.01E-09	5.84E+05	5.85E-04	0.0062
3LIG8	12.79	8.77E-09	4.84E+05	4.19E-03	5.98E-09	5.12E+05	3.05E-03	0.2400
2LIG189	8.81	3.03E-09	1.74E+05	5.24E-04	3.80E-09	1.14E+05	4.37E-04	0.4900
2LIG176	5.68	6.87E-09	1.18E+06	8.05E-03	1.13E-08	5.08E+05	5.73E-03	0.2300

The PD-L1 VHH's (i.e., 2LIG3, 2LIG27, 2LIG97, 2LIG99, 3LIG8, 2LIG189, and 2LIG176) were then cloned into an Actaferon (AFN) format in the pHEN6C expression vector (i.e., pHEN6C-PeIB-PD-L1_VHH-(GGG)₂₀-hIFNa2_R149A-GGS-(His)₆, wherein the PD-L1_VHH is selected from 2LIG3, 2LIG27, 2LIG97, 2LIG99, 3LIG8, 2LIG189, and 2LIG176). Proteins were produced overnight in *E. coli* upon IPTG stimulation and purified from periplasmic extracts using the TALON metal affinity resin (Clontech) according to the manufacturer's instructions.

Biological activity was measured on parental HL116 cells (an IFN responsive cell-line stably transfected with a p6-16 luciferase reporter). Cells were seeded overnight and stimulated for 6 hours with a serial dilution PD-L1 AFN's in the presence or absence of an excess (20 µg/ml) of the corresponding VHH (this latter to mimic the untargeted situation). Luciferase activity was measured on an EnSight Multimode Plate Reader (Perkin Elmer).

As shown in Figures 7A-G, all the PD-L1 VHHs formatted as an AcTaferon were able to induce the IFN luciferase reporter in HL116 cells. The specificity of the AcTaferon activity was assessed by comparing the luciferase reporter activity in the presence of an excess of each respective PD-L1 VHH not linked to an IFN moiety (see Figures 7A-G).

Example 4. *In Vitro* efficacy of human PD-L1 Antibody AcTaferons

The monoclonal antibody atezolizumab (Ate), a PD-L1 targeted monoclonal antibody, was selected as an example to evaluate the specificity of the interferon activity to targeted cells.

Atezolizumab was obtained from InVivogen. Atezolizumab_hIFNa2(R149A) (Ate-IFNa2mut) was generated as follows: the Ab heavy chain was genetically fused to hIFNa2_R149 sequence via a flexible 20*GGG linker in the pMTW expression-vector (resulting vector: pMTW-SIgK-heavy chain-(GGG)₂₀-hIFNa2_R149A-GGS-(His)₉). The light chains were cloned in the same vector (resulting vector: pMTW-SIgK-light chain). Both plasmids were co-transfected in ExpiCHO cells (ThermoFisher) according to the manufacturers guidelines. The resulting Ate-IFNa2mut was purified from the medium using the Ni Sepharose excel resin (GE Healthcare). The effect of the Ate-IFNa2mut was demonstrated *in vitro* using the same assay as described in previous Example 3 using an excess of atezolizumab (Ate) (50 µg/ml) to demonstrate specificity (see Figure 8).

Figure 8 shows that the Ate-IFNa2mut was able to induce the IFN luciferase reporter in HL116 cells. The specificity of the Ate-IFNa2mut activity was assessed by comparing the luciferase reporter activity in the presence of an excess of atezolizumab (Ate) not linked to an IFN moiety.

Example 5. *In Vivo* efficacy of human PD-L1 Antibody AcTaferons

The PD-L1 VHH 2LIG99 was selected to evaluate the antitumor efficacy of human PD-L1 VHH targeted AcTaferon (AFN) *in vivo*.

Human RL follicular lymphoma cell line (RL) tumor model in mice with humanized immune system: Mice with a humanized immune system were generated according to the following protocol. Mononuclear cells were collected following density gradient centrifugation using Lymphoprep from HLA-A2+ human cord blood samples. Human CD34+ hematopoietic stem cells (HSC) were subsequently isolated by MACS technology and examined for CD34+ purity and CD3+ contamination using FACS. HSC's with a CD34 purity of >80% were then intrahepatically injected in 2-3 day old NSG mice that underwent myeloablative irradiation treatment at 100 cGy. At 8-12 weeks post HSC injection, human cell engraftment was analyzed with panleukocyte human and mouse CD45 markers using FACS and mice with >5% human CD45 cells, of total viable blood lymphocytes, were selected for tumor implantation. Twelve weeks post HSC injection, mice were subcutaneously injected with 2×10^6 RL tumor cells. Five days later, mice were treated with Flt3L injected peritoneally on a daily basis until day 18. Treatment with PBS (control) or 2LIG99-based AFN (i.e., AFN comprising 2LIG99 linked to hIFNa2_R149A) was initiated by perilesional administration as of day 9 (when tumors had reached sizes of about 10 mm²) post tumor injection.

As shown in Figure 9, the 2LIG99-based AFN had anti-tumoral effects *in vivo*.

Example 6. *In Vitro* efficacy of human PD-1 AcTaferons

The efficiency of human PD-1 (programmed death-1) targeting of AcTaferons (AFN) was examined by quantification of STAT1 phosphorylation in PD-1 or empty vector transfected Hek293T cells in FACS.

Two different PD-1 VHH's were selected for analysis:

102C3:

QVQLQESGGGLVQAGKSLRLSCAASGSIFSIIHAMGWFRQAPGKEREFVAAITWSGGITYYEDSVKGRFTISRDN
AKNTVYLQMNSLKPEDTAIYYCAADRAESSWYDYWGQGTQVTVSS (SEQ ID NO: 1246); and

102C12:

QVQLQESGGGLVQAGKSLRLSCAASGSIIHAMGWFRQAPGKEREFVAVITWSGGITYYADSVKGRFTISRDN
AKNTVYLQMNSLKPEDTAIYYCAGDKHQSSWYDYWGQGTQVTVSS (SEQ ID NO: 1247).

These PD-1 VHH's were cloned into an AcTaferon (AFN): AFN's were cloned in the pHEN6C expression vector as follows: pHEN6C-PeIB-PD-1_VHH-(GGG)₂₀-hIFNa2_R149A-GGS-(His)₆ (wherein PD-1_VHH is 102C3 or 102C12; i.e., 102C3 AFN and 102C12 AFN). Proteins were produced overnight in *E. coli* upon IPTG stimulation

and purified from periplasmic extracts using the TALON metal affinity resin (Clontech) according to the manufacturer's instructions.

Hek293T cells were transfected with an empty vector or a human PD-1 expression plasmid using the standard calcium phosphate technique. Two days after transfection, cells were re-suspended, washed twice with FACS buffer (2% FBS, 1 mM EDTA in PBS) and stimulated with a serial dilution of 102C3 AFN or 102C12 AFN or wild type IFN α 2 (positive control) for 15 minutes at 37°C. After fixation (10 minutes, 37°C, Fix Buffer I; BD Biosciences) and permeabilization (30 minutes, on ice, Perm III Buffer; BD Biosciences) and washing, cells were stained with anti-STAT1 pY701 Ab (BD Biosciences). Samples were acquired on a FACSCalibur (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences) and analyzed using the FlowJo software (FlowJo).

The percentages of pSTAT1 positive cells were quantified in FACS and plotted in function of the concentration. Figures 10A-C show that 102C3 AFN and 102C12 AFN efficiently induce STAT1 phosphorylation in PD-1 expressing Hek293T cells, but to a much lesser extent in MOCK transfected cells. This in contrast to wild type IFN α 2, which is equally active on PD-1 and MOCK transfected HEK293T cells.

EQUIVALENTS

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

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

INCORPORATION BY REFERENCE

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

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

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

CLAIMS

What is claimed is:

1. A PD-L1 binding agent comprising an amino acid sequence having at least 90% identity with one of SEQ ID NOs: 301, 287, 290, 300, 305, 306, and 309.
2. A PD-L1 binding agent comprising at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:
 - (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 199, 181, 178, 183, 189, 196, 201, and 206;
 - (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 259, 260, 241, 242, 245, 246, 263, and 264; and
 - (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 268, 272, and 277.
3. The PD-L1 binding agent of claim 1 or 2, wherein the targeting moiety is a single-domain antibody.
4. The PD-L1 binding agent of claim 3, wherein the targeting moiety comprises a VHH, a humanized VHH, or a camelized VHH.
5. The PD-L1 binding agent of any one of the above claims, wherein the PD-L1 binding agent comprises one or more signaling agents.
6. The PD-L1 binding agent of claim 5, wherein the signaling agent is selected from one or more of an interferon, an interleukin, and a tumor necrosis factor, any of which are optionally modified.
7. The PD-L1 binding agent of any one of the above claims, wherein the PD-L1 binding agent comprises one or more additional targeting moieties.
8. The PD-L1 binding agent of claim 7, wherein the one or more additional targeting moieties recognize and optionally functionally modulate a tumor antigen.
9. The PD-L1 binding agent of claim 8, wherein the one or more additional targeting moieties recognize and optionally functionally modulate an antigen on an immune cell.
10. The PD-L1 binding agent of claim 9, wherein the immune cell is selected from a T cell, a B cell, a dendritic cell, a macrophage, neutrophil, and a NK cell.
11. The PD-L1 binding agent of any of the above claims, wherein the PD-L1 binding agent recruits cytotoxic T cells to tumor cells or to the tumor environment.
12. The PD-L1 binding agent of any of the above claims, wherein the PD-L1 binding agent recognizes and binds PD-L1 and substantially functionally modulates its activity or does not substantially functionally modulate its activity.

13. A recombinant nucleic acid composition encoding the PD-L1 binding agents of any one of the above claims.
14. A host cell comprising a nucleic acid of claim 13.
15. A PD-1 binding agent comprising at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:
 - (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2-23;
 - (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 24-54; and
 - (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 55-69.
16. A PD-L1 binding agent comprising at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:
 - (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 177-207;
 - (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 208-266; and
 - (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 267-285.
17. The PD-1 or PD-L1 binding agent of claim 15 or 16, wherein the targeting moiety is a single-domain antibody.
18. The PD-1 or PD-L1 binding agent of claim 17, wherein the targeting moiety comprises a V_HH, a humanized V_HH, or a camelized V_HH.
19. The PD-1 binding agent of claim 18, comprising an amino acid sequence having at least 90% identity with one of SEQ ID NOs: 70-83, 1246, or 1247.
20. The PD-L1 binding agent of claim 18, comprising an amino acid sequence having at least 90% identity with one of SEQ ID NOs: 286-316.
21. The PD-1 or PD-L1 binding agent of any one of claims 15-20, wherein the PD-1 or PD-L1 binding agent comprises one or more signaling agents.
22. The PD-1 or PD-L1 binding agent of claim 21, wherein the signaling agent is selected from one or more of an interferon, an interleukin, and a tumor necrosis factor, any of which are optionally modified.
23. The PD-1 or PD-L1 binding agent of any one of claims 15-22, wherein the PD-1 or PD-L1 binding agent comprises one or more additional targeting moieties.
24. The PD-1 or PD-L1 binding agent of claim 23, wherein the one or more additional targeting moieties recognize and optionally functionally modulate a tumor antigen.
25. The PD-1 or PD-L1 binding agent of claim 24, wherein the one or more additional targeting moieties recognize and optionally functionally modulate an antigen on an immune cell.

26. The PD-1 or PD-L1 binding agent of claim 25, wherein the immune cell is selected from a T cell, a B cell, a dendritic cell, a macrophage, neutrophil, and a NK cell.
27. The PD-1 or PD-L1 binding agent of any one of claims 15-26, wherein the PD-1 or PD-L1 binding agent recruits cytotoxic T cells to tumor cells or to the tumor environment.
28. The PD-1 or PD-L1 binding agent of any one of claims 15-27, wherein the PD-1 or PD-L1 binding agent recognizes and binds PD-1 or PD-L1 and substantially functionally modulates its activity or does not substantially functionally modulate its activity.
29. A recombinant nucleic acid composition encoding the PD-1 or PD-L1 binding agents of any one of claims 15-28.
30. A host cell comprising a nucleic acid of claim 29.
31. The PD-1 or PD-L1 binding agent of any one of claims 15-28, wherein the PD-1 or PD-L1 binding agent is suitable for use in a patient having one or more of: cancer, infections, immune disorders, and/or autoimmune diseases.
32. A method for treating or preventing cancer, comprising administering to a patient in need thereof an effective amount of a chimera comprising a targeting moiety comprising an antigen or receptor recognition domain targeting PD-1 or PD-L1 and a signaling agent, selected from one or more of an interferon, an interleukin, and a tumor necrosis factor.
33. The method of claim 32, wherein the signaling agent is modified.
34. The method of claim 32 or 33, wherein the PD-1 or PD-L1 binding agent is a PD-1 or PD-L1 binding agent of any one of claims 1-12 or 15-28.
35. The method of any one of claims 32-34, wherein the PD-1 binding agent comprises at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:
 - (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 2-23;
 - (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 24-54; and
 - (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 55-69.
36. The method of any one of claims 32-34, wherein the PD-L1 binding agent comprises at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:
 - (a) CDR1 comprises an amino acid sequence selected from any one of SEQ ID NOs: 177-207;
 - (b) CDR2 comprises an amino acid sequence selected from any one of SEQ ID NOs: 208-266; and
 - (c) CDR3 comprises an amino acid sequence selected from any one of SEQ ID NOs: 267-285.

37. The method of any one of claims 32-36, wherein the cancer is selected from one or more of basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (e.g. that associated with brain tumors), and Meigs' syndrome.

38. A PD-1 or PD-L1 binding agent of any one of the above claims for use in the treatment of one or more of: cancer, infections, immune disorders, and/or autoimmune diseases.

39. Use of a PD-1 or PD-L1 binding agent of any one of the above claims for the manufacture of a medicament for treating one or more of: cancer, infections, immune disorders, and/or autoimmune diseases.

40. A chimeric protein comprising:

(a) a PD-L1 binding agent of any one of claims 1-12 or 16-28; and

(b) a modified human IFN- α 2, said modified human IFN- α 2 having one or more mutations that confer improved safety as compared to a wild type IFN- α 2; and

wherein the targeting moiety and the modified signaling agent are optionally connected with one or more linkers.

41. The chimeric protein of claim 40, wherein the modified human IFN- α 2 comprises one or more mutations at positions R120, M148, R149, and L153.

42. The chimeric protein of claim 40, wherein the modified human IFN- α 2 comprises one or more mutations selected from R120E, R149A, and L153A.

43. The chimeric protein of claim 40, wherein the modified human IFN- α 2 comprises a R120E mutation and either a R149A or a L153A mutation.

44. A chimeric protein comprising:
- (a) a PD-L1 binding agent of any one of claims 1-12 or 16-28; and
 - (b) a modified human IFN- β , said modified human IFN- β having one or more mutations that confer improved safety as compared to a wild type IFN- β ; and
- wherein the targeting moiety and the modified signaling agent are optionally connected with one or more linkers.
45. The chimeric protein of claim 44, wherein the modified human IFN- β comprises one or more mutations at positions W22, R27, L32, R35, V148, L151, R152, and Y155.
46. The chimeric protein of claim 45, wherein the modified human IFN- β comprises one or more mutations selected from W22G, R27G, L32A, L32G, R35A, R35G, V148G, L151G, R152A, R152G.

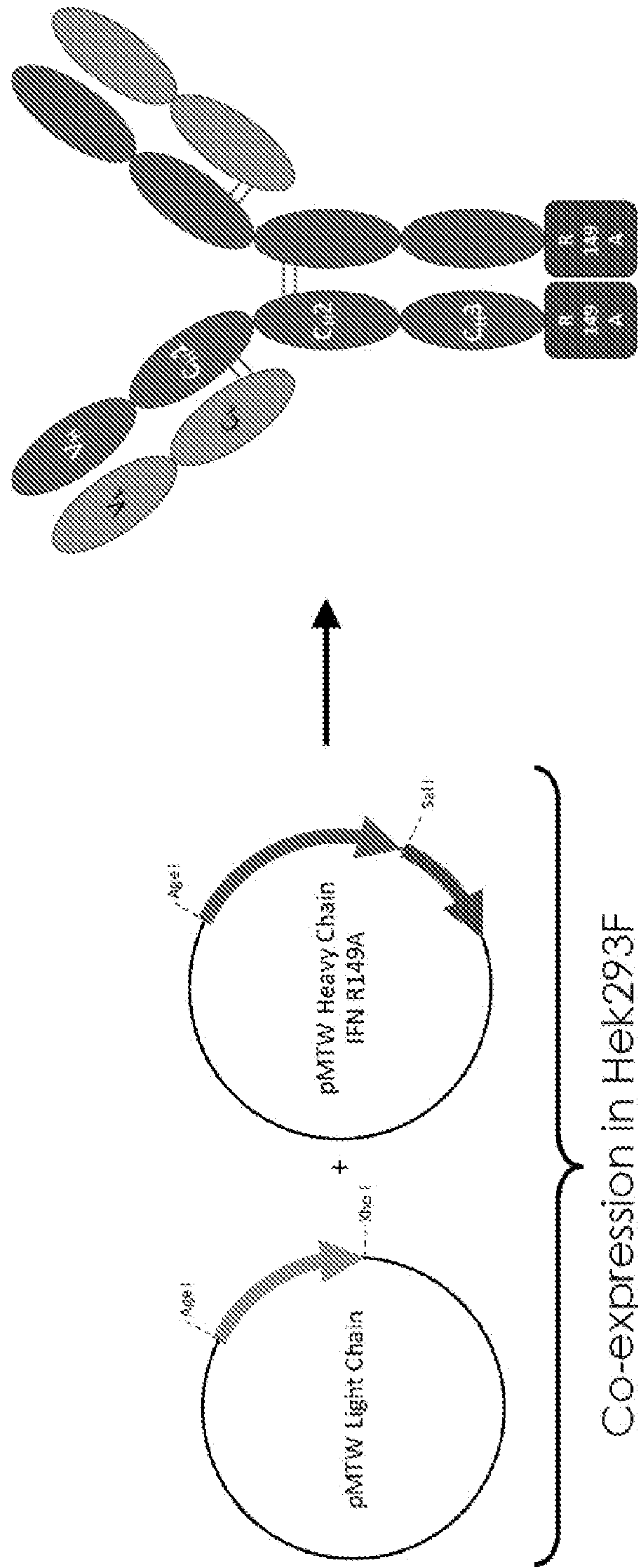


Figure 1

Figure 2

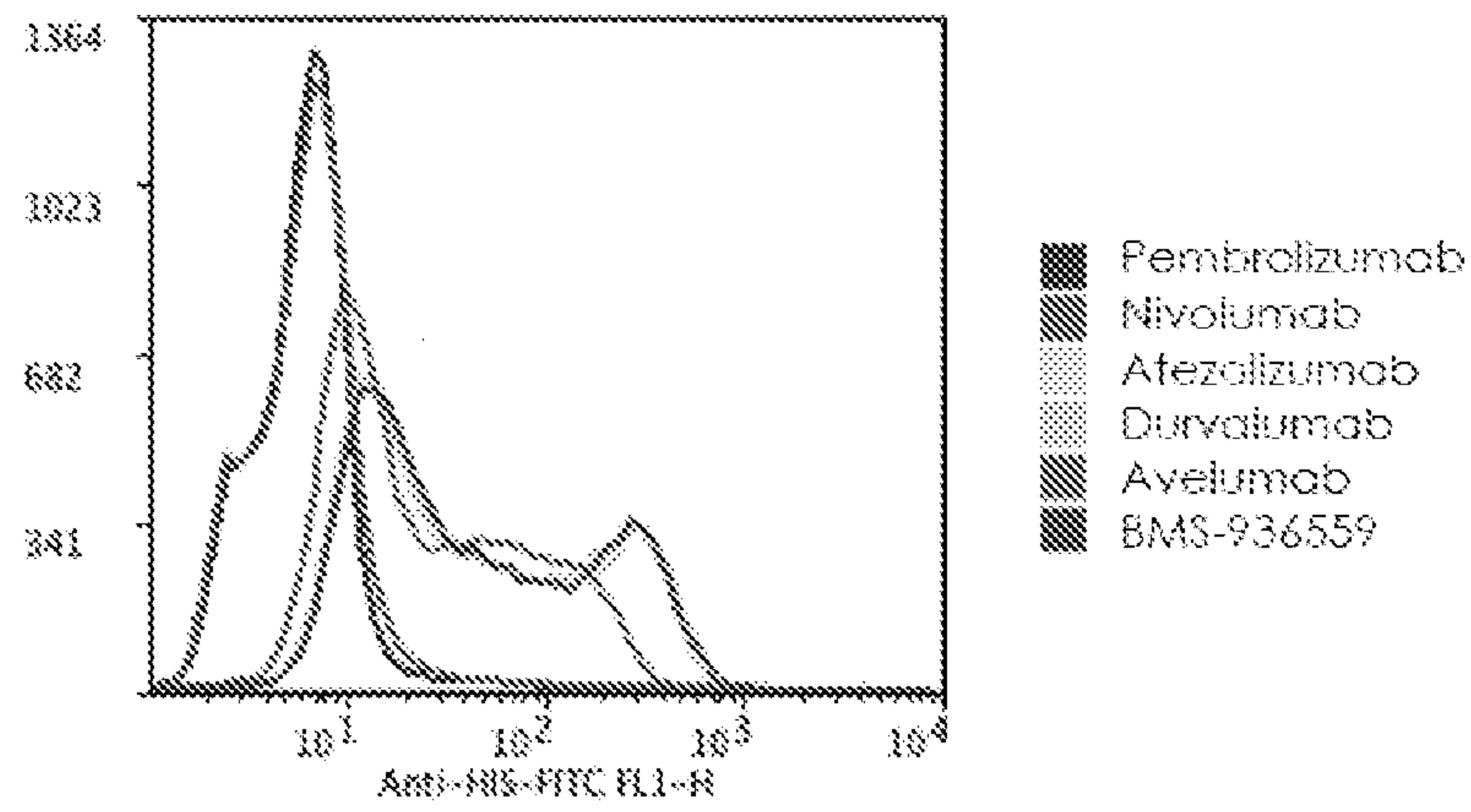
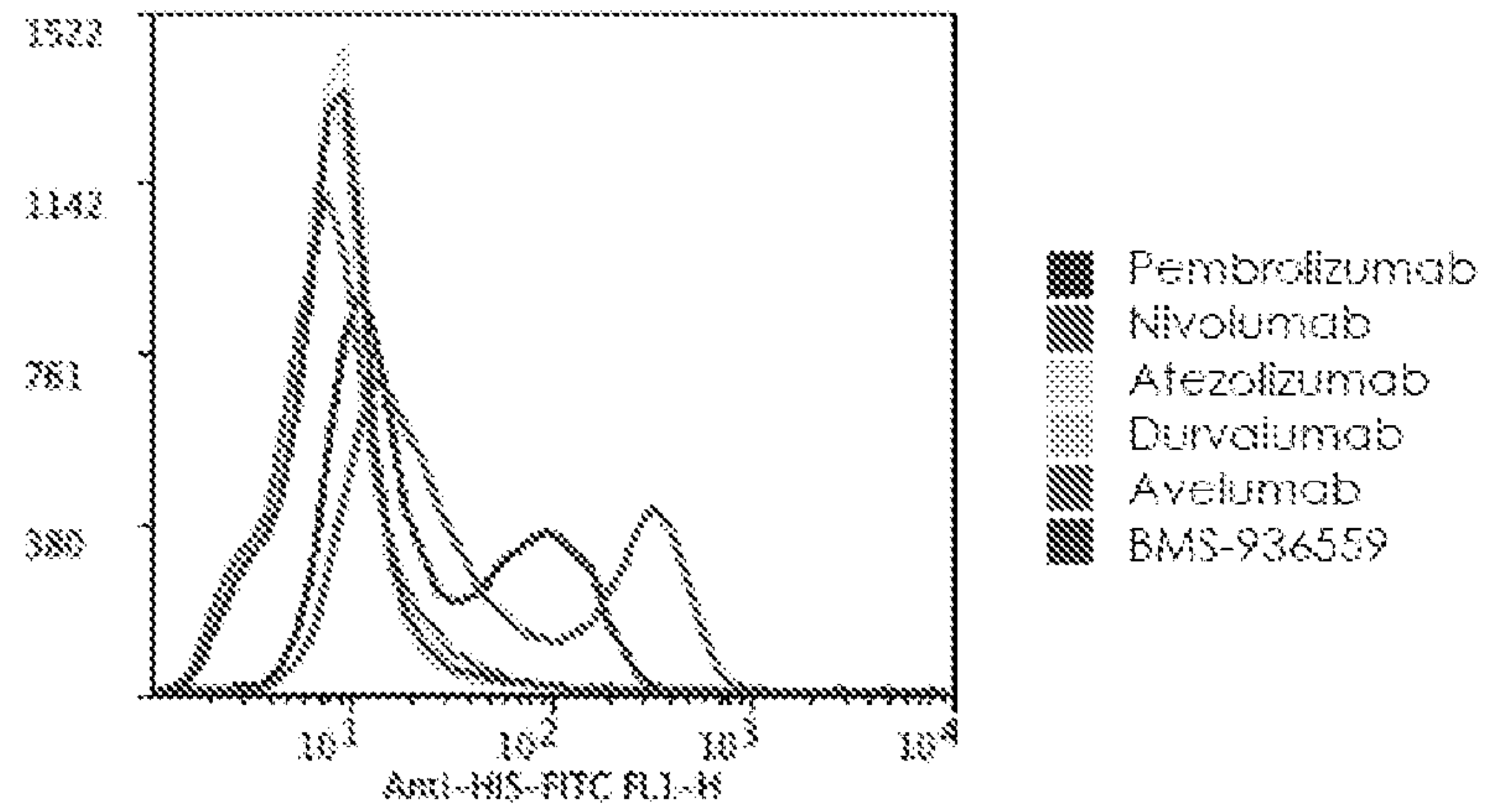
Binding to PD-L1 transfected Hek293T cells*Binding to PD-1 transfected Hek293T cells*

Figure 3

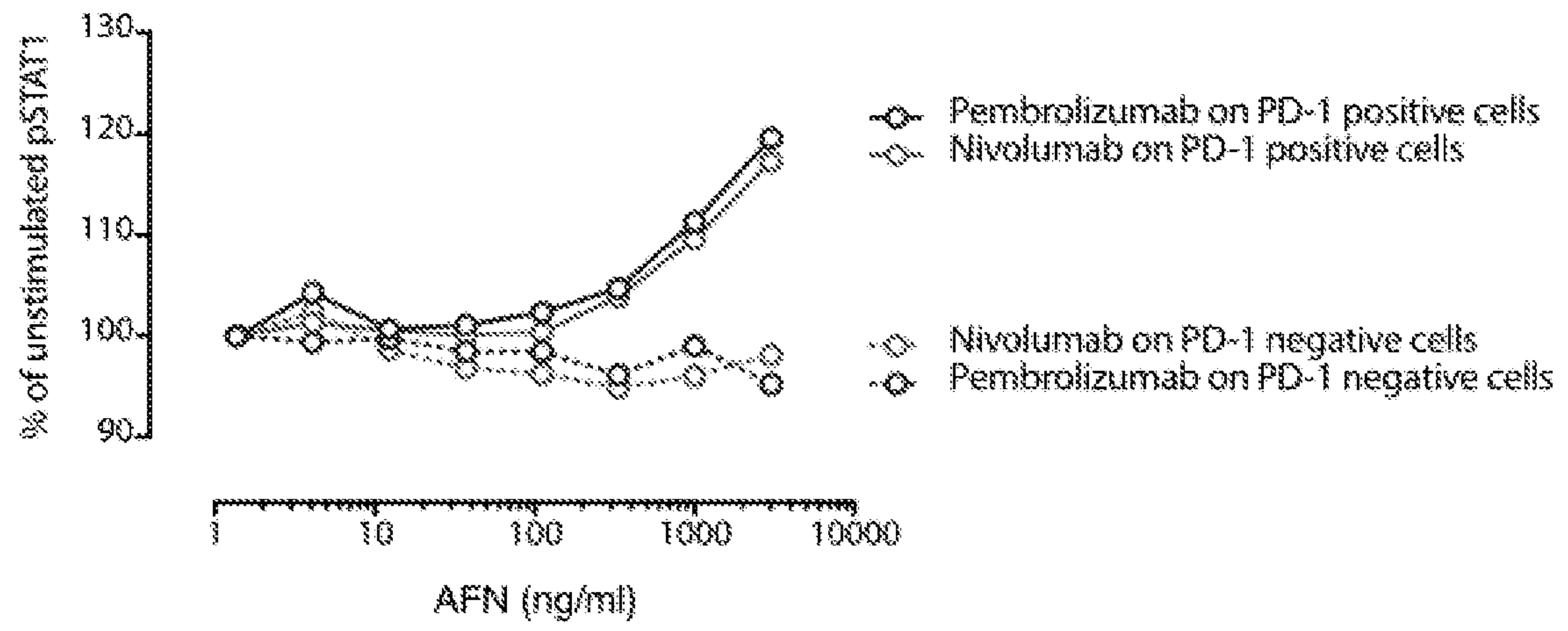


Figure 4

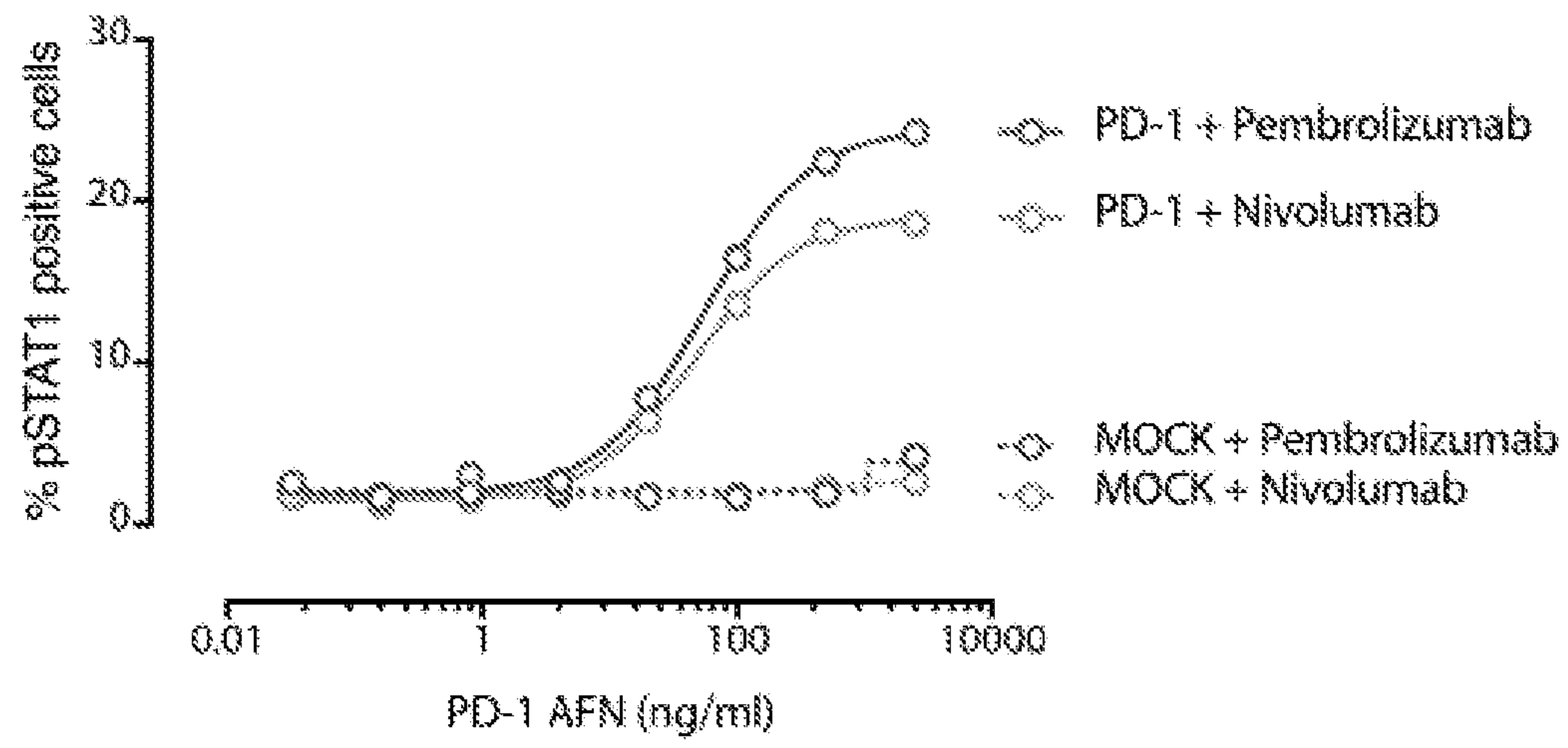


Figure 5

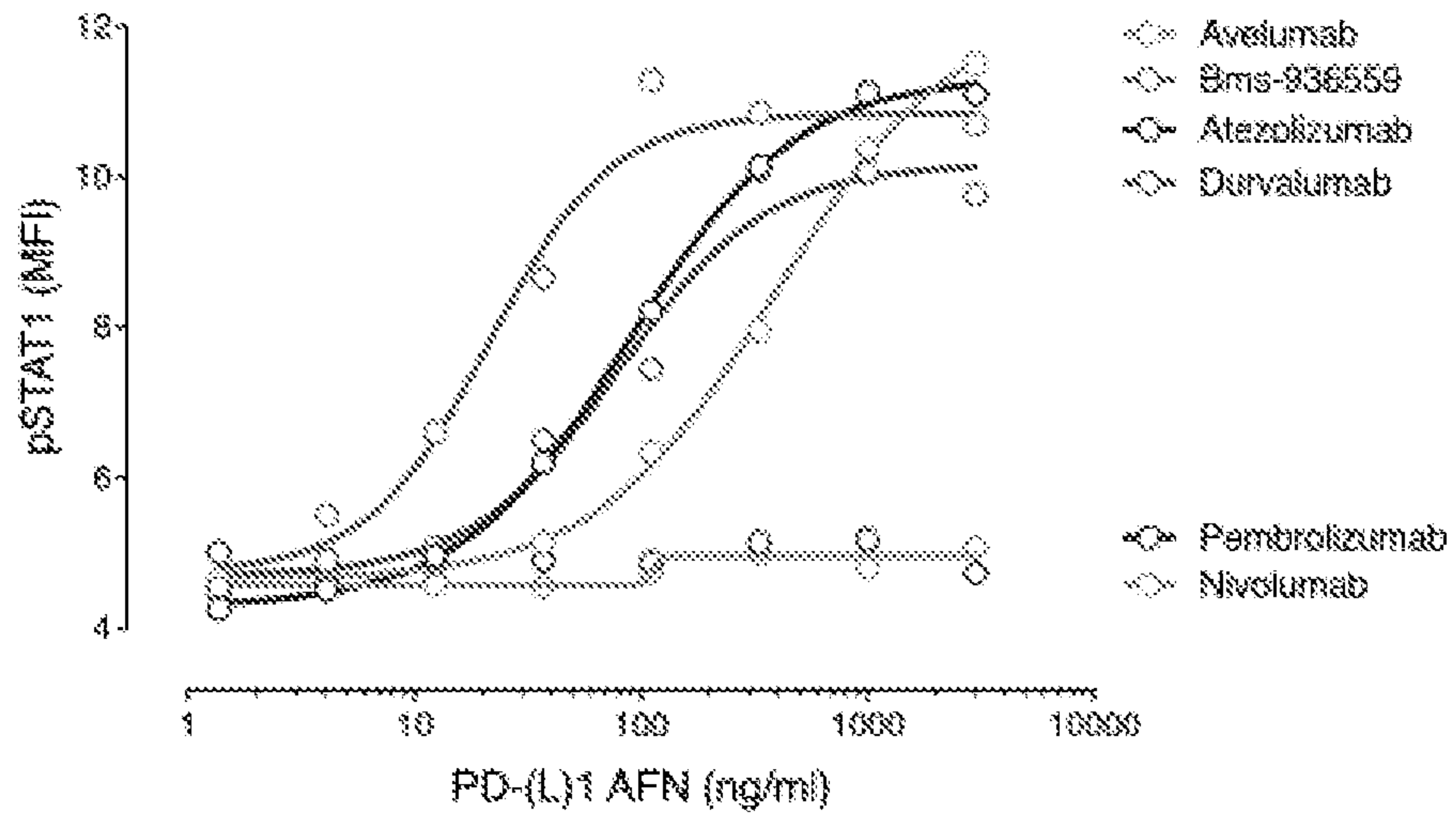


Figure 6

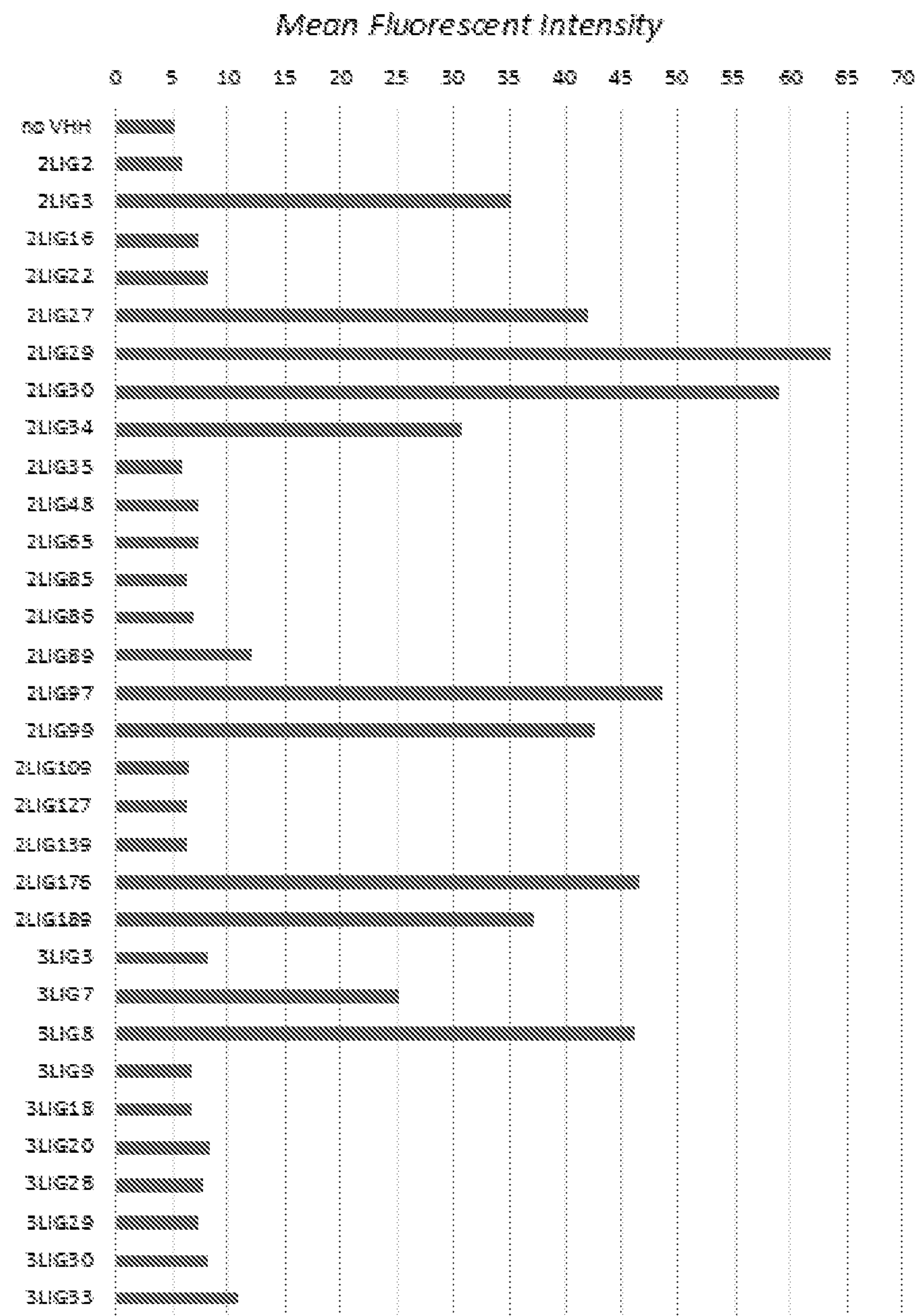


Figure 7A

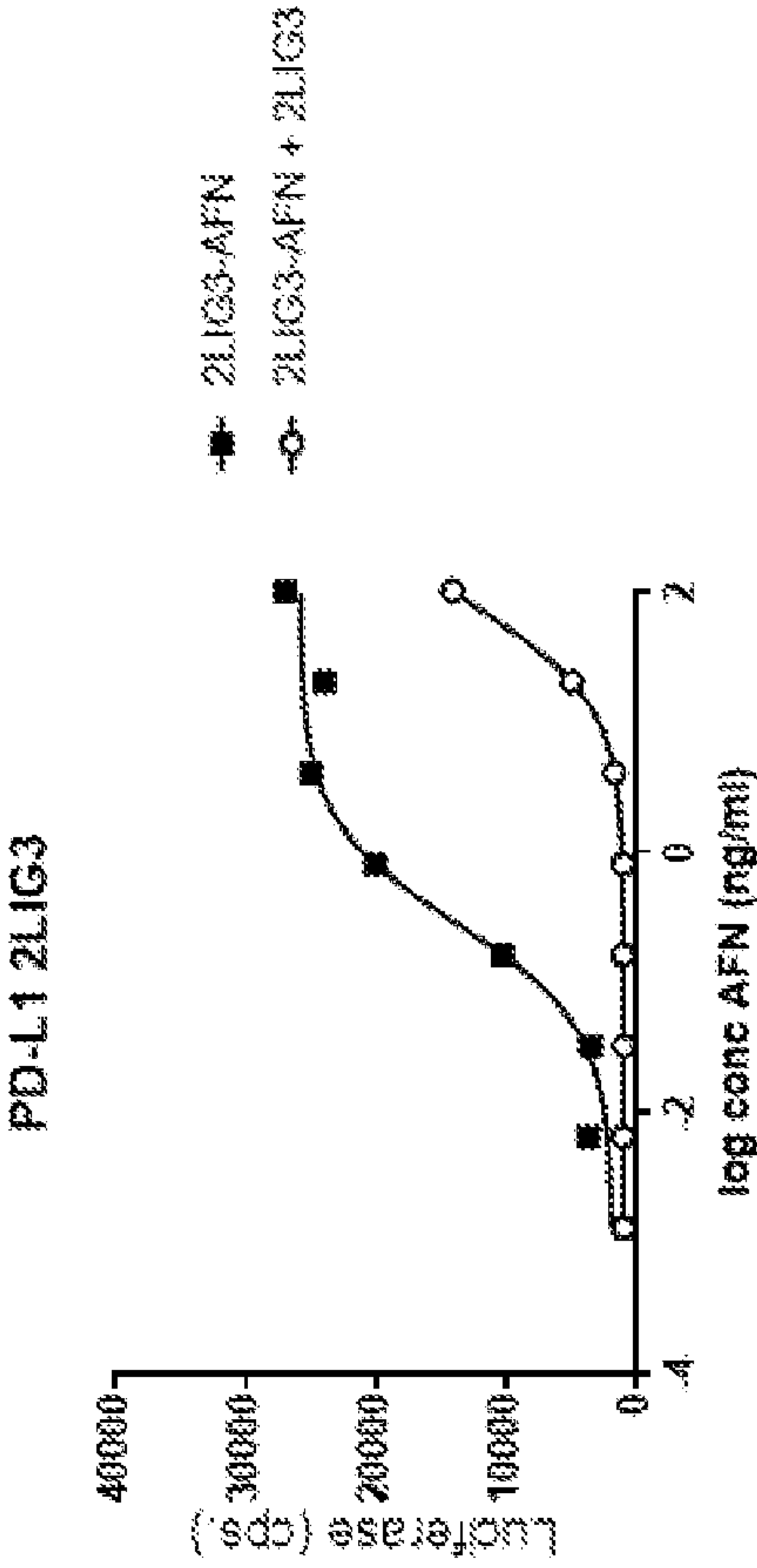


Figure 7B

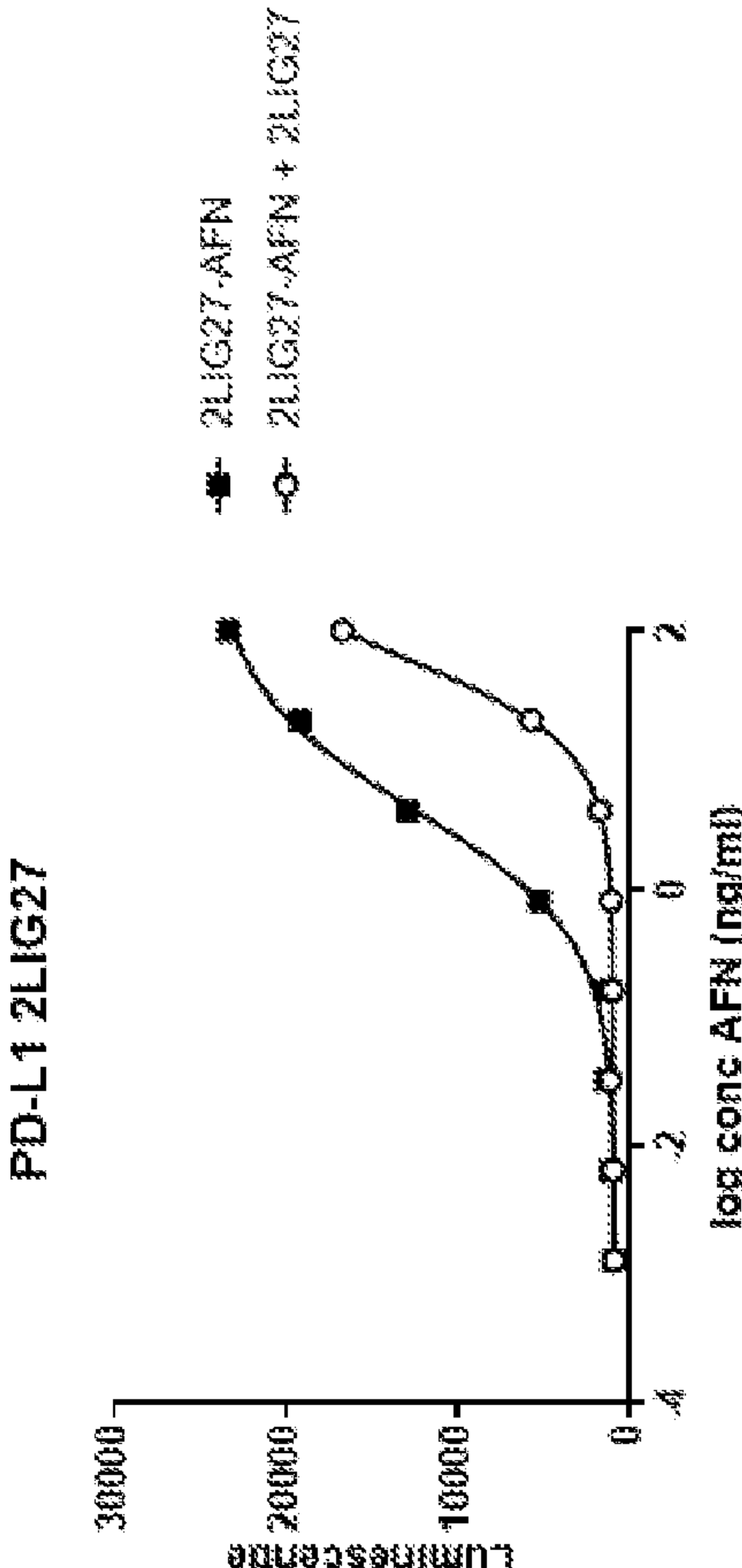


Figure 7C

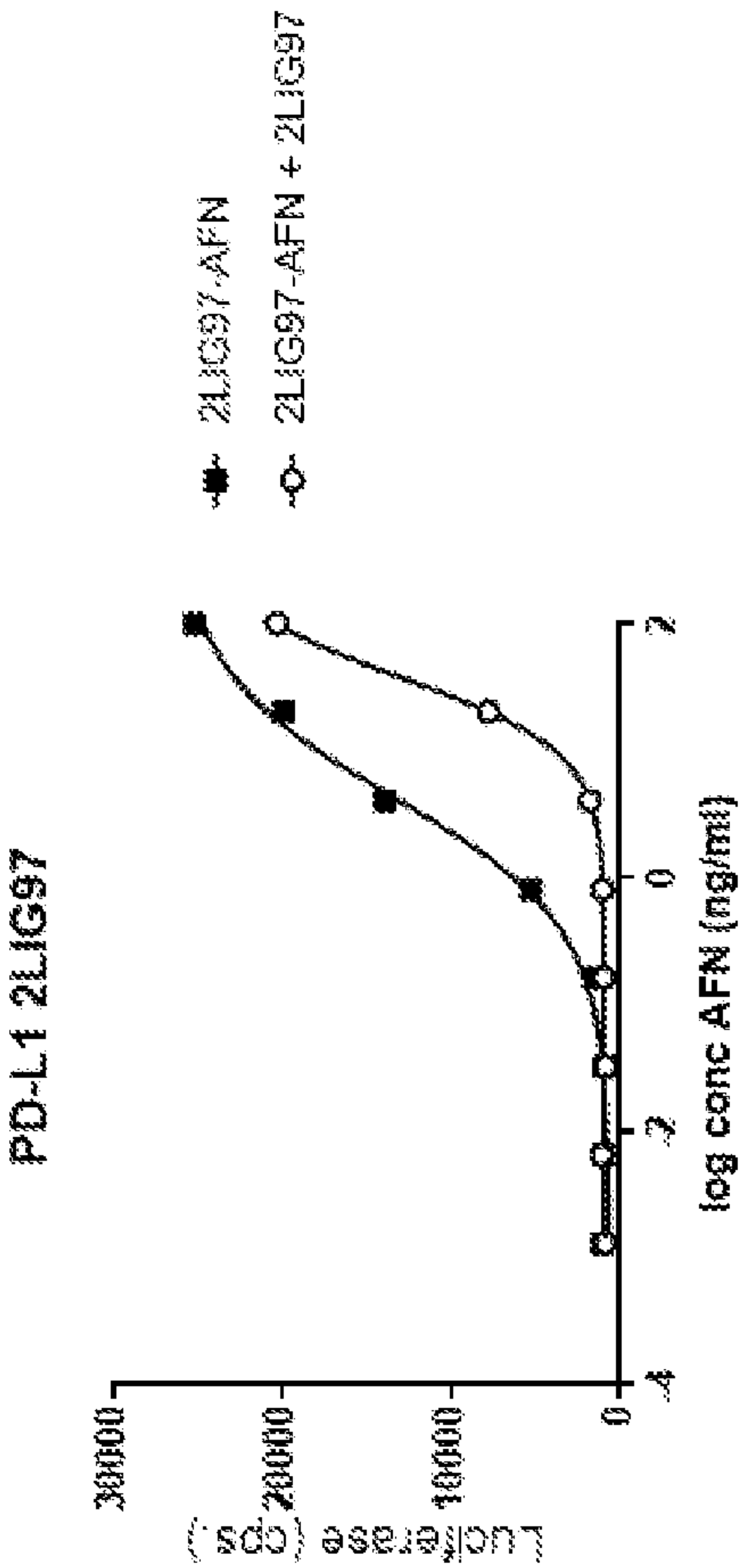


Figure 7D

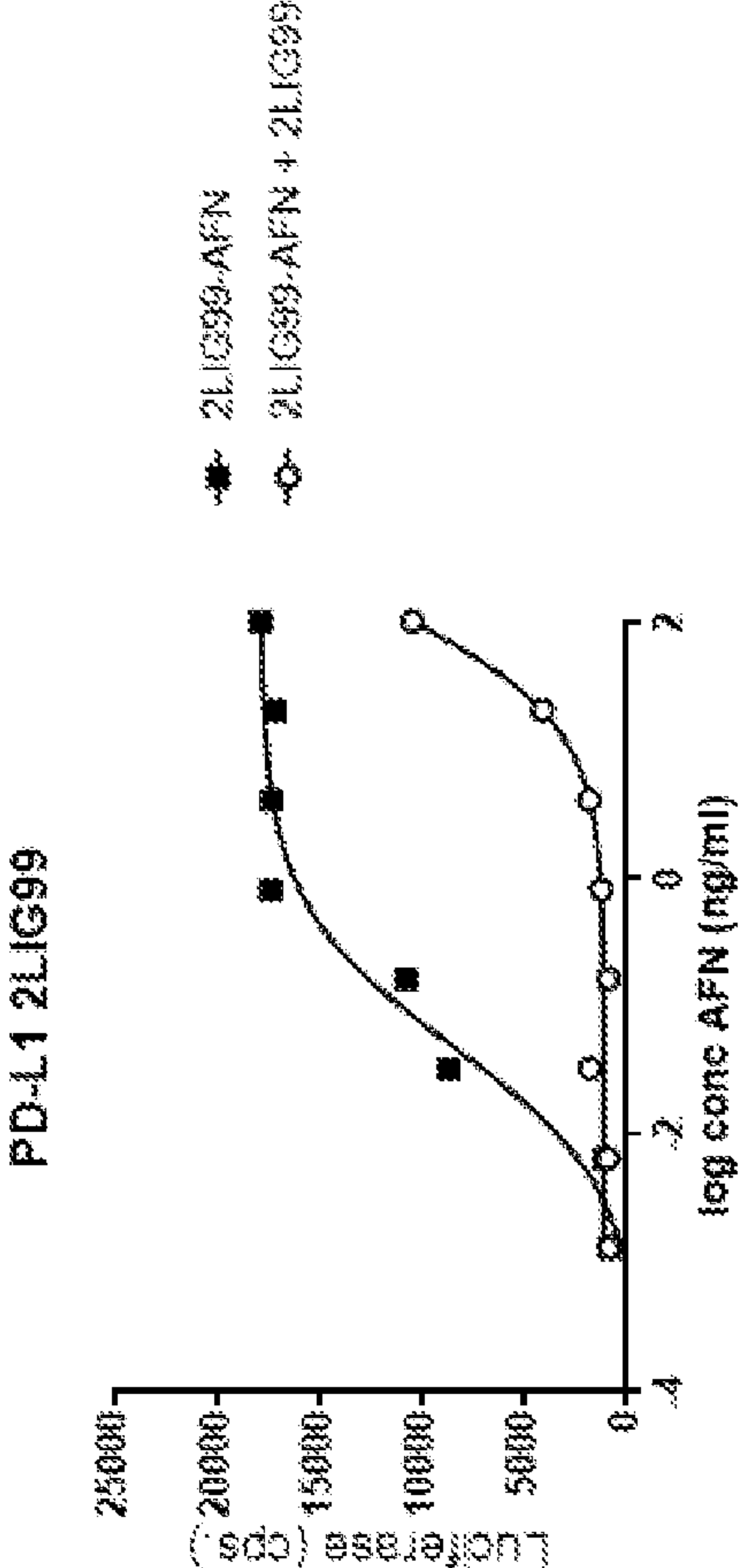


Figure 7E

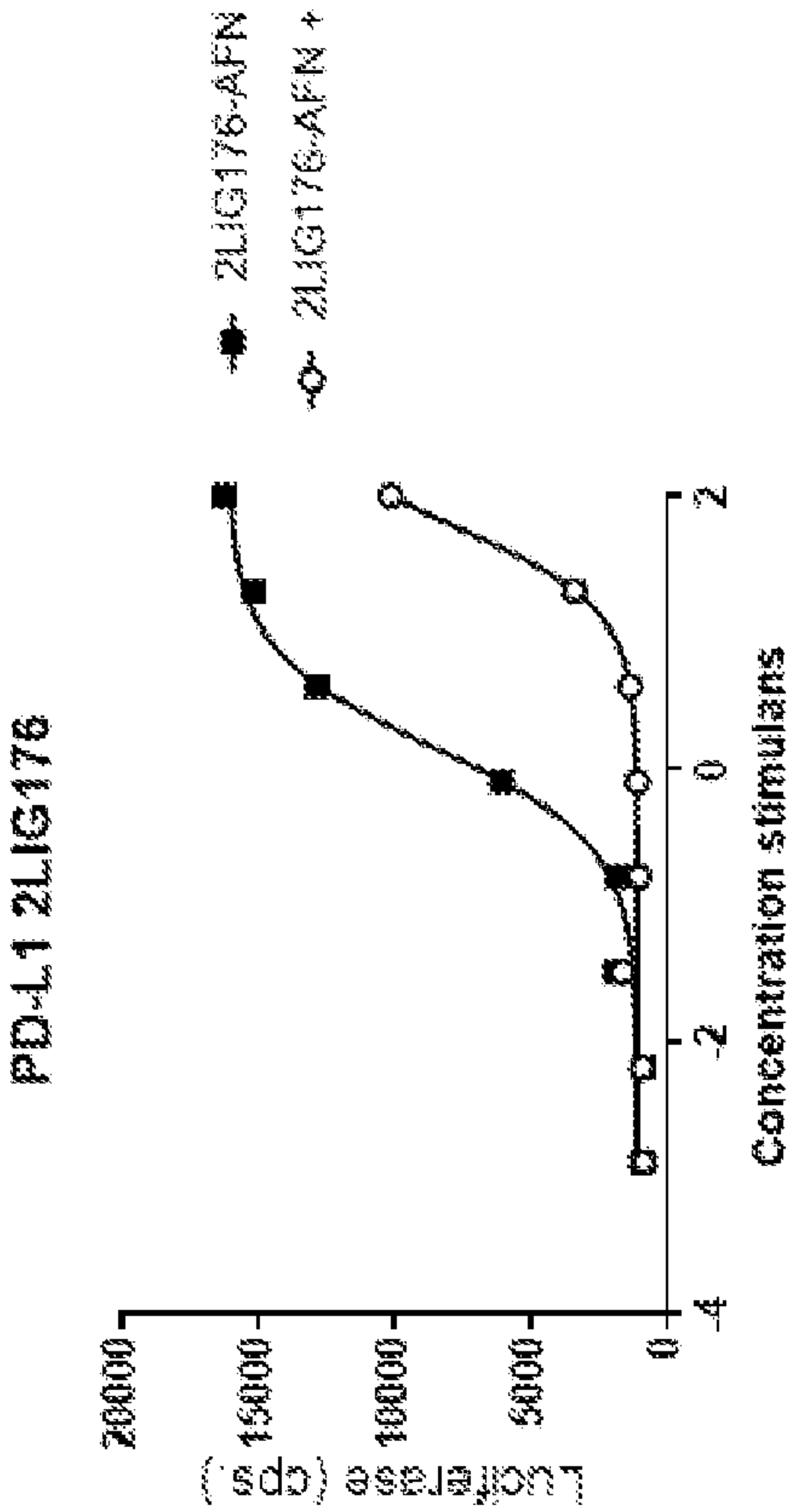


Figure 7F

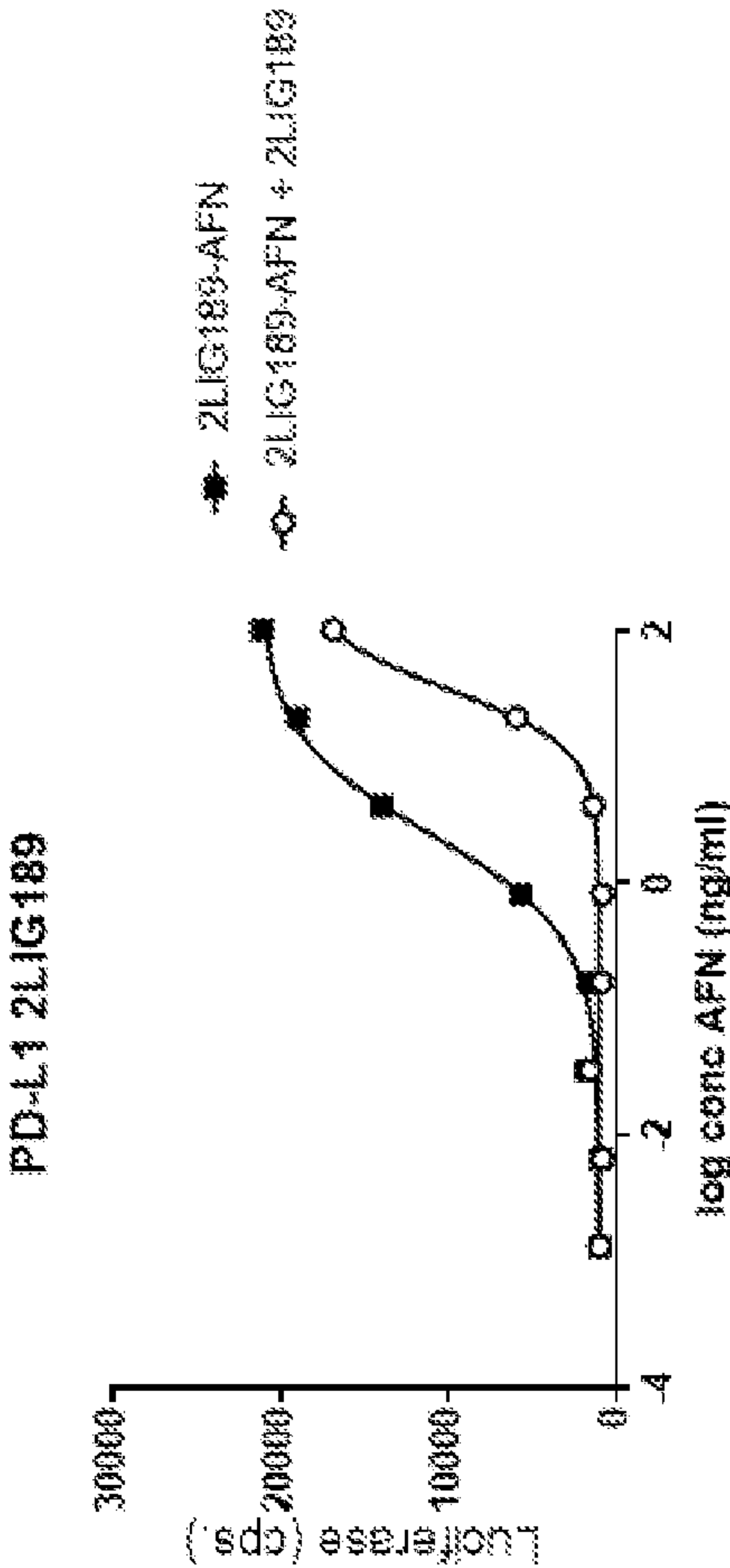


Figure 7G

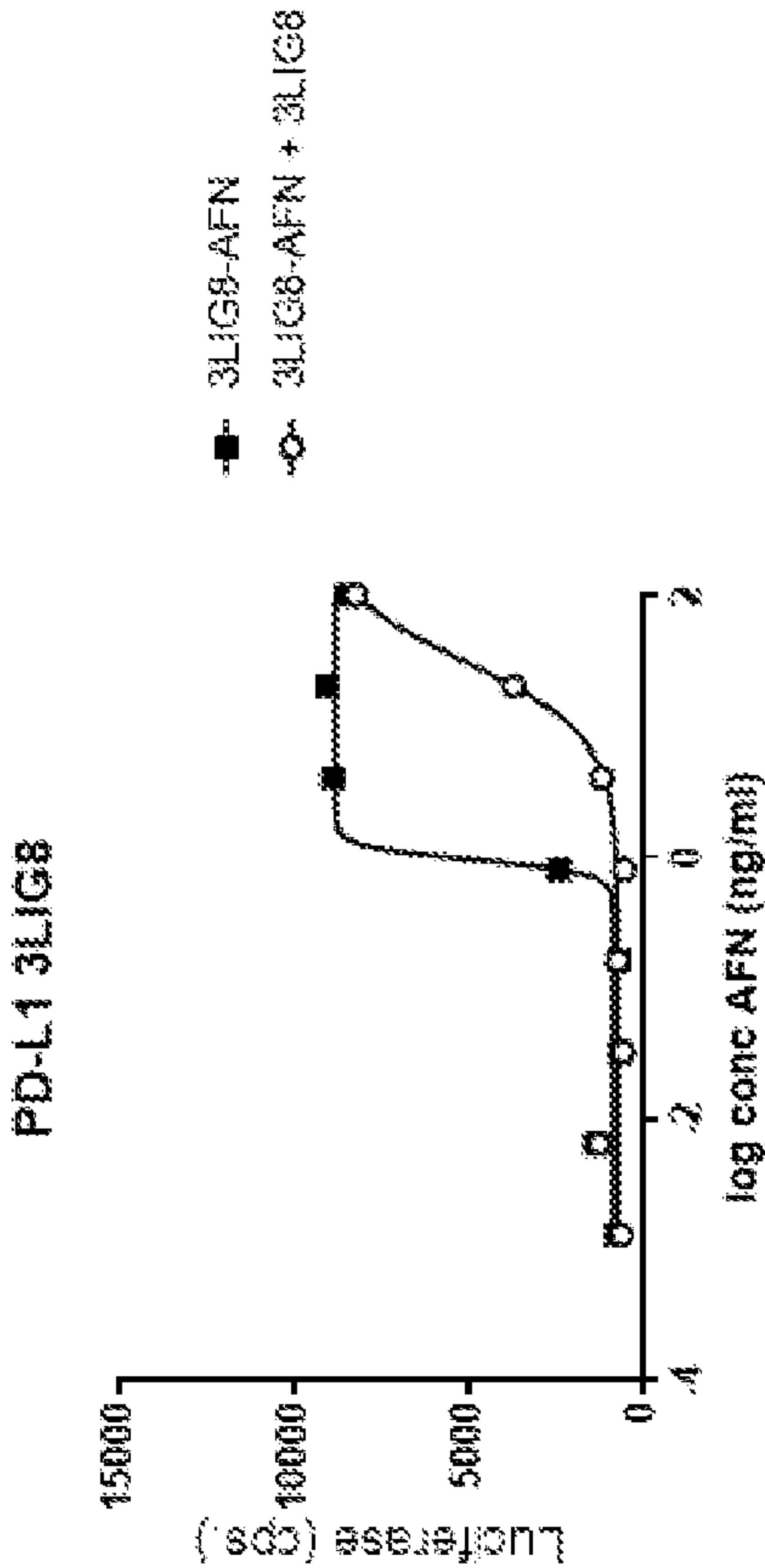


Figure 8

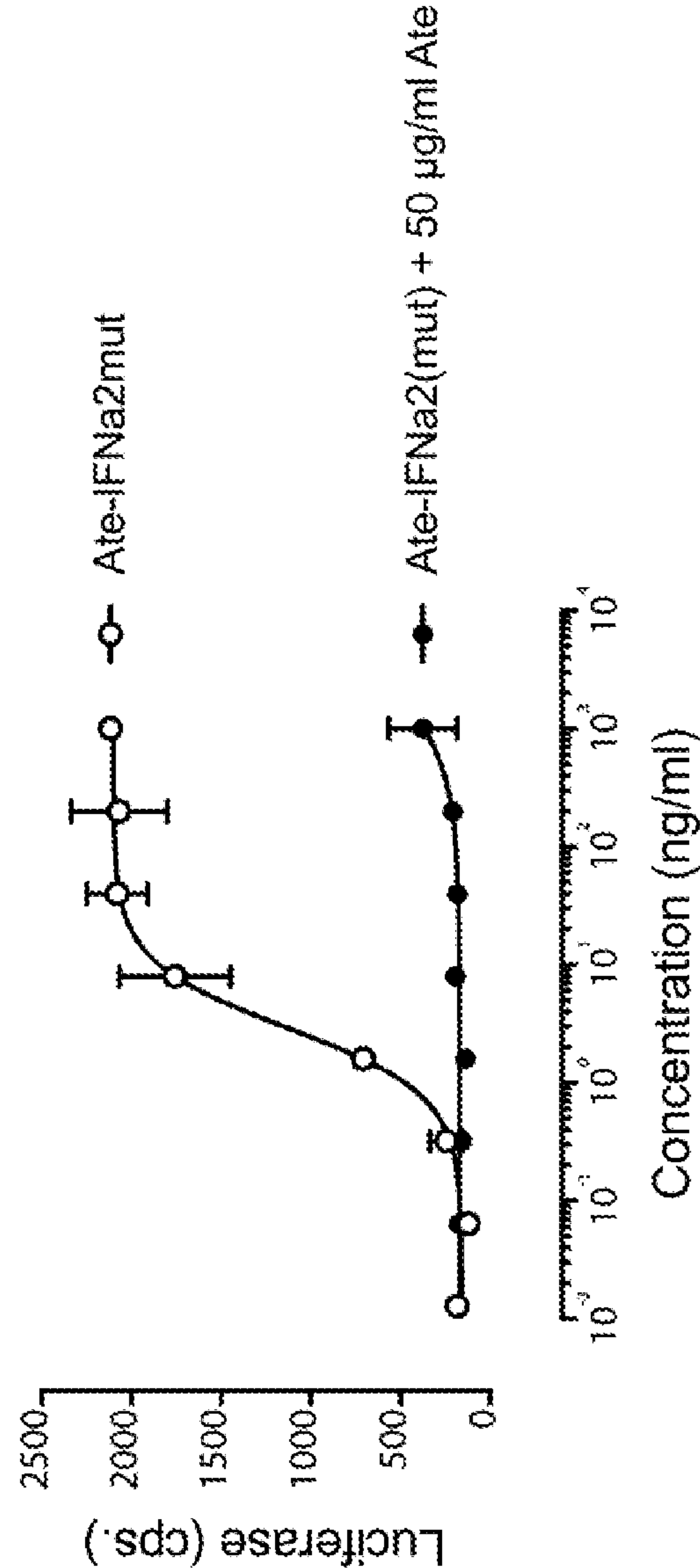


Figure 9

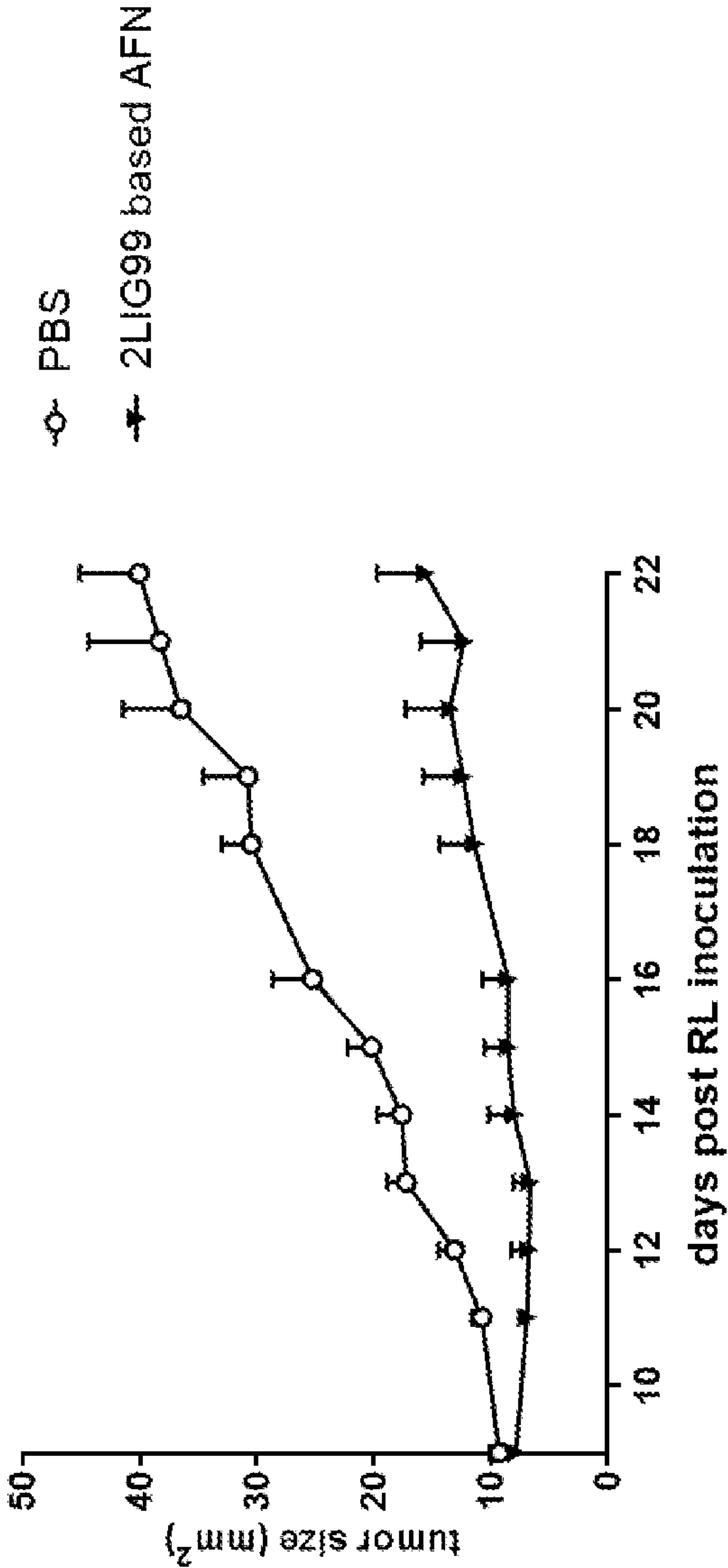


Figure 10A

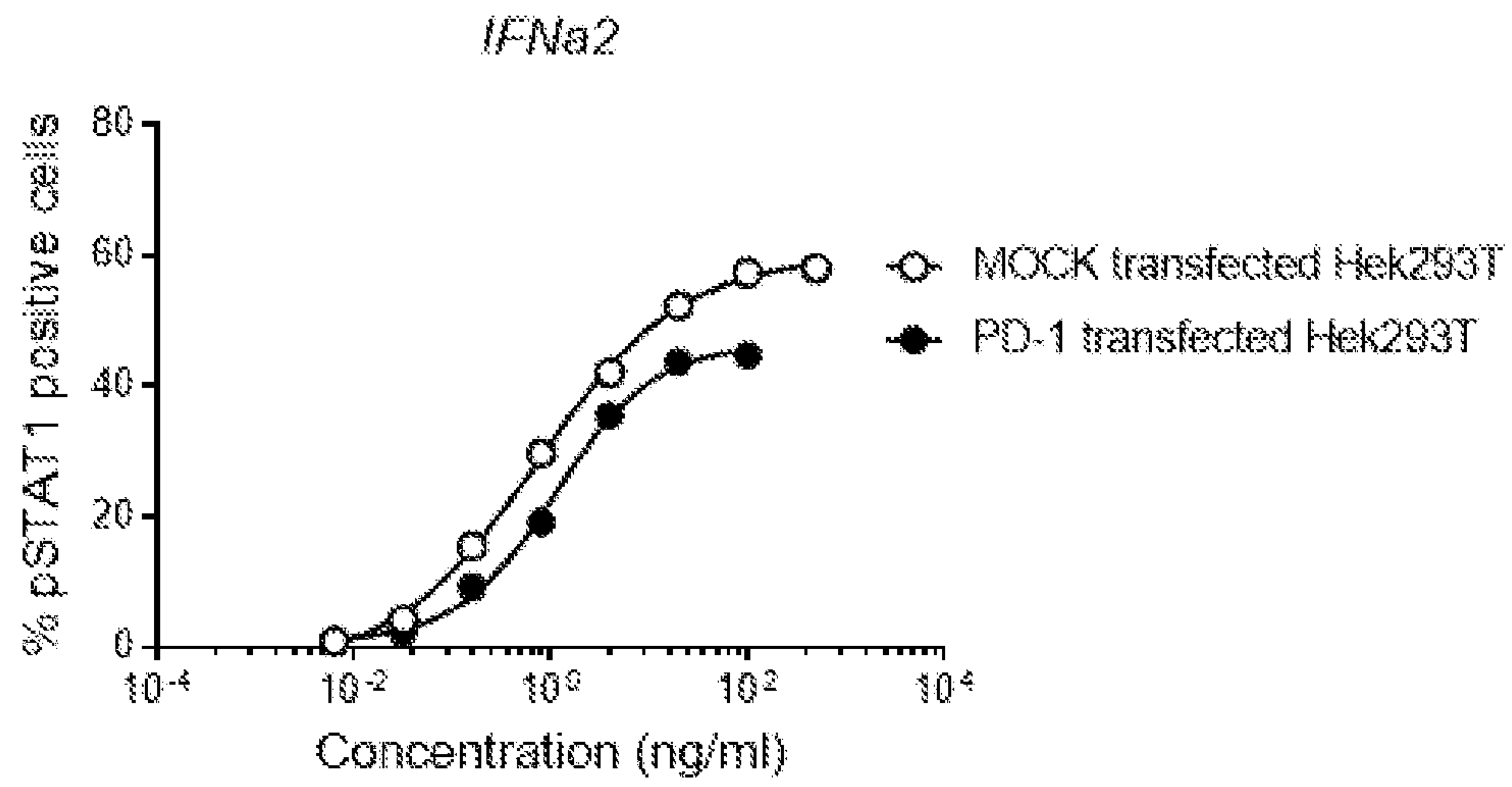


Figure 10B

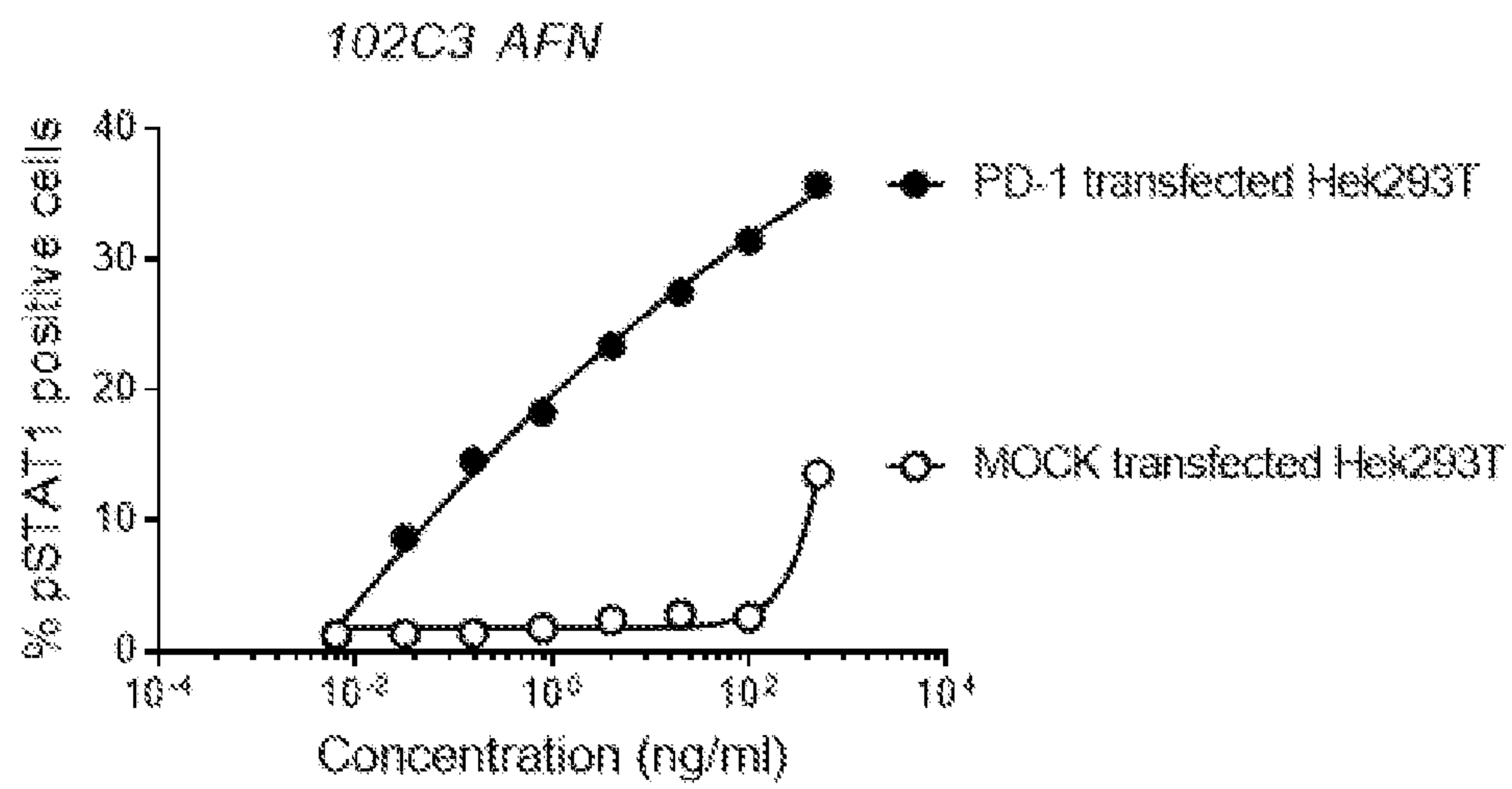


Figure 10C

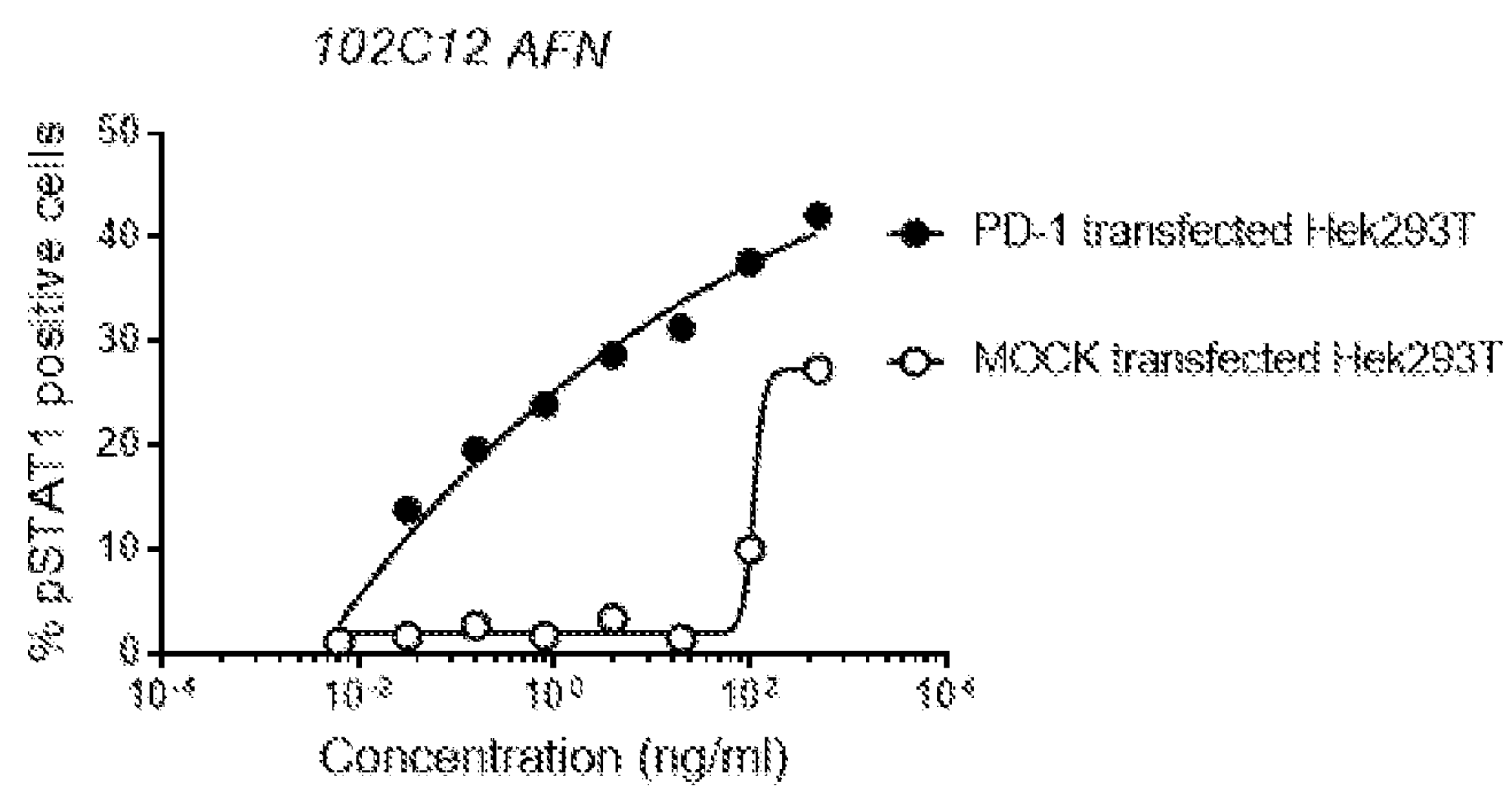


Figure 1

